MICROENVIRONMENT IN DISEASE AND AGING

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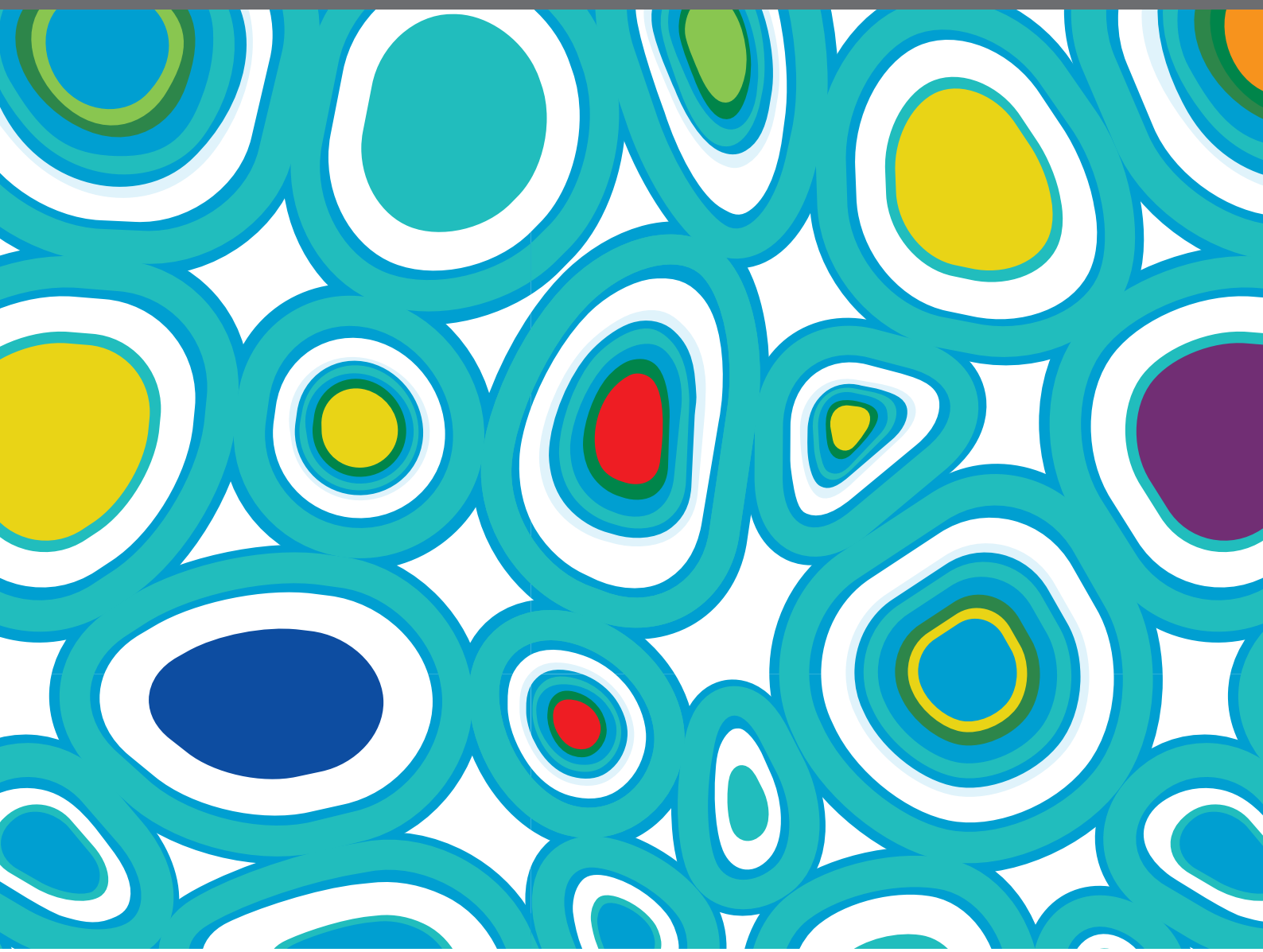

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MICROENVIRONMENT IN DISEASE AND AGING

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REVIEW
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Exosome-Based Cell-Cell Communication in the Tumor Microenvironment

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Tumors are not isolated entities, but complex systemic networks involving cell-cell communication between transformed and non-transformed cells. The milieu created by tumor-associated cells may either support or halt tumor progression. In addition to cell-cell contact, cells communicate through secreted factors via a highly complex system involving characteristics such as ligand concentration, receptor expression and integration of diverse signaling pathways. Of these, extracellular vesicles, such as exosomes, are emerging as novel cell-cell communication mediators in physiological and pathological scenarios. Exosomes, membrane vesicles of endocytic origin released by all cells (both healthy and diseased), ranging in size from 30 to 150 nm, transport all the main biomolecules, including lipids, proteins, DNAs, messenger RNAs and microRNA, and perform intercellular transfer of components, locally and systemically. By acting not only in tumor cells, but also in tumor-associated cells such as fibroblasts, endothelium, leukocytes and progenitor cells, tumor- and non-tumor cells-derived exosomes have emerged as new players in tumor growth and invasion, tumor-associated angiogenesis, tissue inflammation and immunologic remodeling. In addition, due to their property of carrying molecules from their cell of origin to the peripheral circulation, exosomes have been increasingly studied as sources of tumor biomarkers in liquid biopsies. Here we review the current literature on the participation of exosomes in the communication between tumor and tumor-associated cells, highlighting the role of this process in the setup of tumor microenvironments that modulate tumor initiation and metastasis.

Keywords: exosomes, cancer, tumor microenvironment, extracellular vesicles (EVs), cell-cell communication

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INTRODUCTION

The tumor microenvironment is anything but simple. Be it the primary or the metastatic tumor, its complex and heterogeneous microenvironment is comprised of a network of both cellular and acellular constituents. While the former consists of tumor cells and diverse non-transformed cells, such as cancer-associated fibroblasts, macrophages, and endothelial cells, the latter is formed by secreted factors and components of the extracellular matrix (ECM). In its intricacy, the tumor microenvironment has emerged to be a key modulator of tumor progression by providing inhibitory or stimulatory growth signals (Bissell and Hines, 2011).

Interactions amongst neighboring cells in the primary tumor are essential for tumor growth and development, and besides direct cell-cell contact, intercellular communication also happens

through a complex system involving secreted factors. Besides local cell-to-cell communication, secreted factors play a key role in the interaction amongst cells located far apart from each other (Becker et al., 2016; Fu et al., 2016; Kalluri, 2016; Peinado et al., 2017). In the group of secreted factors, we will here focus on the roles of exosomes in setting up and modifying tumor microenvironments.

The study of exosomes and other extracellular vesicles (EVs) is a relatively new field of research that picked up steam in the last couple of decades. The first indication of the existence of EVs came in 1946, when Chargaff and West showed that a platelet-free plasma fraction maintained clotting properties, which was diminished after a high-speed ultracentrifugation that pelleted a particulate fraction with coagulatory activity (Chargaff and West, 1946). After two more studies in the 1950's on the role of alike fractions in promotion of coagulation (Hougie, 1955; O'Brien, 1955), in 1967 Peter Wolf published his seminal work showing by electron microscopy the "coagulant material in minute particulate form" pelleted by ultracentrifugation. Wolf called these lipid rich particles originated from platelets "platelet-dust," and also suggested that these lipid particles might be normally liberated in circulating blood (Wolf, 1967). Decades later, platelet-derived microparticles were in fact shown to be the most abundant extracellular vesicle population in peripheral blood (Zmigrodzka et al., 2016). In the early 1980's, two articles were published reporting that these particles could also be produced by tumor cells (Dvorak et al., 1981, 1983). Later, in 1987, while studying reticulocyte maturation, Johnstone and colleagues introduced for the first time the term "exosomes" to describe these particles (Johnstone et al., 1987). By then, exosomes were commonly seen as "trashbags" for excreting cellular components, but in 1998 Sebastian Amigorena's group proposed a role for exosomes in the communication between cells of the immune system, thus making exosome's debut as a mediator of cell-cell communication (Zitvogel et al., 1998). Few years after, in 2001, it was shown that platelet-derived EV could transfer antigens like CD41 from platelets to the cell membranes of CD34+ hematopoietic stem-progenitor cells, demonstrating the ability of EVs to horizontally transfer information (Janowska-Wieczorek et al., 2001; **Figure 1A**). Platelet-derived EV were also shown to induce tumor chemotaxis, proliferation, invasion and expression of angiogenic factors, contributing to the formation of distant metastasis (Janowska-Wieczorek et al., 2005, 2006; Toth et al., 2008; Dashevsky et al., 2009). In addition, they have been shown promote thrombus formation and contribute to metastasis (Falanga et al., 2003), being plasmatic levels of these EVs associated aggressiveness, prognosis and survival of oncologic patients (Kim et al., 2003; Helley et al., 2009; Italiano et al., 2010; Voloshin et al., 2014).

Being still an immature scientific field, EVs' research still faces several basic challenges. The nomenclature of distinct types of EVs, the lack of good established markers and the technical difficulties and heterogeneity of isolation protocols, for instance, have in the last few years been subject of extensive evaluations and debates. In an attempt to improve standardization in the field, the International Society for Extracellular Vesicles recently released guidelines for the analysis of EVs and the reporting of the results (Lotvall et al., 2014), a standardization initiative followed by others, such as the EV-TRACK Consortium (EV-TRACK Consortium et al., 2017). EVs can be classified according to size and cellular origin (endosome- or plasma membrane-derived). Differently from larger EVs, which are released directly from the plasma membrane of both living and dying cells, such as microvesicles and apoptotic bodies, and from vesicular artifacts composed of disrupted membrane fragments spontaneously released by cells undergoing necrosis, known as microsomes (Witwer et al., 2013), exosomes are small EVs, ranging in size from 30 to 150 nm, with a multivesicular endosomal origin actively secreted by all cell types upon fusion of Multivesicular Bodies with the plasma membrane (Becker et al., 2016; Kalluri, 2016). However, most present-day EV's isolation protocols do not discriminate EVs according to their origin, and only recently Kowal and colleagues showed that only the subpopulation bearing the three Tetraspanins CD9, CD63, and CD81 corresponds to endosome-derived exosomes. Another source of confusion is the fact that current isolation protocols provide enriched fractions of EVs, but not pure fractions (Kowal et al., 2016). Thus, many studies to date consider small vesicles as exosomes regardless of cellular origin, and for the purposes of this review we do the same. Also, various nomenclatures have been used to describe small EVs over the years, and for the sake of clarity and according to the protocols the authors used for EV isolation we will call these small EVs exosomes, even when authors use other terms to refer to these vesicles.

Although not yet completely characterized, the process of exosomes biogenesis and release to the extracellular microenvironment relies on several energy-dependent active steps mediated by, for instance, SNAREs and Rabs and Ras GTPases (Pfeffer, 2007). During this process, exosomes are packed with proteins, lipids, DNAs, messenger RNAs (mRNAs), microRNAs (miRNA) and other non-coding RNAs (Becker et al., 2016; **Figure 1B**). As in other scenarios of cellular stress, including hypoxia and ER stress, exosomes production and composition has been described as markedly affected in oncologic settings, where the role of exosomes as mediators of cell-to-cell communication has been broadly studied. Exosomes' concentration is frequently higher in the blood of cancer patients when compared with healthy control human blood, and the cargo of exosomes can change according to the patient's health status (Kalluri, 2016). Here we will discuss the anti- and pro-tumorigenic effects of tumor and non-tumor cells-derived exosomes in tumorigenic and metastatic processes, focusing on how exosomes mediate interactions amongst tumor cells, leukocytes, fibroblasts, tumor-associated vasculature, and stem/progenitor cells relevant to the tumor microenvironment.

EXOSOMES IN TUMOR-TUMOR COMMUNICATION

The proliferation of tumor cells, a process indispensable for cancer progression, relies on soluble growth factors. As mentioned above, cells also convey information to the microenvironment through molecules packed in exosomes and

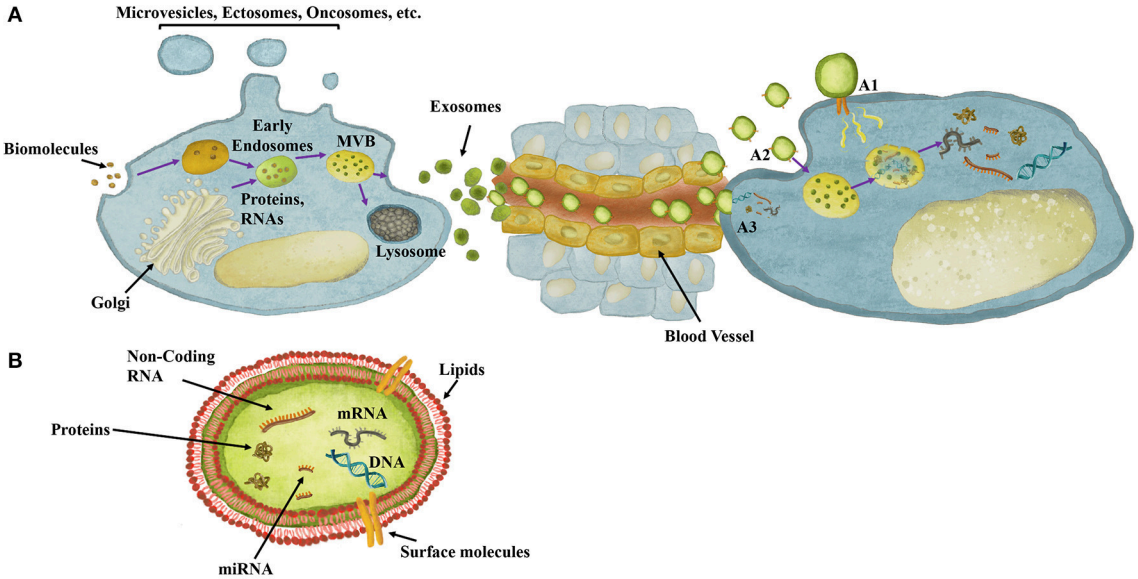

FIGURE 1 | Exosomes role in cell-cell communication and their content. **(A)** Exosomes are extracellular vesicles composed of biomolecules derived from, for instance, Golgi and endocytosis, which are processed through endosomal compartments into multivesicular bodies (MVB). MVBs can either fuse with lysosomes for degradation or with the plasma membrane for release of exosomes to the extracellular milieu. Once released, exosomes can act both locally or travel through the circulation reaching distant sites. Exosomes mediate cell-cell communication though different mechanisms. **(A1)** Exosomes may dock at the plasma membrane of the target cell and activate intracellular signaling by ligand-receptor interaction. **(A2)** Exosomes may be endocytosed by phagocytosis, micropinocytosis or receptor-/raft-mediated endocytosis, and fuse with the delimiting membrane of an endocytic compartment, releasing their content into the cytoplasm of the recipient cells. **(A3)** Exosomes may be directly taken up by membrane fusion, releasing their content into the cytoplasm. **(B)** Exosomes structure, involving a double-layered lipid membrane vesicle containing every basic cellular biomolecule, including Proteins, DNA, mRNA and miRNA.

other EVs through complex signaling networks that are only beginning to be unveiled. Studies involving several distinct cancer cells showed that tumor-derived exosomes can induce tumor cell proliferation. For example, an autocrine induction of cellular proliferation was observed in chronic myeloid leukemia (Raimondo et al., 2015) and in human gastric cancer (Qu et al., 2009; Pan et al., 2017) through, for instance, the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) and MAPK/ERK signaling pathways (Qu et al., 2009) and transference of a long noncoding RNA (Pan et al., 2017). Still in gastric cancers, the signaling for tumor cell proliferation through MAPK can be mediated by exosomal CD97 (Li C. et al., 2015). In human bladder cancer, induction of cell proliferation was observed when the human bladder cancer cells T24 and 5637 were treated with T24 tumor cell-derived exosomes, in a mechanism also mediated by activation of the Akt and ERK pathways (Yang et al., 2013). In addition, glioblastoma-derived exosomes were shown to induce proliferation of the human glioma U87 cell line (Skog et al., 2008) in a mechanism dependent of the CLIC1 protein (Setti et al., 2015). In a narrower context related to prostate cancer treatment, namely the diminished availability of androgens caused by androgen-deprivation therapy, prostate cancer LNCaP cells cultured in the presence of androgens secrete exosomes enriched in CD9, which in turn induce the proliferation of androgen-deprived LNCaP cells (Soekmadji et al., 2016). Another example involves the promotion of *in vivo* growth of murine melanomas by systemic treatment of mice with melanoma-derived exosomes, which a ccelerated growth and inhibited apoptosis of melanoma tumors *in vivo* (Matsumoto et al., 2017).

In addition to the effects on cell proliferation, tumor-derived exosomes can also modify the migratory status of recipient malignant cells. Nasopharyngeal carcinoma-derived exosomes carrying Epithelial to Mesenchymal transition (EMT)-inducing signals, including TGF-β, Hypoxia-Inducible Factor 1 alpha (HIF1α) (Aga et al., 2014), Matrix Metalloproteinases (MMPs) (You et al., 2015), Notch1, LMP1 Casein Kinase II and Annexin A2 (Yoshizaki et al., 2013; Jeppesen et al., 2014; Kruger et al., 2014; Ung et al., 2014; Cha et al., 2015), were shown to enhance the migratory capacity of the tumor recipient cells. Another example involves exosomes derived from hypoxic prostate cancer cells, which induced increased invasiveness and motility of naïve human prostate cancer cells (Ramteke et al., 2015).

In addition to several works reporting their pro-tumorigenic effects, exosomes were also shown to play a role in tumor-tumor communication by transferring chemoresistance. Since Corcoran and colleagues reported that exosomes can transfer Docetaxel resistance in prostate cancer (Corcoran et al., 2012), similar phenomena have been described in distinct tumor contexts, such as in lung, breast and liver cancers (Takahashi et al., 2014; Xiao et al., 2014; Kong et al., 2015). Indeed, in lung cancer the transfer of Cisplatin resistance is mediated by production of exosomes containing low levels of miRNA miR-100-5p by donor resistant cells, which in turn leads to an increased expression

of the mammalian target of Rapamycin (mTOR) protein and chemoresistance in the recipient cells (Qin et al., 2017). In breast cancer, miRNA packed in exosomes from drug-resistant cells can modify the expression of specific target genes, including Sprouty2 (targeted by miR-23a), PTEN (targeted by miR-222), APC4 (targeted by miR-452) and p27 (targeted by miR-24), modulating chemoresistance in recipient cells that incorporate these exosomes (Chen et al., 2014a; Mao et al., 2016). In fact, exosomal miR-222 plays a key role in this process (Chen et al., 2014b; Yu et al., 2016), as the silencing of miR-221/222 prevents the transmission of resistance (Wei et al., 2014). Besides miRNAs, the transfer of exosomal mRNAs that encode proteins that confer drug resistance may lead to chemoresistance in the recipient cell. GSTP1 exosomal mRNA from breast cancer cells resistant to Adriamycin, for instance, confer resistance to previously sensitive cells. Importantly, identification of GSTP1 in circulating exosomes from peripheral blood of patients was correlated with worst prognosis in breast cancer patients treated with Adriamycin (Yang et al., 2017).

EXOSOMES IN TUMOR-FIBROBLAST COMMUNICATION

An ideal metabolic and physiological environment for tumor growth requires a supportive stroma. Fibroblasts are the most abundant cells in the majority of solid tissues, participating in responses to environmental cues and constituting a frequent target of tumor-derived signals (Olumi et al., 1999; Orimo et al., 2005; Hu et al., 2015). Amongst these signals, exosomes produced by tumor cells have been described as important modulators of the activation status of fibroblasts and to play a major role in the setup of tumor microenvironments (**Table 1**). One of the factors involved in the activation of these cells, frequently named Cancer-Associated Fibroblasts (CAFs), is Transforming Growth Factor beta (TGF-β) (Tomasek et al., 2002), which can be carried to the extracellular milieu by exosomes and induce differentiation of CAFs (Webber et al., 2010, 2015). In addition, prostate cancer-derived exosomes containing miR-100, −21, and −139, were shown to induce RANKL and Metalloproteinases expression in CAFs, playing a potential role in prostate cancer progression and metastasis (Sanchez et al., 2016). Furthermore, under hypoxic conditions, prostate cancer cells release exosomes containing nearly three times more proteins than those in normoxic conditions, which induce activation of CAFs (Ramteke et al., 2015), and have been associated with the promotion of EMT, stemness, and angiogenesis by prostate cancer cells (Giannoni et al., 2010; Fiaschi et al., 2013). Tumor-derived exosomes were also described as regulators of metabolism in the tumor microenvironment, as breast cancer tumors could suppress glucose uptake by non-tumor cells, including lung fibroblasts, via secretion of exosomes containing miR-122, increasing glucose availability and facilitating metastasis (Fong et al., 2015).

The genetic profile of fibroblasts that take up exosomes also interferes with the effectiveness of the cell-to-cell communication mediated by exosomes. For example, fibroblasts deficient in BRCA1, a tumor suppressor gene with role in DNA repair, internalize bigger amounts of serum-derived exosomes when compared to wild type counterparts (Hamam et al., 2016). In addition, these cells were shown to suffer malignant transformation when exposed to sera-derived exosomes from oncologic patients. It suggests that oncosuppressor genes can prevent integration of exosome information (including those from tumor cells) and protect these cells from pro-tumorigenic messages (Abdouh et al., 2017).

It has also been shown that tumor-derived exosomal effects in fibroblasts is also influenced by the aggressiveness of carcinoma cells. For example, when internalized by stromal fibroblasts, mammary carcinoma-derived exosomes carrying the Neuroblast Differentiation-associated protein AHNAK induce phenotype shifting of CAF, which is frequently associated with cancer progression. Notably, the expression of AHNAK varies according to the aggressiveness of the tumor, being low in benign epithelial breast cells, intermediate in localized malignant cells and high in metastatic cells (Silva et al., 2016).

Tumor-Fibroblast communication mediated by exosomes is not limited to local tissues, as it has also been described at distant tumor-associated microenvironments, such as pre-metastatic niches. For example, once internalized by fibroblasts, breast, and colorectal cancers and leukemia cells-derived exosomes containing the transcript of the enzyme Telomerase hTERT mRNA contributed to the establishment of pre-metastatic niches by increasing fibroblast proliferation and lifespan (Gutkin et al., 2016). In the context of malignant-ascites, gastric and ovarian tumor-derived exosomes may participate in the transformation of normal mesothelial cells into CAF by Mesothelial-Mesenchymal transition. Also, the augmented expression of CAF markers, such as Fibroblast Activation Protein, α-SMA and Fibronectin, can induce TGF-β1 expression, increasing peritoneum fibrosis and preparing this site for metastasis (Wei et al., 2017). Similar fibrotic effects have been found in exosomal CD151 and Tetraspanin 8 (Tspan8), which are essential components in the crosstalk between cancer initiating cells and their respective tumor-associated cells by, for instance, contributing to ECM remodeling (Yue et al., 2015). In addition, exosomes derived from lung-tropic tumors, such as some types of breast cancers, express high levels of Integrins α6β1 and/or α6β4, which allow them to specifically bind to lung fibroblasts, induce upregulation of S100A4, A6, A10, A11, A13, and A16, and lead to the formation of lung-pre-metastatic niches supportive of metastasis (Hoshino et al., 2015).

The crosstalk between stromal and tumor cells is bi-directional, and hence CAF-derived exosomes may act in tumor cells as well as in other non-tumor cells of the tumor microenvironment (**Table 1**). In esophageal cancer, for instance, it was shown that CAF-derived exosomes containing several microRNA species, including miR-33a, miR-326, play a role in tumor progression, influencing tumor cell adhesion, endocytosis, and cell-cell junctions (Nouraee et al., 2016). Furthermore, pancreatic CAFs, cells intrinsically resistant to the chemotherapeutic agent Gemcitabine, secrete chemoresistance-inducing exosomes when grown in the presence of this drug. This process, mediated by exosomal miR-146a and Snail, has been shown to mediate transfer of resistance to chemosensitive L3.6

TABLE 1 | Exosomal-mediated phenotypes associate with cancer progression.

Process	Exosomal factor	Effect	References
TUMOR-DERIVED EXOSOMAL FACTORS			
Cancer progression and metastasis	TGF-β	Differentiation of fibroblast into CAF	Webber et al., 2010, 2015
	miR-100, −21, −139	ECM remodeling and modulation of cell migration	Sanchez et al., 2016
	TGF-β2, TNF 1α, IL-6, TSG101, Akt, ILK1, β-Catenin	Activation of prostate CAF by promotion of EMT, stemness and angiogenesis	Giannoni et al., 2010; Fiaschi et al., 2013; Ramteke et al., 2015
	Neuroblast Differenciation-Associated protein (AHNAK)	Differentiation of fibroblast into CAF	Silva et al., 2016
Metabolic Environment	miR-122	Suppression of glucose uptake	Fong et al., 2015
Establishment of Pre-metastatic niches	hTERT mRNA	Increased fibroblast proliferation and lifespan	Gutkin et al., 2016
	CD151, Tetraspanin 8	Fibrosis and ECM remodeling	Yue et al., 2015
	Integrin α6β1, α6β4	Lung-pre-metastatic niche formation mediated by S100A4, A6, A10, A11, A13, and A16	Hoshino et al., 2015
FIBROBLAST-DERIVED EXOSOMAL FACTORS			
Cancer progression and/or metastasis	EphA2	Induction of proliferation	Takasugi et al., 2017
	Wnt10b	Induction of breast cancer metastasis to liver	Chen et al., 2017
	miR-21, −278e, and −143	Activation of EMT in breast cancer	Donnarumma et al., 2017
	miR-33a, −326	Influence in tumor cell adhesion, endocytosis and cell-cell junction	Nouraee et al., 2016
Metabolic Switch	miRNA- 302d, −29b, −22, −155, −25, −29a, −23a, −21, −16, −222, −24, −199a, −125b, −144	Down-regulation of genes related to OXPHOS by CAF exosomes	Zhao et al., 2016
	Lactate, Acetate, Citrate, Pyruvate, α-Ketoglutarate, Fumarate, Malate, aminoacids such as Gln, Arg, Glu, Pro, Ala, Thr, Ser, Asn, Val, Leu, Phe, Ile, Gly, or lipids such as Stearate and Palmitate	Modulation of glycolysis, tricarboxylic acid cycle, lipid and protein synthesis by CAF exosomes	Zhao et al., 2016
Chemoresistance	miR-146a, Snail	Induction of CAF chemoresistance	Richards et al., 2017

and chemoresistant PANC1 and AsPC1 pancreatic cancer cells (Richards et al., 2017). Moreover, it was shown that inhibition of exosomes secretion by CAF prevented chemoresistance in cases of colorectal carcinoma (Hu et al., 2015). The growth of cancer cells can also be influenced by the altered secretome of senescent cells. In fact, exosomes from senescent fibroblasts induce proliferation of MCF-7 human breast cancer cells, in a mechanism mediated by EphA2 (Takasugi et al., 2017). CAF-derived exosomes are also capable to support tumor growth by providing nutrients to malignant cells. In fact, by modulating mitochondrial oxidative phosphorylation and glycolysis, CAF-derived exosomes can contain complete metabolites that may be used under nutrient deprivation stress conditions by tumor cells (Zhao et al., 2016).

Another important mediator in CAF differentiation and tumor biology is P85-α, as the downregulation of this factor promotes not only cancer progression via EMT by the Wnt10b paracrine pathway, but also metastatic progression. In addition, P85-α downregulation in fibroblasts is associated to Wnt10b upregulation in fibroblast-derived exosomes, which in turn induce breast cancer metastatic progression to liver (Chen et al., 2017). EMT is also modulated by CAF-derived exosomes containing miRs-21, −278e, and−143, influencing breast cancer cell phenotype and aggressiveness (Donnarumma et al., 2017). Taking advantage of this communication route involving modulation of tumor biology by fibroblast-derived exosomes, Kamerkar et al. showed that normal fibroblast-like mesenchymal cells can be engineered to carry interfering RNA or Short Hairpin RNA. Specifically, it was shown that engineered cells can produce exosomes capable to preferentially bind to tumor cells, target oncogenic KRAS and suppress cancer in multiple mouse models of pancreatic cancer (Kamerkar et al., 2017).

EXOSOMES IN TUMOR-ENDOTHELIAL CELLS COMMUNICATION

Endothelial cells (ECs) are key components of the tumor microenvironment not only by providing a conduit to nutrients, but also by representing a source of trophic factors. In this

context, exosomes also play an instrumental role in tumor-EC communication (**Table 2**). For instance, the process of neovascularization was shown to be modulated by Myeloid leukemia-derived exosomes enriched in Vascular Cell Adhesion Molecule (VCAM)-1 and Intercellular Adhesion Molecule (ICAM)-1 (Taverna et al., 2012). In addition, increased vascularization has been associated with packaging of miR-92a in leukemia-derived exosomes (Umezu et al., 2013) and of CO-029/D6.1A Tetraspanin in pancreatic cancer-derived exosomes (Gesierich et al., 2006). It was also demonstrated that upregulation of Heparanase in tumor cells, including myeloma and breast cancers, is associated with increased exosomes production and exosomal packaging of Syndecan-1, Vascular Endothelial Growth Factor (VEGF) and Hepatocyte Growth Factor, which lead to increased endothelial invasion through the ECM (Thompson et al., 2013). Additionally, skin cancer-derived exosomes can promote angiogenesis by delivering Epidermal Growth Factor receptor (EGFR) (Al-Nedawi et al., 2009) and miR-9 to ECs (Gajos-Michniewicz et al., 2014). Furthermore, melanoma-derived exosomes were shown to condition sentinel lymph nodes prior to the installation of melanoma cells and further metastasis through upregulation of Collagen 18 and Laminin 5, and production of angiogenic growth factors (Hood et al., 2011).

Tumor-derived exosomes are also known to influence the integrity of vascular barriers, which is frequently associated with metastatic dissemination. Melanoma-derived exosomes, for instance, induce pulmonary vascular leakiness (Peinado et al., 2012) and upregulate genes related to tumor cell recruitment, such as Stabilin 1, Vitronectin, Integrins, and Ephrin receptor β4, in lymph nodes (Hood et al., 2011), creating pre-metastatic niches supportive of metastasis. In addition, breast cancer-derived exosomes enriched in miR-105 targets ECs tight junctions by modifying the expression of Claudin 5, Zonula Occludens protein 1, and Occludin, promoting metastasis by destroying vascular endothelial barriers (Zhou M. et al., 2014). Another example involves brain tumor-derived exosomes containing miR-181c, which modulates ECs actin dynamics and promote breakdown of the blood-brain barrier by 3-Phosphoinostide-dependent Protein Kinase-1 degradation (Tominaga et al., 2015). Similarly, exosomes produced by glioblastoma cells containing high levels of VEGF-A induce ECs permeability and angiogenesis *in vitro* (Treps et al., 2017).

Hypoxia is another important factor in modulating tumor-EC communication. For example, hypoxic glioblastoma cells release exosomes that interact with ECs, stimulating proliferation and angiogenesis *in vitro* and *in vivo* (Skog et al., 2008; Kucharzewska et al., 2013), and also triggering Tissue Factor/Factor VIIa-dependent activation of hypoxic ECs (Milia et al., 2002; Svensson et al., 2011). Moreover, hypoxic colorectal cancer cells secrete Wnt4-enriched exosomes that promote Beta-Catenin nuclear translocation and proliferation of ECs (Huang and Feng, 2017), while hypoxic breast cancer and hepatocellular carcinoma cells were shown to release pro-angiogenic exosomes enriched in miR-210 (King et al., 2012) and miR-23a (Sruthi et al., 2017), respectively. In addition, exosomes produced by the human squamous carcinoma cell lineage A431 under hypoxic or reoxygenation conditions were shown to modulate the tumor microenvironment by facilitating angiogenesis, and as consequence, metastasis (Park et al., 2010).

EXOSOMES IN TUMOR-LEUKOCYTES COMMUNICATION

One of the hallmarks of cancer is the ability of tumor cells to employ different strategies to evade the host immune surveillance (Hanahan and Weinberg, 2011). Contemporary evidence points toward a central role of tumor-derived exosomes in modulating the immune response and influencing cancer development by mediating the dialogue between immune and cancer cells (Czernek and Düchler, 2016). In fact, tumor-derived exosomes have been shown to hijack the immune surveillance program of the host by amplifying tumor-derived signals, including those involved in inflammation, and in certain cases, tumorigenesis (Grivennikov et al., 2010; Cavallo et al., 2011) and escape of

TABLE 2 | Effect of tumor-derived exosomes in endothelial cell (EC) biology.

Process	Exosomal factor	Effect	References
Angiogenesis	miR-92a and CO-029/D6.1A Tetraspanin	EC tube formation	Gesierich et al., 2006; Umezu et al., 2013
	Syndecan-1, Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor	EC invasion	Thompson et al., 2013
	Epidermal Growth Factor Receptor (EGFR) and miR-9	Angiogenesis	Al-Nedawi et al., 2009; Gajos-Michniewicz et al., 2014
	MMP9, Pentraxin 3, IL-8, PDGF-AB/AA, CD26, Plasminogen Activator Inhibitor 1, Caveolin 1, Tissue Factor, Factor VIIa, Wnt4, miR-210, miR-23a	ECs proliferation and angiogenesis	Svensson et al., 2011; King et al., 2012; Kucharzewska et al., 2013; Huang and Feng, 2017; Sruthi et al., 2017
Integrity of vascular barriers	miR-105	Destruction of endothelial barriers and promotion of metastasis	Zhou W. et al., 2014
	miR-181c	Breakdown of blood-brain barrier	Tominaga et al., 2015
	VEGF-A	Increase in ECs permeability and angiogenesis	Treps et al., 2017

tumor cells from the immune system (Wieckowski et al., 2009; Whiteside, 2013).

It is also known that tumor-derived exosomes are not only key players in the immune editing of the primary tumor niche, but also in the pre-metastatic and metastatic niches, as they can outsmart stromal and immune players into overcoming the immune response (Hanahan and Weinberg, 2011), fostering the setup of pro-metastatic microenvironments (Syn et al., 2016). In this section, we will dissect how exosomes can mediate cell-cell communication between the cellular components of the immune system and tumor cells and play a role in tumor biology (**Figure 2**).

Exosomes Roles in Macrophages and Neutrophils Polarization

All types of immune cells are potentially sensitive to tumor-derived exosomes immunomodulation effects. However, exosomes can induce different levels of modification in each of these cells, as it is the case with macrophages. Activated macrophages, for instance, display a remarkable phenotypic plasticity according to environmental cues, being usually divided into a continuum of M1 and M2 functionally polarized states. In general, based on Th1/Th2 polarization, M1 macrophages are pro-inflammatory and tumoricidal (classically activated), while M2 are anti-inflammatory and tumor supportive (alternatively activated) (Martinez et al., 2008; Gautier et al., 2012; Xue et al., 2014; You et al., 2015). In a tumor microenvironment, macrophages can be educated into Tumor-Associated Macrophages (TAMs) displaying M2 characteristics, promoting angiogenesis and releasing pro-tumorigenic growth factors, chemokines and cytokines (Mantovani et al., 2004; Rogers and Holen, 2011; Quatromoni and Eruslanov, 2012; Schiavoni et al., 2013). In this setting, tumor-derived exosomes have been shown to play a key role in the polarization status of macrophages. For instance, colorectal cancer-derived exosomes induce pro-tumorigenic macrophage phenotypes by using cytoskeleton centric proteins as functional units. In fact, cytoskeleton rearrangement is a primordial characteristic of macrophage activation and maturation (Chen Z. et al., 2016). Another example involves epithelial ovarian cancer, where exosomes containing miR222-3p induced a shift in the activation status of macrophages into a M2 polarization, in a process mediated by down-regulation of the SOCS3/STAT3 pathway (Ying et al., 2016). Furthermore, miR-25-3p- and miR-92a-3p-rich exosomes from human liposarcoma cell lines were observed to induce IL-6 secretion by TAMs, leading to

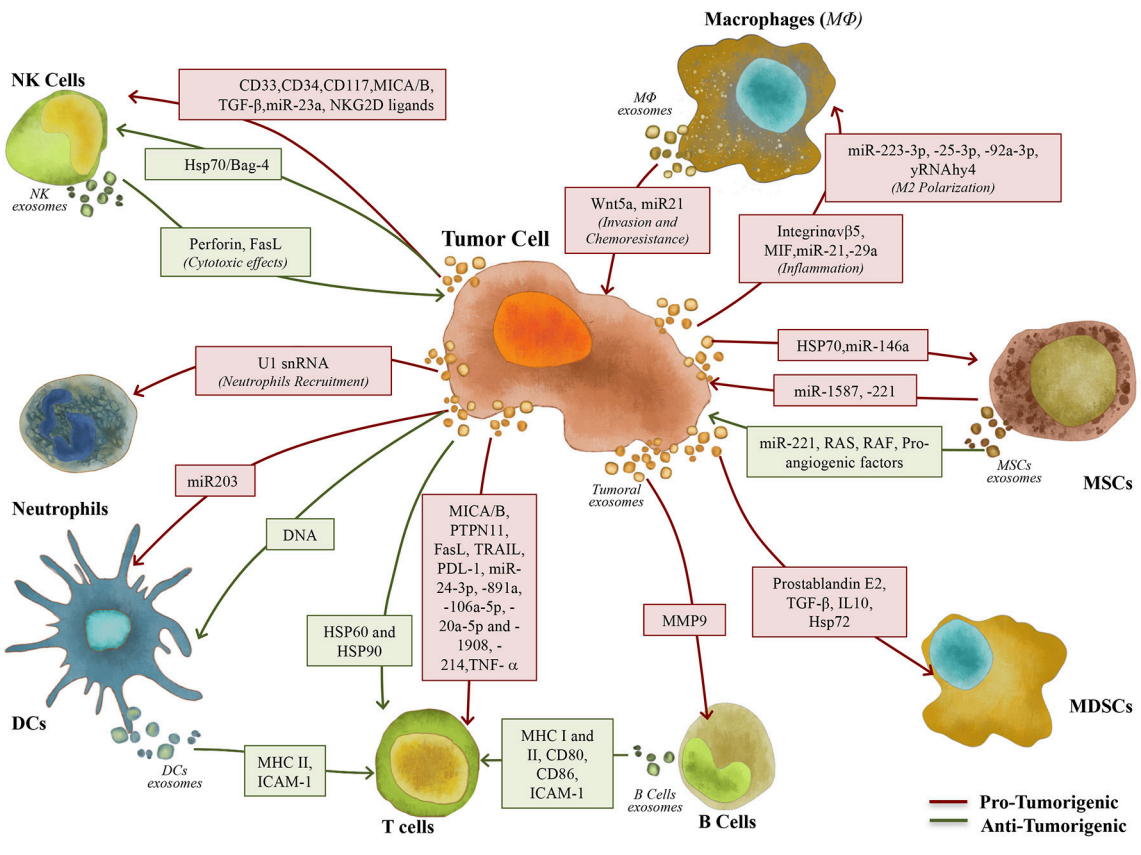

FIGURE 2 | Exosomes role in the communication between tumor and immune/stem cells. Exosomes play a key role in the interaction between tumor and immune/stem cells, including Macrophages (MΦ), Natural Killer (NK) cells, Neutrophils, Dendritic Cells (DCs), B and T cells, Myeloid-Derived Suppressor Cells (MDSCs) and Mesenchymal Stem Cells (MSCs), contributing to the setup of pro- (red arrows) and anti-tumorigenic (green arrows) responses.

an increase in liposarcoma cellular proliferation (Casadei et al., 2017). In chronic lymphocytic leukemia (CLL), tumor-derived exosomes containing noncoding Y RNA hY4 were shown to induce CLL-associated phenotypes in recipient monocytes, including the release of cytokines, such as C-C motif chemokine ligand 2 (CCL2), CCL4, and IL-6, and the expression of PD-L1, suggesting a potential exosome-based mechanism of immune escape (Haderk et al., 2017). In addition to modulate macrophage polarization, tumor-derived exosomes were shown to influence macrophages migration. For example, Bandari et al. showed in a recent report that when exposed to commonly utilized anti-tumor agents, such as Bortezomib, Carfilzomib, or Melphalan, myeloma cells produce Heparanase-rich exosomes that induce migration and TNF-α secretion by macrophages (Bandari et al., 2018).

Tumor-derived exosomes are frequently related to NF-κB activation in macrophages and promotion of pro-tumorigenic microenvironments. For instance, gastric cancer-derived exosomes were shown to induce NF-κB activation in macrophages, leading to an increase in the expression of pro-inflammatory factors such as IL-6 and TNF-α, in turn promoting the proliferation of gastric cancer cells (Wu et al., 2016). Similar observations were obtained by Chow et al., who showed that breast cancer-derived exosomes also stimulate the NF-κB pathway in macrophages, leading to the secretion of the pro-inflammatory cytokines IL-6, TNF-α, GCSF, and CCL2 (Chow et al., 2014). Another example involves lung tumor-derived exosomes containing miR-21 and miR-29a. These exosomes were shown to bind to Toll-like receptor (TLR)7 and TLR8, leading to NF-κB activation and secretion of the pro-metastatic inflammatory cytokines TNF-α and IL-6. In turn, these cytokines were shown to induce the formation of a pulmonary microenvironment supportive of lung metastatic burden (Fabbri et al., 2012).

TAMs are also known to induce inflammatory responses, playing a key role in the setup of tumor microenvironments supportive of metastasis formation and progression. For example, once taken up by hepatic resident macrophages, in a mechanism mediated by exosomal Integrin αVβ5 (Hoshino et al., 2015), pancreatic cancer-derived exosomes containing high levels of Macrophage Migration Inhibitory Factor (MIF) (Costa-Silva et al., 2015) were shown to induce upregulation of secreted factors associated with liver fibrosis, such as TGFβ (Costa-Silva et al., 2015), and pro-inflammatory genes involved with metastasis, such as S100A8 and S100P (Lukanidin and Sleeman, 2012; Hoshino et al., 2015). In response to this inflammatory microenvironment, hepatic stellate cells shift into an activated state marked by upregulation of Fibronectin expression. This Fibronectin-rich microenvironment then promotes the accumulation of bone marrow-derived macrophages and the formation of a pre-metastatic microenvironment supportive of liver metastasis (Costa-Silva et al., 2015).

In addition to mediate immune responses, macrophages can also modulate tumor-derived exosomes biodistribution. Subcapsular sinus $CD169^+$ macrophages, for example, form a physical layer that block tumor-derived exosomes' dissemination in spleen and lymph nodes (Saunderson et al., 2014). In agreement with this, high density of $CD169^+$ macrophages in lymph nodes positively correlates with longer overall survival in patients with colorectal carcinoma (Ohnishi et al., 2013). Pucci et al., however, showed that this macrophage barrier can be disrupted by melanoma-derived exosomes, permitting the entrance of these exosomes in the lymph node cortex, where they interact with B cells and foster tumor progression by inducing autoantibodies production (Pucci et al., 2016).

Tumor-derived exosomes are not the only players in tumor-macrophage communication, as macrophages-derived exosomes can also exert effects in tumor cells biology. For instance, macrophages-derived exosomes can induce tumor invasion by transferring Wnt5a from macrophages to cancer cells, leading to the activation of the β-Catenin-independent Wnt signaling pathway, in a process that defines a new strategy of malignant invasion by breast cancer (Menck et al., 2013). Moreover, TAM-derived exosomes were shown to modulate chemoresistance. In fact, Zheng et al. showed that exosomes produced by TAM containing miR-21 could confer Cisplatin resistance to gastric cancer cells, in a process mediated by down-regulation of PTEN (Zheng et al., 2017). Wu and colleagues highlighted the relevant role of exosomes in the tumor microenvironment, unraveling an additional layer of complexity in the tumor-host communication network. In this work, it was shown that TAM-derived exosomes could suppress migration of endothelial cells by targeting the miR-146b-5p/ TRAF6/NF-kB/MMP2 pathway. Interestingly, ovarian cancer-derived exosomes were able to revert this inhibitory effect by transferring lncRNAs (Wu et al., 2017).

Neutrophils are also known as key mediators of innate immune response, as they are essential to protect the host against infection and to support tissue repair (Mayadas et al., 2014). Similar to macrophages, neutrophils also display phenotypic plasticity, which is influenced by different tumor-derived signals and that can exert pro- or anti-tumor effects. Indeed, tumors can manipulate neutrophils early in their differentiation process, creating a diverse repertoire of functional polarization states (Fridlender et al., 2009; Coffelt et al., 2016). In this setting, exosomes have been shown as emerging mediators of tumor-neutrophil interactions. For instance, breast cancer-derived exosomes can promote tumor growth by inducing bone marrow-derived neutrophils recruitment to tumor sites (Bobrie et al., 2012). Furthermore, neutrophils have emerged as crucial mediators in the pre-metastatic niche development (Wculek and Malanchi, 2015), as breast cancer-derived exosomal RNAs can activate host stromal TLR3, inducing neutrophil recruitment, which is critical for the setup of pre-metastatic niches (Kenific et al., 2016; Liu et al., 2016).

Exosomes' Role in Suppression of Natural Killers' Cytolytic Response

Natural killer (NK) cells are another important component of the immune system, as these innate lymphoid cells are able to assemble a rapid cytotoxic activity ("ready to kill"), allowing them to control microbial infections and tumor progression in a process regulated by a balance of activating and inhibitory

signals (Morvan and Lanier, 2015). Different reports state that the cytotoxicity of NK cells is significantly impaired in oncologic settings, in part due to the immune suppressive effect of tumor-derived exosomes. This immune suppressive effect has been associated with altered expression of NK cell activating surface receptors. For instance, exosomes derived from plasma of acute myelogenous leukemia (AML) patients, containing CD33, CD34, CD117, MICA/MICB, and TGF-β, were shown to decrease cytotoxic activity of NK cells, in a process involving Smad phosphorylation and down-regulation of NKG2D receptor in NK cells (Szczepanski et al., 2011; Whiteside, 2013), which was reversible by IL-15 (Szczepanski et al., 2011). Another example involves the work of Berchem et al., who showed that tumor-derived exosomes produced by hypoxic cells are qualitatively different from normoxic counterparts. Using various tumor models, they revealed that hypoxic tumor-derived exosomes were able to negatively regulate NK cell functions, in a mechanism also mediated by transfer of TGF-β1 and decrease of NKG2D levels in NK cells. The authors also described miR-23a and TGF-β as an immunosuppressive factor transferred to NK cells that directly targets CD107a expression, leading to a decrease of anti-tumor responses (Berchem et al., 2016). In fact, in the presence of tumor-derived exosomes, NKG2D is one of the most profoundly affected NK receptors, which have MHC class I-related chain A (MICA) and B (MICB) as crucial ligands for its induction (Mincheva-Nilsson and Baranov, 2014). Accordingly, Groh et al. showed that cancer patients exhibit a decrease of NKG2D surface expression on circulating NK cells in comparison with healthy individuals (Groh et al., 2002). Furthermore, Ashiru et al. demonstrated that the shedding of the most frequently expressed MICA allele in human populations, MICA*008, in tumor-derived exosomes induce down-regulation of NKG2D in NK's cell surface, leading to an impaired cytotoxic function (Ashiru et al., 2010). Moreover, a recent study showed that prostate tumor-derived exosomes with NKG2D ligands selectively induce a dose-dependent downregulation of cell surface NKG2D in both NK cells and CD8+ T cells, leading to impaired cytotoxic function of both immune cell types (Lundholm et al., 2014). Tumor-derived exomes bearing NKG2D ligands (such as MIC-A/B and ULBP 1 and 2) were also shown to act as decoys, impairing NKG2D-mediated NK-cell cytotoxicity and facilitating the immune evasion of leukemia/lymphoma cells (Hedlund et al., 2011).

An additional mechanism of immune evasion was described by Liu et al., who showed that murine mammary-derived exosomes were able to inhibit IL-2-stimulated NK cells proliferation and block IL-2-mediated activation of NK cells, thus abolishing their cytotoxic response to tumor cells (Liu et al., 2006). In addition, tumor-derived exosomes containing death receptor ligands, such as FasL, on their membrane were also found to induce the apoptosis of NK cells, similarly to what happens with T cells (Andreola et al., 2002; Saito et al., 2013). On the other hand, anticancer drugs have been described to induce chemoresistant hepatocellular carcinoma cells to release exosomes that elicit anti-tumor NK cell responses, in a mechanism mediated by exosomal Heat Shock Proteins (Lv et al., 2012). Similar to other tumor-immune cell-cell communication settings, NK cells have also been shown to influence tumor cells biology though production of exosomes. It was illustrated in a recent work of Zhu et al., showing that NK cells-derived exosomes can induce cytotoxic effects in melanoma cells *in vitro*, representing a potentially novel antitumor strategy (Zhu et al., 2017).

Exosomes Role in Impairment of Cytotoxic Lymphocytes (CTL) Response and Induction of Immune Tolerance/Immune Regulator Cells

The centerpiece of antitumor immunity is the effective response of $CD4^+$ and $CD8^+$T cells. In this setting, tumor-derived exosomes have been also described as potent mediators of proliferation, activation, and apoptosis of these cells, enabling tumor evasion from immune surveillance. For instance, T cell death has been shown to be induced by tumor-derived exosomes through both extrinsic and intrinsic apoptotic pathways. In fact, induction of T cells apoptosis by tumor-derived exosomes occurs through receptor-mediated pathways involving Fas Ligand (FasL), TRAIL, and PDL-1 (Abusamra et al., 2005; Kim et al., 2005; Wieckowski et al., 2009). In addition, Abusamra et al. demonstrated that prostate cancer-derived exosomes expressing FasL trigger T-cell apoptosis in a dose-dependent fashion (Abusamra et al., 2005). In another example, head and neck squamous cell carcinoma-derived exosomes were shown to induce activated $CD8^+$T cells apoptosis through Caspase 3 cleavage, mitochondrial Cytochrome C release, loss of mitochondrial membrane potential, DNA fragmentation and early membrane changes, including Annexin V binding (Czystowska et al., 2011). The PI3K/AKT pathway is also targeted by exosomes in activated $CD8^+$ T cells, as tumor-derived exosomes were shown to cause Akt dephosphorylation in a time-dependent manner, leading to downregulation of the anti-apoptotic proteins Bcl-2, Bcl-xL, and Mcl-1 and upregulation of the pro-apoptotic protein Bax (Czystowska et al., 2009, 2011). Furthermore, the production of tumor-derived exosomes containing MICA/B and FasL is associated with impairment of the effectives of both innate and adaptive immunity (Abusamra et al., 2005; Lundholm et al., 2014). Therefore, it has been suggested that such exosomes are able to modulate lymphocyte functions by mimicking "activation-induced cell death" (AICD) (Blanchard et al., 2002; Perone et al., 2006; Taylor and Gercel-taylor, 2011). Another example involves melanoma-derived exosomes, which were shown to suppress $CD8^+$T cell proliferation and viability by delivering PTPN11 (protein and mRNA) (Wu et al., 2017). In addition, human nasopharyngeal carcinoma-derived exosomes can inhibit T cell proliferation and differentiation into Th1 and Th17 cells, promoting regulatory T cell (Treg) generation, decreasing ERK, STAT1, and STAT3 phosphorylation and increasing STAT5 phosphorylation in the recipient T cells. These tumor-derived exosomes also increased the secretion of the pro-inflammatory cytokines IL-1β, IL-6, and IL-10, and decreased the release of IFNγ, IL-2, and IL-17, both in $CD4^+$ and $CD8^+$T cells. The content of exosomes from patient sera and nasopharyngeal carcinoma cell lines

was further explored, showing to be enriched in miR-24-3p, −891a, −106a-5p, −20a-5p, and−1908. The same work showed that these miRNA clusters downregulate the MARK1 signaling pathway, affecting T cells proliferation and differentiation (Ye et al., 2014).

Unresponsiveness of $CD8^+$ T cells can also be achieved by damaging the TCR (T-cell receptor) signaling pathway, which mediate TCR–MHC–peptide interactions and T cell activation, in a mechanism involving CD3ζ chain transfer of activating signals to the nucleus. Soderberg et al. demonstrated that melanoma-derived exosomes transfer TNF-α to $CD4^+$ and $CD8^+$T cells, affecting the TCR–CD3 complex, and causing T cell disruption (Soderberg et al., 2007). In agreement, several studies also reported that tumor-derived exosomes from cancer patients mediate inhibition of CD3ζ chain expression in T cells, impairing T cell activation (Taylor et al., 2003; Kim et al., 2005; Taylor and Gercel-taylor, 2011).

Another critical step in the anti-tumor immune response is the $CD4^+$ and $CD8^+$T cells homing to the primary tumor site (Taylor et al., 2003; Kim et al., 2005; Taylor and Gercel-taylor, 2011). Lee et al. showed that by shedding ICAM1 in the membrane of their exosomes, cancer cells prevent the interaction between lymphocytes and endothelial cells, thus decreasing the recruitment of adaptive immune cells (Lee et al., 2010).

Besides impairment of CTL responses, another mechanism present in tumor cells which promote escape from immune surveillance involves interference in the differentiation process of naïve immune cells toward an immunosuppressive phenotype. For example, tumor-derived exosomes are capable of inducing expansion of $CD4^+CD25^+Foxp3^+$ Treg cells and their suppressor activity, and to trigger $CD8^+$T cells apoptosis (Wieckowski et al., 2009). Additionally, exosomes produced by various types of human and murine tumors were reported to transfer miR214 to peripheral $CD4^+$ T cells, stimulating immune evasion by downregulating PTEN and promoting Treg expansion (Yin et al., 2014).

In a recent study, Muller et al. showed that tumor-derived exosomes can also induce differential regulation of key immune function-related genes in conventional $CD4^+$ T, $CD8^+$ T, and Treg. In $CD4^+$ T cells, for instance, inhibitory genes were upregulated by tumor-derived exosomes, which led into a loss of CD69 on their surface and a functional decline of these cells. On the other hand, exosomes induced CD39 expression and adenosine production in Treg, while downregulating mRNA expression levels of the genes involved in the control of immmunosuppressive pathways (Muller et al., 2016). Collectively, by exploiting diverse mechanisms acting in concert, tumor-derived exosomes contribute to an immunosuppressive environment and peripheral tolerance, which play pivotal role in tumor growth and progression.

Exosomes Role in DCs Differentiation and Maturation

Dendritic cells (DCs) are versatile meditators of the immune system, forming a remarkable network that shapes innate and adaptive immunity, according to peripheral signals (Merad et al., 2013). The DCs importance is associated with their function as professional antigen-presenting cells (APCs), since they are able to perform antigen presentation and initiation of primary T cell response, including those frequently directed against tumor cells (Liu et al., 2017).

The presence of tumor-derived exosomes during the generation of DCs was associated with low expression of co-stimulatory molecules and production of inhibitory cytokines by these cells, in a process followed by suppression of T cells proliferation and anti-tumor cytotoxicity (Valenti et al., 2006). In addition, tumor-derived exosomes taken up by immature DCs were shown to inhibit the maturation process of these cells (Yang et al., 2011).

The antigen recognition capacity of DCs can also be modulated by tumor-derived exosomes, which affect the expression of pattern recognition receptors (PRRs) in DCs. Pancreatic cancer-derived exosomes, for instance, can downregulate the expression of TLR4 (via miR-203) in DCs, inducing the production of TNF-α and IL-12 and inhibiting DCs-mediated antitumor responses triggered by TLR4 (Zhou M. et al., 2014). In summary, increasing number of reports have been showing that tumor-derived exosomes can also mediate host immunosuppression by modulating the differentiation, maturation and function of DCs.

Exosomes Role in B Cells

B cells are important players in the tumor-induced modulation of immune response, being the second most abundant tumor-infiltrating lymphocytes (Yuen et al., 2016). In 1996, Raposo et al. reported for the first time that B-lymphocytes secrete exosomes, and that these particles contain MHC class II able to perform antigen presentation and to induce antigen-specific CD4+ T cell responses (Raposo et al., 1996). In addition to the several reports of exosomes production by B cells, and of the potential roles of these particles in the immune system (Escola et al., 1998; Clayton et al., 2005; Rialland et al., 2006; Buschow et al., 2010), these cells were also reported to be influenced by tumor-derived exosomes. In fact, esophageal cancer-derived exosomes containing HSP90 can influence naïve B cells to develop into an immunosupressive regulatory phenotype expressing TGF-β (Li Y. et al., 2015). Furthermore, melanoma and lymphoma cells were shown to release exosomes that induce IL-10 production in splenic B cells. In turn, IL-10 promote the generation of regulatory B cells which inhibit T cell activity (Yang et al., 2012), suggesting a novel mechanism of induction of inhibitory B cells based on exosome-driven pathways.

Exosomes Involvement in the Stimulation of Immunity against Cancer

Exosomes produced by cancer cells are known to predominantly induce immune suppression and to support tumorigenesis. However, several reports show that these nanoparticles are also capable of stimulating immunity (Reiners et al., 2014; Czernek and Düchler, 2016; Liu et al., 2017; Yoshimura et al., 2017). This dual role of tumor-derived exosomes is mainly due to their ability to express tumor-associated antigens. In addition, exosomes are considered ideal sources of antigens for APCs

education, especially due to its easy collection from the peripheral circulation by the use non-invasive methods. In fact, early reports showed that antigens in tumor-derived exosomes can be transferred to DCs and induce specific anti-tumor immune responses (Andre et al., 2002; Robbins and Morelli, 2014). For instance, DCs pulsed with hepatocellular carcinoma cell (HCC)-derived exosomes led to an increase in IFNγ levels and CD8+ T cells, and in the reduction of IL10 and TGF-β levels in HCC-bearing mice (Rao et al., 2016). Another example involves cancer cells treated with anti-tumor agents, which can release DNA-containing exosomes capable of activating DCs though the STING-dependent pathway and reinforce antitumor immunity (Kitai et al., 2017).

Additionally, tumor-derived exosomes can enhance anti-tumor immunity through transfer of cytokines or heat shock proteins (Liu et al., 2017). In fact, HSP60 and HSP90 are abundant in exosomes derived from heat-shocked mouse B lymphoma cells, being these exosomes associated with increased antitumor immune responses by T cells (Chen et al., 2006). In line with these findings, tumor-derived HSP70/Bag-4+ exosomes stimulate NK cell activity, inducing granzyme B release and pancreatic cancer cell apoptosis (Gastpar et al., 2005). Based on these anti-tumor effects generated by tumor-derived exosomes, several lines of research have been focusing in developing exosome-based tumor vaccines. Common strategies involve genetic modification of exosomes-producing cells in order to modify exosomes content, including IL2 (Yang et al., 2007) and IL18 (Dai et al., 2008) and improve exosome-driven immunogenicity. In addition, other strategies are based on stimulating the release of exosomes that act on NK cell toxicity by stressing tumor cells with, for instance, anti-cancer drugs (Lv et al., 2012).

The ability to induce effective immunity is not limited to tumor-derived exosomes, as immune cells can also release exosomes able to act on the immune system and elicit antitumor responses. DC-derived exosomes, for instance, can act as antigen-presenting particles (Zitvogel et al., 1998) and stimulate antigen-specific cytotoxic T lymphocytes *in vivo* (Raposo et al., 1996; Zitvogel et al., 1998). In addition, DC-derived exosomes containing ICAM-1 and B cells-derived exosomes containing MHC Class I and II molecules, co-stimulatory molecules (CD80 and CD86) and ICAM-1 were shown to promote antigen-presenting function in recipient immune cells (Clayton et al., 2001; Théry et al., 2001; André et al., 2004; Segura et al., 2005). These exosomes can modulate immune responses either directly, by exposing these molecules, or indirectly, by conveying internal components to surrounding cells. Taken the effects mentioned above, DC-derived exosomes represent an important strategy to suppress tumor growth through novel cell-free vaccination approaches (Tian and Li, 2017).

To date, DC-based cellular immunotherapy has exhibited several limitations, such as fluctuations in the molecular composition of DCs, challenges in defining its composition, low levels of membrane expression of peptide-MHC-II complexes, presence of immunosuppressive cytokines able to convert DCs into tolerogenic state, hindrances to store live DCs and problems regarding stability management over longer periods (Palucka and Banchereau, 2012; Pitt et al., 2016). When compared to DC-based cellular immunotherapy protocols, DC exosomes-based strategies present significant advantages, as exosomes have better defined molecular composition for each patient, higher levels of peptide–MHC-II complexes and higher stability for storage due to their lipid composition (Pitt et al., 2016; Yoshimura et al., 2017). Although further studies are still necessary, important progress has been made in clinical applications of DC exosomes-based vaccines. Indeed, patients with advanced malignancies, including those with non-small cell lung cancer (Morse et al., 2005; Besse et al., 2016), metastatic melanoma (Escudier et al., 2005) and colorectal cancer (Dai et al., 2008), that were vaccinated with DC-derived exosomes displayed activation of T and NK cell-based immune responses. In addition, two phase I (Escudier et al., 2005; Morse et al., 2005) and one phase II clinical trial (Besse et al., 2016) using DC-derived exosomes have now been completed, showing the feasibility and safety of this approach.

EXOSOMES IN TUMOR-STEM/PROGENITOR/NON-TRANSFORMED CELL COMMUNICATION

Besides the well-known effects in differentiated cells, tumor-derived exosomes can also induce pro-tumorigenic microenvironments by mediating tumor-stem/progenitor cell communication. Melanoma-derived exosomes, for instance, "educate" bone marrow-derived cells via the horizontal transference of the oncoprotein MET, which leads to mobilization of vasculogenic and hematopoietic bone marrow progenitor cells to ensure vascular proliferation and immunosuppression at the pre-metastatic niches (Peinado et al., 2012). Tumor-stem/progenitor cell communication was also described in scenarios of bone metastasis, as exosomes from the bone-metastatic prostate cancer PC3 cells were shown to modulate both osteoclastogenesis and osteoblast proliferation, influencing the process of bone metastasis. In turn, osteoblast-derived exosomes were shown to promote PC3 prostate cancer cell proliferation (Morhayim et al., 2015).

In addition, tumor-derived exosomes were shown to manipulate the process of myeloid precursor cells differentiation toward myeloid-derived suppressor cells (MDSCs), which are known to contribute to tumor progression by allowing immune evasion (Nagaraj and Gabrilovich, 2007). Exosomes derived from breast carcinomas, for instance, were shown to be taken up by bone marrow cells and to switch the differentiation pathways of these cells toward MDSCs via Prostaglandin E2 and TGF-β, promoting accumulation of COX2, IL6, VEGF and Arginase1 by MDSCs (Xiang et al., 2009). Similarly, glioma stem cell-derived exosomes were shown to act on MDSCs via IL10 and Arginase 1 upregulation and HLA-DR downregulation in CD14+ monocytic MDSCs, leading to inhibition of T cell activation (via CD25 and CD69) and decrease of Th1 cytokine production (Domenis et al., 2017). In agreement with these findings, tumor-derived exosomes prevented the process of monocytes maturation by switching differentiation toward altered $CD14^{+}$

monocytes expressing low or absent levels of HLA-DR, which was shown to suppress T cell proliferation and cytolytic functions (Valenti et al., 2006; Temme et al., 2010). Additionally, Chalmin et al. showed that HSP72+ exosomes derived from several tumor models can stimulate the suppressive function of MDSCs via Stat3 activation, leading to IL-6 production in a MyD88/TLR2-dependant mechanism (Chalmin et al., 2010). This finding was reinforced by the involvement of MyD88, a molecule involved in the propagation of signals generated by the TLR family and production of IL-6, TNF-α, and CCL2, in the recruitment and activity of MDSCs by tumor-derived exosomes (Liu et al., 2010). In another example, Wang and colleagues described that multiple myeloma-derived exosomes can create a pro-tumorigenic microenvironment in the bone marrow by inducing MDSCs expansion and activity, endothelial cell growth via the STAT3/p53 pathway and immunosuppression via upregulation of Nitric Oxide Synthase (Wang et al., 2016). Finally, melanoma-derived exosomes can prevent bone marrow myeloid precursors differentiation into DCs via induction of IL-6, favoring the MDSC phenotype (Valenti et al., 2006).

Cancer-derived exosomes can also elicit changes in mesenchymal stem cells (MSCs), which contribute to promote and sustain pro-tumorigenic inflammatory niches. For instance, $HSP70^{+}$ lung tumor-derived exosomes induce activation of NF-kB and secretion of IL-6, IL-8, and MCP1 by MSCs, in a TLR2-mediated signaling, leading MSCs into a more inflammatory and tumor supportive phenotype (Li et al., 2016). In a recent report, De Veirman et al. showed that myeloma-derived exosomes can transfer miR-146a to mesenchymal cells, leading to the secretion of several cytokines and chemokines by these recipient cells, including CXCL1, IL6, IL-8, IP-10, MCP-1, and CCL-5, and to the promotion of myeloma cells migration and survival (De Veirman et al., 2016). Another example involves exosomes produced by KMBC cholangiocarcinoma cells that induce IL-6 upregulation in MSCs, which in turn promotes the proliferation of KMBC cells (Haga et al., 2015). In addition, ovarian and breast cancer-derived exosomes can induce MSCs differentiation into CAFs, resulting in increased metalloproteinase activity and expression of a vast array of factors involved in the remodeling of epithelial adherent junction pathways, thus enhancing tumor cell invasiveness and aggressiveness (Clayton et al., 2007; Cho et al., 2011a,b). Similarly, prostate cancer-derived exosomes can induce a process of tumor mimicry through the expression of epithelial neoplastic and vascular markers by adipose tissue-derived MSC, together with the acquisition of aberrant cytogenetic variations and Mesenchymal-to-Epithelial transition (Abd Elmageed et al., 2014). Moreover, breast cancer-derived exosomes also act on adipose tissue-derived MSCs, inducing the secretion of SDF-1, VEGF, CCL5 and TGF-β, which was associated with differentiation of these MSCs into tumor-associated myofibroblasts (Cho et al., 2011a).

Like other exosomes-based tumor-stroma interaction circuits, tumor-MSCs communication is also bidirectional. For instance, exosomes released by MSCs can upregulate the expression of VEGF in tumor cells via activation of the ERK1/2 and p83 mitogen-activated protein kinase pathways, increasing ECs proliferation and angiogenesis, therefore supporting tumor growth (Zhu et al., 2012). Another example involves exosomes enriched in miR-1587 produced by glioblastoma-associated MSC, which were shown to increase the proliferation and clonogenicity of tumor-initiating glioma stem-like cells (Figueroa et al., 2017). Interestingly, bone marrow, umbilical cord, and adipose tissue MSC-derived exosomes displayed differential effects on the proliferation of U87MG glioblastoma cells, indicating that the origin of the stem cells might be of relevance toward its pro-tumorigenic effects (Del Fattore et al., 2015). Furthermore, exosomes produced by bone marrow-derived MSC containing high levels of miR-221 induced proliferation, migration, invasion and adhesion of gastric cancer cells. In addition, exosomal expression of miR-221 was also associated with poor prognosis in patients with gastric cancer (Ma et al., 2017).

Another relevant aspect influenced by exosomal tumor-stem/progenitor cell communication is chemoresistance. Although chemoresistance frequently originates from selection of resistant tumor cell clones after drug exposure, drug resistance can also rise from the transference of biomolecules produced by stromal cells to exosomes. In pre-clinical models, for example, bone marrow stromal cell-derived exosomes induced tumor migration, proliferation and drug resistance by reducing the expression of apoptosis-related proteins by multiple myeloma cells (Wang et al., 2014). In addition, MSC-derived exosomes were found to induce resistance to 5-Fluouracyl and Cisplatin via activation of the RAS/RAF/MEK/ERK pathway in gastric cancer cells (Ji et al., 2015). And, finally, exosomal RNA produced by stromal cells were shown to activate RIG-I anti-viral signaling in breast cancer cells, leading to the expansion of therapy resistant breast cancer cells in a mechanism involving NOTCH3 induction (Boelens et al., 2014).

Tumor-derived exosomes have also been shown to act in non-transformed differentiated cells counterparts, inducing the expression of malignant features by these recipient cells. For instance, Chen et al. showed that exosomes are able to mediate intercellular communication between neoplastic and normal cells, promoting a pro-inflammatory phenotype in the latter. Specifically, it was shown that exosomes from arsenite-transformed liver cells activate the IL6, IL8/STAT3 pathway, enhancing miR155 expression and inflammatory properties in normal liver cells (Chen C. et al., 2016). Moreover, exosomes derived from the MatLyLu metastatic prostate tumor cells were capable to induce higher levels of proliferation in normal prostate epithelium cells than exosomes from the less aggressive non-metastatic Dunning G prostate tumor cells (Halin Bergstrom et al., 2016).

SUMMARY

Exosomes are biological active vesicles and professional carriers of information in intercellular communication. In an oncologic scenario, they play a pivotal role in shaping the tumor microenvironment by affecting tumor growth and proliferation, mediating the crosstalk between tumor and tumor-associated

cells and molding the host immune response. As emerging components in tumor-host crosstalk, exosomes modulate elementary steps of tumor progression, such as growth, invasion and immunosurveillance. In addition to effects in local tumor microenvironments, exosomes released from tumors were shown to mediate distant cell-cell communication processes which result in the setup of pro-tumorigenic microenvironments supportive of metastatic dissemination. This is achieved, for instance, though modulation of fibroblast activation, ECM production, angiogenesis and immunesurveillance. As both tumor- and stroma-derived exosomes are found abundantly at the peripheral circulation, they represent a precious opportunity to access non-invasively and in real time the biological status of the tumor microenvironment. Taking into account the relevance of non-tumor cells in cancer progression, this may provide a new source of biological markers with application in not only prediction of prognosis, but also in disease follow-up during and after therapeutic intervention. In addition to the current efforts in investigating the application of exosomes as anti-tumor tools (such as DC-derived exosomes), the further comprehension of the basic biology of exosomes, including the identification of exosomal components relevant for tumor progression, may represent an opportunity for novel therapeutic strategies based on the targeting of pro-tumorigenic exosomes-based cell-cell communication. However, in spite of the substantial expansion of the field, especially in the last decade, the roadmap to understand the exosomes communication system with the organism is still far from being fully understood. Besides the need for more information on fundamental issues, such as exosomes biogenesis and the mechanisms of exosomal cargo delivery, further effort is still necessary in the standardization of methods involving exosomes purification and characterization. The establishment of standard methods for exosomes isolation, as well as of a consensus regarding biological and physical characteristics that define a group of extracellular vesicles as exosomes, such as the one proposed by the EV-TRACK Consortium (EV-TRACK Consortium et al., 2017), are essential. This is of great relevance, as the myriad of methods for exosomes isolation described in the literature (such as ultracentrifugation, immuneprecipitation and size exclusion chromatography) has the potential to complicate the reproducibility of current and future works and the overall establishment and progress of this flourishing research field.

AUTHOR CONTRIBUTIONS

BC-S: organization of content and structure, writing and reviewing; SC: writing exosomes role in tumor-fibroblast and tumor-endothelial cells communication, figures preparation; JM: writing exosomes role in tumor-immune system communication, preparation of figures; NC: writing exosomes role in tumor stem/progenitor cells communication. MS: writing introduction and the role of exosomes in tumor-tumor communication.

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REVIEW
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Feeling Stress: The Mechanics of Cancer Progression and Aggression

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The tumor microenvironment is a dynamic landscape in which the physical and mechanical properties evolve dramatically throughout cancer progression. These changes are driven by enhanced tumor cell contractility and expansion of the growing tumor mass, as well as through alterations to the material properties of the surrounding extracellular matrix (ECM). Consequently, tumor cells are exposed to a number of different mechanical inputs including cell–cell and cell-ECM tension, compression stress, interstitial fluid pressure and shear stress. Oncogenes engage signaling pathways that are activated in response to mechanical stress, thereby reworking the cell's intrinsic response to exogenous mechanical stimuli, enhancing intracellular tension via elevated actomyosin contraction, and influencing ECM stiffness and tissue morphology. In addition to altering their intracellular tension and remodeling the microenvironment, cells actively respond to these mechanical perturbations phenotypically through modification of gene expression. Herein, we present a description of the physical changes that promote tumor progression and aggression, discuss their interrelationship and highlight emerging therapeutic strategies to alleviate the mechanical stresses driving cancer to malignancy.

Keywords: cancer progression, cell contractility, mechanical stresses, tissue tension, solid stress, ECM stiffness, therapeutic targets

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INTRODUCTION

The tumor microenvironment is a dynamic landscape composed of cancer cells surrounded by the extracellular matrix (ECM) and a host of stromal cells, including fibroblasts, immune cells, blood and lymphatic vascular cells, and other tissue-specific cells (e.g., adipocytes). From transformation of the normal tissue, on through progression of the primary tumor to invasion, dissemination, and metastasis, the physical context of the tumor changes dramatically (Kumar and Weaver, 2009). These changes to the tumor microenvironment are driven by enhanced tumor cell contractility, expansion of the growing tumor mass, and alterations to the material properties of the surrounding ECM. Indeed, the physical properties of a tissue, such as ECM stiffness and architecture, can have a profound influence on cellular behavior and ultimately, tissue organization and function.

Cancer progression is associated with changes to both the cellular responses to chemical and mechanical signals as well as to the material properties of the ECM components of the transformed tissue (reviewed in Butcher et al., 2009; Kumar and Weaver, 2009). Cells are exposed to a number of

different mechanical stresses (**Figures 1**, **2A**) that activate downstream signaling pathways through a process termed mechanotransduction. Importantly, oncogenes engage many of the same signaling pathways that are activated in response to mechanical stress (reviewed in Yu et al., 2011), thereby reworking the cell's intrinsic response to exogenous mechanical stimuli and enhancing cytoskeletal contractility. Active tumor cell engagement with this mechanically-evolving microenvironment results in changes to cytoskeletal structure, cellular shape, differentiation, survival/death, proliferation, adhesion, and migration which can drive tumor progression and aggression (Butcher et al., 2009; Yu et al., 2011). Herein we present a description of the mechanical stresses that promote tumor progression and aggression, discuss their interrelationship, and highlight emerging therapeutic strategies to alleviate the mechanical stresses driving cancer to malignancy.

TUMOR CELL CONTRACTILITY

Cells respond to mechanical stresses by altering their intracellular tension (**Figure 2**). This response is achieved through coordinated cytoskeletal rearrangement and actomyosin contraction. Mechanotransduction is the process through which cells sense and respond to mechanical signals (e.g., ECM rigidity, compression, tension) by translating these mechanical stimuli into biochemical signals. Cellular interpretation of these biochemical signals influences cell morphology, behavior and function.

Mechanotransduction and Actomyosin Contractility

Mechanotransduction is essential for a number of normal biological processes, such as hearing (reviewed in Schwander et al., 2010), and pathologies, such as cancer. Focal adhesion and adherens junction protein complexes mediate the bi-directional physical communication between cell-ECM and neighboring cells, respectively. In this way, ECM rigidity and intercellular dynamics modify intracellular tension, which feeds back to influence tissue stiffness and morphology. Application of forces to the cell membrane can also lead to opening of mechanically-activated ion channels (e.g., Piezo1 and Piezo2) and actomyosin contractility (Maroto et al., 2012, reviewed in Honore et al., 2015).

Cells interact with the ECM via integrin receptors and, in response to mechanical signals from the ECM, integrins become activated and oligomerized (Butcher et al., 2009). Subsequently, the adhesion plaque protein talin undergoes a conformational change, which fosters intermolecular interactions. Once the integrin-adhesion plaque association has been activated, the focal complexes begin to assemble and eventually mature into focal adhesions (Butcher et al., 2009). Focal adhesions are composed of multiple mechanosensors (e.g., talin, vinculin), signaling molecules [e.g., focal adhesion kinase (FAK), SRC, phosphatidylinositol-3 kinase (PI3K)], adapter proteins (e.g., paxillin) and actin linker proteins (e.g., filamin, alpha-actinin) which physically connect the integrins to the cytoskeleton (reviewed in Wozniak et al., 2004). On stiff substrates, physical resistance to cellular tension enhances talin stabilization through vinculin binding and also potentiates FAK activation (**Figure 2B**). Focal adhesion formation results in Rho/Rho kinase (ROCK) activation and actomyosin contraction. Reinforcement / maturation of the focal adhesion complex further increases contraction via the assembly of actin stress fibers (**Figure 2B**). In addition, via interplay with growth factor receptors (GFRs) and G-protein coupled receptors (GPCRs), focal adhesion formation also activates signaling downstream of both FAK and SRC (e.g., MAPK) (Butcher et al., 2009; Yu et al., 2011).

The magnitude of these transmitted mechanical signals is regulated by the stabilization of the focal adhesions (greater number or size), which leads to augmented actomyosin

Mechanical stress

When external forces are applied to the surface of an object, such as a cell membrane, the object is said to be under stress.

Stress is a measure of the object's internal resistance to deformation. It is calculated as the amount of force over a given area (N/m^2) and is expressed in Pascals (Pa).

There are three types of mechanical stresses that cells encounter: **tensile**, **compressive** and **shear**.

The effects of these stresses on tumor progression and aggression are dependent on the magnitude, duration and direction of the applied forces, as well as the material properties (e.g., viscoelasticity, stiffness) of the cellular and extra-cellular tissue components .

Types of mechanical stress and sources within the tumor microenvironment.

Stress	Source of stress
Tensile	Cellular actomyosin contraction in response to ECM stiffness
Compressive	Tumor expansion due to continuous cell proliferation within a confined space
Shear	Blood and interstitial fluid flow

FIGURE 1 | Mechanical stress.

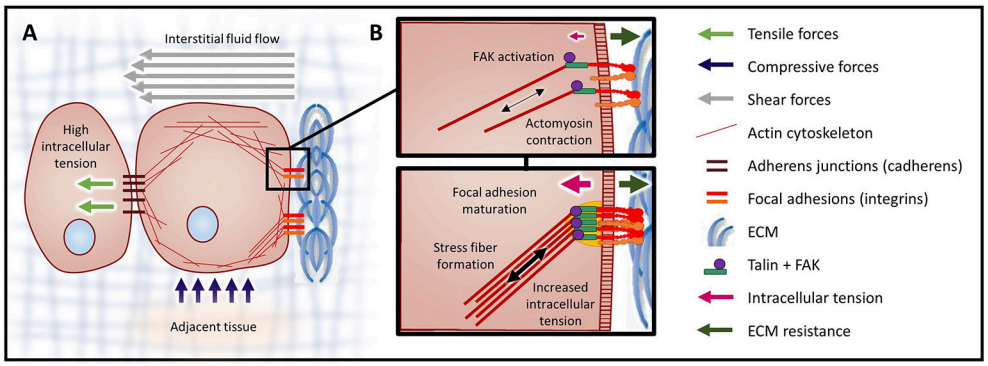

FIGURE 2 | Mechanical forces and cellular contractility. **(A)** Forces exerted onto a cell can cause three types of mechanical stress: tensile (stretch), compressive, and shear. Forces that induce tensile and compressive stress are applied perpendicular to the cell surface, while forces causing shear stress are exerted parallel to the cell surface. **(B)** Integrin clusters activated through ECM binding are bound by talin, initiating actin polymerization and producing intracellular tension. In response to high tensile force (a consequence of elevated ECM rigidity), FAK is activated (top). Activation of FAK leads to the recruitment of additional linker proteins, focal adhesion maturation, and increased actomyosin contraction via the assembled actin stress fibers (bottom).

contractility and traction force generation. Focal adhesion formation and FAK activation synchronizes the immediate response (traction force generation) and the sustained response (altered gene expression) through coordinated actomyosin contraction and signaling pathway activation, respectively. Increased cellular tension, integrin signaling and crosstalk with GFR and GPCR signaling pathways, ultimately lead to enhanced tumor cell growth, survival, and invasion (Provenzano and Keely, 2011).

Similar to the focal adhesions, the adherens junction complexes that communicate intercellular tension are composed of receptors (i.e., cadherins), mechanosensors (i.e., catenins), linker proteins, and signaling molecules (i.e., SRC). Adherens junctions and focal adhesion complexes crosstalk though shared protein components and physical interconnection via the actin cytoskeleton (Oldenburg et al., 2015, reviewed in Mui et al., 2016). Indeed, actomyosin contractility-induced cell tension can promote tissue stiffness and β-catenin mediated interfollicular epidermal hyperplasia and tumor growth (Samuel et al., 2011).

In addition, mechanotransduction can occur through mechanically-activated ion channels (e.g., Piezo1), which respond to external stimuli either directly or indirectly. Mechanically-activated ion channels, which are regulated by GTPases, were among the first mechanosensor proteins described. Like other chemical mechanotransduction pathways, signals are amplified within the cell and crosstalk with other signaling pathways. These proteins regulate many cellular processes, such as cell polarity (Huang et al., 2015) and proliferation (Basson et al., 2015) that are perturbed in cancer. Recently, Gudipaty and colleagues demonstrated that stretch-induced epithelial cell division and extrusion require activation of Piezo1 (Gudipaty et al., 2017). Mechanically-activated ion channels, including Piezo1, have been shown to be dysregulated in both solid (Maroto et al., 2012; McHugh et al., 2012; Yang et al., 2014; Fels et al., 2016) and non-solid tumors (Morachevskaya et al., 2007; Nam et al., 2007; Pottosin et al., 2015), implicating this mode of mechanotransduction as a potential driver of tumor progression.

Reciprocity of Intracellular Tension and Gene Expression

Sustained cellular responses to mechanical stimuli depend ultimately upon altering gene expression. For example, expression of integrins, matrix metalloproteases (MMP), and ECM proteins is increased in response to stiff substrates (Delcommenne and Streuli, 1995; Nukuda et al., 2015). These gene expression changes enable cells to modify the compliance of their microenvironment by remodeling the ECM via increased tension and altered matrix composition and arrangement. Force-induced gene expression changes can also affect the mechanical properties of the tumor cell themselves by altering the expression of cytoskeletal proteins (McGrail et al., 2015).

The magnitude of intracellular tension generated by actomyosin contractility also depends on the cytoskeletal composition (type and organization of actin, intermediate filament and microtubule proteins), which reflects the cell type and phenotypic state of the cell (Butcher et al., 2009; McGrail et al., 2015). The cytoskeletal architecture, intracellular tension and force-generating capabilities of transformed cells are notably changed from the normal epithelium. Intermediate filaments, which provide mechanical support to the cell, differ in their expression profile between normal and transformed epithelial cells (Kumar and Weaver, 2009). Furthermore, epithelial-to-mesenchymal transformation (EMT), a proposed feature of highly metastatic tumor cells, is characterized by a switch from keratin to vimentin intermediate filament expression (reviewed in Kumar and Weaver, 2009; Wirtz et al., 2011). Changes to the cellular cytoskeleton, such as during transformation or EMT, drastically alter the shape and the amount of tension exerted on neighboring cells and the ECM. EMT results in cells with increased migration, invasion and dissemination potential (Yu et al., 2011).

In addition to mechanical signals being transduced to the nucleus through chemical mechanotransduction pathways (mechanosignaling), it is possible that physical signals may be directly transduced to the nucleus through physical anchoring of the cytoskeletal networks with the nuclear lamina via the linker of nucleoskeleton and cytoskeleton (LINC) complex (Crisp et al., 2006; Wirtz et al., 2011). This paradigm maintains that as actin is tethered to both the cell membrane as well as the nuclear membrane, tensional forces can be transmitted to the nucleus (via the LINC complex), neighboring cells (via adherens junctions) and the ECM (via focal adhesions). Interestingly, it was shown in isolated nuclei that the nucleus also responds to mechanical tension by inducing nuclear stiffening (Guilluy et al., 2014); however, what role this plays in the regulation of gene expression is currently unknown.

Oncogene and Growth Factor Modulation of Cell Contractility

Mechanotransduction pathways crosstalk with other chemical signal transduction cascades (e.g., GFR and GPCR signaling pathways) to influence transient responses and persistent cellular behaviors though modulation of gene expression (Butcher et al., 2009; Yu et al., 2011). For instance, stimulation of the thrombin GPCR Par1 causes RhoA/Rap1 mediated activation of integrin signaling, phosphorylation of FAK and ERK1/2, and increased glioblastoma cell proliferation (Sayyah et al., 2014). In invasive melanoma cells, cytokine signaling-induced activation of the JAK1/STAT3 pathway leads to increased ROCK-dependent actomyosin contractility, that feeds back to reinforce STAT3 signaling and promote ECM remodeling by tumor-associated stromal fibroblasts (Sanz-Moreno et al., 2011). Thus, mechanical forces collaborate with biochemical signals to modulate cell, and ultimately, tissue behavior.

Following transformation, a tumor cell's intrinsic response to tension is often altered by oncogene engagement with mechanotransduction components. For example, EGFR-transformed breast epithelial cells have elevated ERK-Rho activity and increased myosin-mediated cell contractility (Paszek et al., 2005). Furthermore, HRAS activation of ROCK drives the progression of squamous cell carcinoma by inducing actomyosin contractility, tissue stiffness (through collagen deposition) and cell proliferation (Samuel et al., 2011). Moreover, the influences of oncogene expression and growth factor/cytokine stimulation can converge onto mechanotransduction pathways to alter tumor progression. In a KRAS-driven murine pancreatic ductal carcinoma (PDAC) model, loss of TGF-beta signaling leads to activation of GPCR-mediated JAK/STAT3 pathway signaling and stimulation of ROCK1, that increases tumor cell contractility, induces ECM remodeling and localized stiffening, and promotes focal adhesion maturation to promote tumor progression and aggression (Laklai et al., 2016). These studies highlight the common link and between increased actomyosin contractility of cells within the tumor microenvironment, augmented ECM stiffness and cancer progression across a variety of solid tumors from various organs (i.e., breast, skin, and pancreas).

Tension-Regulated Cellular Processes Drive Tumor Progression

Increased intracellular tension through actomyosin contractility is sufficient to induce cell proliferation, disrupt cell–cell adherens junctions and effect ECM remodeling, all of which can promote loss of tissue polarity, ECM reorganization/stiffening and tumor cell invasion (Butcher et al., 2009; Yu et al., 2011) (**Figure 3**). Transformed cells exert higher traction forces than normal cells when embedded within a compliant matrix resembling physiological stiffness conditions (Wang et al., 2000; Paszek et al., 2005; Butcher et al., 2009). The ability of a cell to invade depends upon both the magnitude and direction of traction forces (Koch et al., 2012).

In addition to a cell's ability to generate traction, metastasis necessitates that cells are able to squeeze through small openings in the ECM and between cells of the endothelium (Wirtz et al., 2011). Cell compliance was demonstrated to be tuned by the extracellular context, as tumor cells stiffen as they invade into 3D collagen gels due to increased actomyosin contractility (Staunton et al., 2016). Thus, it is not surprising that tumor cells may become more compliant than their normal counterparts and that the extent of cellular compliance correlates with metastatic capability (Guck et al., 2005). Interestingly, higher target cell membrane tension enhances perforin-mediated killing by cytotoxic CD8+ T cells (Basu et al., 2016), suggesting that increased compliance of metastatic tumor cells could potentially enable them to evade immune destruction.

While the current perspective is that cells need to be softer to enable migration under spatial constraint, a recent study demonstrated that the nucleus is the greatest impediment to confined migration, not cortical tension (Mekhdjian et al., 2017). This finding suggests that the nucleus, not the cortex, of metastatic tumor cells is softer and that this deformability, together with the ability to exert higher traction force at the integrin adhesions, permits metastatic cells to navigate rapidly through confined stiff spaces. Thus, the ability of cells to migrate through a dense ECM depends on adhesiveness, nuclear volume, contractility, and to a lesser extent cortical cell stiffness (Lautscham et al., 2015). Indeed, the majority of total cell stiffness comes from the nucleus, which is the largest organelle and almost an order of magnitude stiffer than the cytoplasm (Dahl et al., 2004; Tseng et al., 2004). As cells migrate through dense matrices, the nucleus must deform, which can cause nuclear rupture and DNA damage to occur (Denais et al., 2016; Raab et al., 2016). Decreasing nuclear stiffness, through knockdown of lamin A expression, increases cell motility and ability to migrate through dense matrices but impairs the survival of tumor cells exposed to shear stress (Davidson et al., 2014; Mitchell et al., 2015). Thus, greater nuclear compliance coupled with elevated contractile forces enables cells to pull themselves through tight spaces with less risk of nuclear rupture.

Another form of cellular deformation involved in cell migration that depends on actin cytoskeleton rearrangement is invadopodia formation. Invadopodia are linked to tumor cell invasion and metastasis, and augmenting intracellular tension (using the PP1/2 inhibitor calyculin A) increases invadopodia

formation, protease secretion, ECM degradation and an invasive cellular phenotype (Aung et al., 2014; Jerrell and Parekh, 2014). Thus cellular tension generation plays important roles in tumor metastasis.

MICROENVIRONMENTAL STRESSES

Solid Stress

Unchecked proliferation of cancer cells results in rapid expansion of the tumor mass, compression of the tumor interior and distention of the surrounding stromal tissue. The forces exerted by the expanding tumor mass and the resistance to deformation of the surrounding stromal tissue make up what is collectively known as solid stress (reviewed in Jain et al., 2014; **Figure 3**). Recently, several new methods have been developed to measure solid stress in tumors which have demonstrated that the tumor type, tumor size and the properties of the surrounding tissue all influence tumor solid stress (Nia et al., 2016).

Forces and strains propagated outward from the tumor, toward the surrounding stromal tissue, can result in increased ECM tension and remodeling, as well as disruption of tissue structure surrounding the tumor mass (Jain et al., 2014). Elevated ECM tension in these adjacent tissues may be exacerbated by crowding from tumor-associated myofibroblast proliferation and immune cell infiltration/expansion during the desmoplastic and pro-inflammatory stromal responses. Furthermore, changes to the material properties of the ECM (i.e., stiffening due to deposition/remodeling) can also contribute to the growth and solid stress of the tumor. Collagen fibers stiffen under tension causing resistance to further stretching, while hyaluronan can trap interstitial fluid and swell due to hydration, providing resistance to compression and increased intratumoral solid stress (Jain et al., 2014). In order for a tumor to continue to increase in size, it must displace or degrade the surrounding non-malignant tissue. Computational modeling has found that the stiffness of a solid tumor must exceed 1.5 times that of the surrounding tissue in order to continue to expand (Voutouri et al., 2014).

Conversely, externally-applied forces resulting in compression or confinement of the tumor volume may reduce cancer cell proliferation, induce apoptosis/necrosis, augment ECM deposition/organization, and enhance the invasive and metastatic potential of tumor cells (Yu et al., 2011). Indeed, externally-applied compressive stress is sufficient to reduce the volume and proliferative rate of cells in the core of multicellular structures grown in 3D matrices (Helmlinger et al., 1997; Delarue et al., 2014; Mascheroni et al., 2016). Low-proliferating cell populations contribute to treatment resistance and compression of tumor cells themselves may compromise the efficacy of chemotherapeutic agents (Mascheroni et al., 2017). *In vivo*, 1 month of induced compression stress (at levels comparable to those measured in growing tumors), leads to translocation of β-catenin from the adherens junctions to the nucleus, activation of β-catenin target genes, and increased colon crypt size (due to hyperplasia) (Fernández-Sánchez et al., 2015), implicating compression stress as an inducer of tumorigenesis.

Moreover, solid stress compresses blood vessels in the tumor interior, which impairs the oxygen and nutrient supply to the tumor and temporarily impedes cancer progression (Padera et al., 2004). Sustained compression of the vasculature within the tumor results in poor tissue perfusion, hypoxia and the development of a necrotic core (Stylianopoulos et al., 2013). Hypoxia in the tumor core drives metastasis directly by increasing EMT and stem-like features in cancer cells (reviewed in Muz et al., 2015), as well as indirectly, through recruitment of pro-tumor macrophages (reviewed in Condeelis and Pollard, 2006). Together, this increases the invasive and metastatic potential of tumor cells.

Solid stresses at the periphery of the tumor are sufficient to cause compression of blood vessels surrounding the tumor and consequently, vessels in the surrounding normal tissue are deformed to elliptical shapes (Stylianopoulos et al., 2013). Constriction of the lymphatic vasculature within the stroma reduces drainage of extravasated fluid (Jain et al., 2014). Thus, solid stress may promote elevated interstitial fluid pressure as a consequence of reduced interstitial space and compromised collection of fluid due to blocked vessels. In the context of a confined tumor, such as glioblastoma multiforme (GBM), this stress may be intensified by confinement of the brain by the skull. Together, reduced blood flow and increased interstitial pressure hinders efficient drug delivery of chemotherapeutics to the tumor and exacerbates hypoxia (Zhang et al., 2014), which reduces the efficacy of radiation treatment (Mpekris et al., 2015).

Mathematical modeling predicts that changes in the collective tension (a product of cell–cell adhesion) at the tumor-stoma interface can affect the shape of the tumor and this loss of tension can promote collective cell migration and metastasis (Katira et al., 2013). Thus, rate and direction of tumor cell migration may also be affected by the solid stress dynamics.

Shear Stress

The metastatic process subjects tumor cells to a variety of additional microenvironments and forces. As tumor cells escape the primary tumor and transit through the circulation, they are exposed to a number of different solid and fluid forces, many of which elicit shear stress (**Figure 3**). Hemodynamic shear stress, which is caused by the movement of blood along the cell surface, is influenced by both the fluid viscosity and fluid flow velocities (Wirtz et al., 2011). Shear stress can also be caused by solid forces from endothelial cell contact as tumor cells intravasate and extravasate from the vasculature.

For metastasis to occur, tumor cells must survive transit within the circulation, and not surprisingly, tumor cells have been shown to be more resistant to shear stress than normal cells (Mitchell et al., 2015). Survival is dependent on the time spent in circulation and the magnitude of shear stress tumor cells experience (Fan et al., 2016). Physiological resting levels of shear stress [∼5–30 dynes/cm^2 (0.5–3 Pa)] inhibit proliferation and stimulate migration and adhesion of tumor cells (Avvisato et al., 2007; Mitchell and King, 2013; Ma et al., 2017; Xiong et al., 2017), while levels of shear stress similar to exercise conditions [60 dynes/cm^2 (6 Pa)] caused tumor cell death (Regmi et al., 2017). Thus, the ability of a tumor cell to withstand the various dynamic mechanical stresses it will encounter, as it leaves

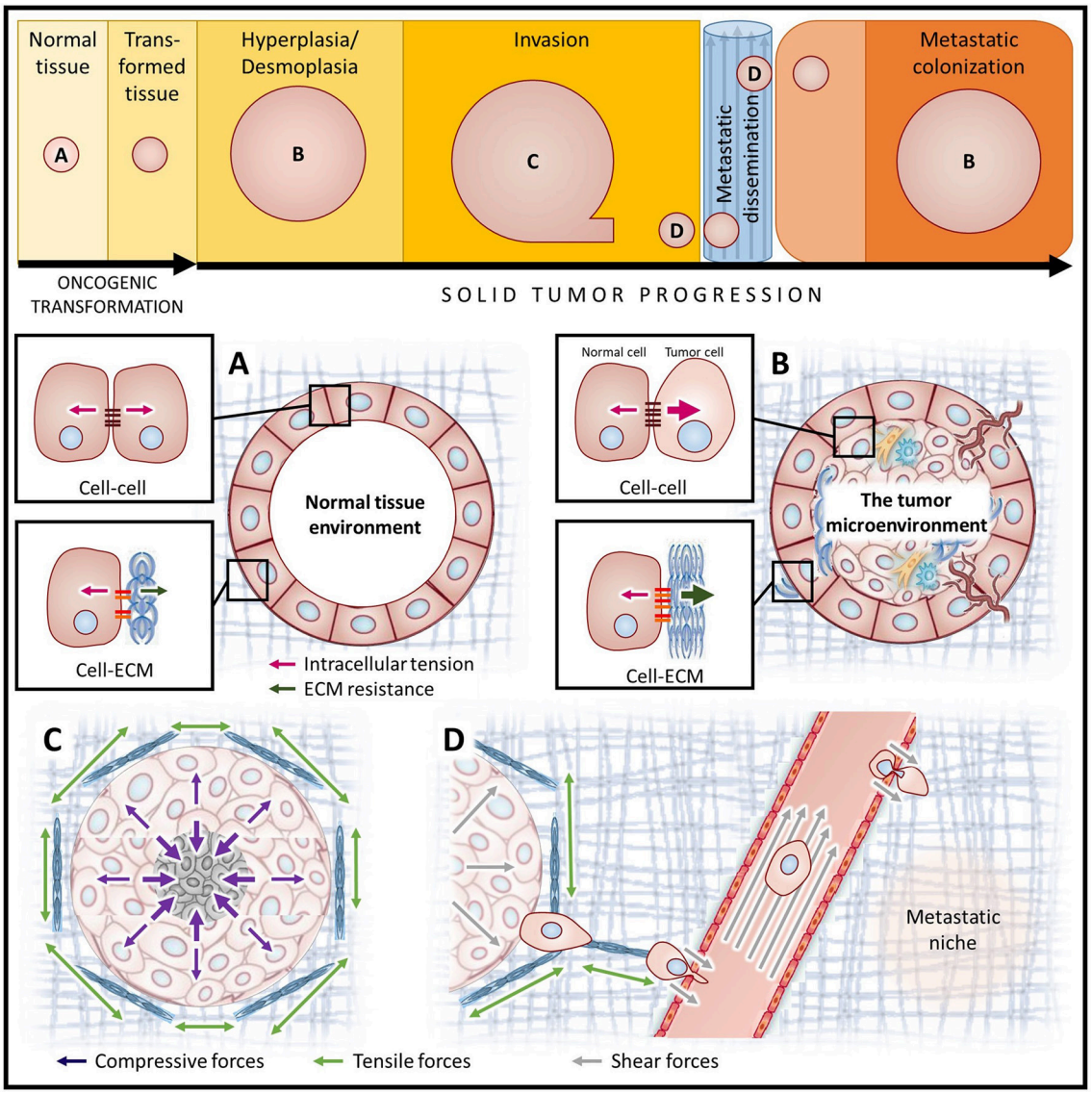

FIGURE 3 | Diverse mechanical stimuli act on tumor cells throughout cancer progression**.** Simplified depiction of oncogenic transformation and solid tumor progression for cancers of epithelial origin (top). Letters indicate the stages of cancer progression focused on in panels **(A–D)**. **(A)** In homeostatic tissues, the forces between cells and the ECM are balanced. **(B)** The tumor microenvironment is composed of cancer cells with augmented contractility (increased intracellular tension), surrounded by a progressively stiffening ECM (increased ECM resistance), and a host of stromal cell types including fibroblasts, immune cells and vascular cell types. **(C)** Tumor expansion confined by the surrounding stroma compresses both the tumor and the adjacent stromal tissue, causing increased interstitial pressure. Augmented ECM rigidity increases stromal resistance to compression and exacerbates solid stress. **(D)** A high interstitial fluid pressure gradient elicits fluid flow from the tumor core to the periphery, promoting metastatic dissemination. Following escape from the primary tumor, cancer cells migrate along tension-oriented collagen fibers toward the vasculature. Tumor cells are exposed to high shear stresses as they intravasate/extravasate between endothelial cells and travel through the circulation *en route* to future secondary tumor sites.

the primary tumor and establishes a secondary tumor site, will require continuous cellular adaptation.

Metastatic sites are not random. Primary tumors from various organs show a preference for colonization of different secondary organs, likely due to a combination of blood flow pattern (mechanical hypothesis) and favorability of the microenvironment (seed and soil hypothesis) (Wirtz et al., 2011). Circulating tumor cell adhesion to the vessel wall is required for extravasation. High shear stress increases the frequency of tumor-endothelial cell contact, but impedes the ability of these cells to form stable cell–cell adhesions (Wirtz et al., 2011). High shear forces require tumor cells to make stronger adhesions in order to extravasate. Thus, often tumor cells extravasate at branch points or after being trapped in small capillaries (Wirtz et al., 2011).

Similar to how cells undergo drastic rearrangement of their cytoskeleton and deformation to squeeze themselves through

dense ECM matrices (Wirtz et al., 2011), actomyosin contraction induced cellular tension likely plays a vital role in tumor cell resistance to shear forces, enabling survival in circulation and during extravasation between endothelial cells. Importantly, hemodynamic forces also affect the phenotype and function of endothelial cells composing the vasculature and thus impact cancer progression via this axis; however, this discussion is outside the scope of this review.

Interstitial Fluid Pressure

In normal tissue, the interstitial fluid pressure is a physiological hydrostatic pressure composed of the pressure from both the free fluid and the fluid immobilized by hyaluronan. However, the interstitial fluid pressure within the tumor microenvironment drastically increases as a consequence of tumor growth, augmented vascular permeability (leakiness) and impaired lymphatic drainage (Stylianopoulos et al., 2013; Jain et al., 2014).

Tumour blood vessels are often tortuous with irregular branching morphologies and leaky due to a lack of pericyte coverage (reviewed in Carmeliet and Jain, 2011). These vascular abnormalities lead to inadequate blood flow rates, reduced oxygen tension (hypoxia) and increased interstitial fluid pressure (Carmeliet and Jain, 2011; Jain et al., 2014). Hyaluronan swelling (due to increasing fluid retention), is another major contributor to interstitial fluid pressure that can also intensify solid stress by providing compressive resistance to the ECM (Jain et al., 2014). Increasing solid stresses can lead to lymphatic vessel crushing and impaired fluid drainage (Padera et al., 2004). Together, these factors increase the interstitial fluid pressure within the tumor and contribute to a difference in fluid pressures between the tumor and the surrounding tissue (Jain et al., 2014).

The difference between the elevated interstitial fluid pressure within the tumor and interstitial pressure of the adjacent peritumoral tissue creates a fluid pressure gradient that causes the outward flow of fluid from the tumor into the surrounding stroma and may facilitate tumor cell escape from the primary tumor (**Figure 3**; Jain et al., 2007). Furthermore, while solid stresses impede blood flow and thereby inhibit perfusion, high interstitial fluid pressure limits the penetration and dissemination of therapeutic agents in solid tumors, impairing treatment efficacy (Boucher and Jain, 1992; Jain et al., 2007, 2014).

ECM MATERIAL PROPERTIES

Cancer progression is associated with changes to tissue structure and mechanical properties of the ECM (**Figure 3**). Increased ECM rigidity (decreased compliance) correlates with cancer progression and is sufficient to perturb normal tissue morphology (Paszek et al., 2005).

ECM remodeling in the tumor involves ongoing production of matrix proteins, their assembly and crosslinking, as well as their turnover by MMPs. This remodeling contributes to ECM stiffening, which occurs primarily through increased collagen deposition (i.e., increased protein concentration), augmented collagen crosslinking (e.g., higher lysyl oxidase (LOX) enzyme expression), and via parallel reorientation of the collagen fibers (Butcher et al., 2009; Kumar and Weaver, 2009; Yu et al., 2011). Importantly, increased collagen abundance and reorganization into thick, linearly oriented fibers correlates with tumor progression and clinical outcome (Acerbi et al., 2015). High ECM stiffness may also predispose individuals to develop certain cancers. Normal breast tissue clinically determined to have high mammographic density (MD) contains stiffer ECM, thicker collagen fibers and more linearized collagen than low MD breast tissue (Acerbi et al., 2015), and was shown to increase the overall lifetime risk of breast cancer development (Boyd et al., 1992; Razzaghi et al., 2012).

In the tumor microenvironment, collagen crosslinking enhances integrin activation, focal adhesion maturation, PI3K/AKT signaling and tumor cell invasion/metastasis (Levental et al., 2009; Pickup et al., 2013; Rubashkin et al., 2014). High LOX expression in mammary tumors drives ECM stiffening, focal adhesion formation and metastasis, which can be abrogated using a LOX inhibitor (Levental et al., 2009; Pickup et al., 2013). Mechanistically, focal adhesion stabilization by vinculin in response to rigid ECM stimulates the activation of PI3K/AKT signaling and promotes tumor cell proliferation and invasion (Levental et al., 2009; Pickup et al., 2013; Rubashkin et al., 2014). Substrate rigidity-induced actomyosin contractility, also increases invadopodia formation and matrix degradation (Aung et al., 2014; Jerrell and Parekh, 2014), cellular responses associated with tumor aggression. In addition to a rigid matrix, integrin clustering can be facilitated by a bulky glycocalyx at the cell surface, which applies tension to matrix-bound integrins (Paszek et al., 2014). Hence, ECM stiffening through collagen crosslinking stimulates tumor cells to generate higher intracellular tension and exert stronger traction forces on their surroundings, which subsequently increases ECM stiffness as a result of applied tension. This positive feedback promotes tumor progression and metastasis.

Tumor progression propagated by ECM rigidity involves altered micro-RNA (miR) expression. Upregulation of oncogenic miR-18a in response to integrin signaling was shown to decrease PTEN expression and promote malignancy, in breast cancer models (Mouw et al., 2014). Similarly, increased ECM stiffness downregulates tumor suppressive miRs, such as miR-203. Downregulation of miR-203 was shown to repress ROBO1, which regulates actin organization and epithelial contraction (Le et al., 2016).

At the tumor periphery, increased ECM tension promotes remodeling that favors linear reorientation of collagen fibers. This ECM organization, along with compression-induced solid stress and a larger interstitial fluid pressure gradient (resulting in increased fluid flow), may promote tumor cell escape from the primary tumor. While cell migration through the ECM is impeded by protein density (small pore size) (Wirtz et al., 2011), metastasis is facilitated by oriented collagen fibers and paracrine signals from immune cells that guide the directional migration of tumor cells toward the vasculature (Condeelis and Pollard, 2006; Leung et al., 2017). Accordingly, ECM stiffening and tumor grade are associated with increase immune cell infiltration (Acerbi et al., 2015).

Remodeling of the ECM can cause the release and activation of growth factors (e.g., TGF-beta) in the stroma which further potentiate tumor progression. Matrix stiffness and TGF-beta can both drive EMT and promote tumor metastasis by increasing MMP secretion and cell migration (Wei et al., 2015). Likewise, increased ECM stiffness and TGF-beta release also cause fibroblast-to-myofibroblast conversion. Tumor associated myofibroblasts display stronger actomyosin contractility than their resident fibroblast precursors and exacerbate the desmoplastic stromal response by secreting and remodeling ECM proteins (Kumar and Weaver, 2009). Infiltrating immune cells also contribute to the desmoplastic response by secreting a variety of cytokines that activate the surrounding tumor and stromal cells. Intriguingly, tumor-activated macrophages are more compliant than resting macrophages (Yu et al., 2011).

Tumor-associated vasculature displays heightened actomyosin contractility (Yu et al., 2011) and endothelial cells plated on different ECM substrates have different responses to tension (Collins et al., 2014). As increased cell-ECM tension can disrupt cell–cell adhesions, augmented ECM stiffness and changes to the ECM composition may promote hyper-permeability of the already leaky blood vasculature and further impair tissue drainage by the lymphatic vasculature.

POTENTIAL THERAPEUTIC TARGETING

In order to save lives, novel approaches to cancer prevention and treatment are needed. Given the wealth of evidence supporting physical forces as drivers of tumor growth and metastasis, and the differences in the mechanical properties of cells and matrix components between normal tissue and cancer tissue, it is reasonable to target the mechanical features of tumors for therapeutic intervention. This could be achieved through modulation of the mechanical properties of the stroma or inhibition of the cellular responses to increased stromal stiffening. Discussed next are prospective cancer therapeutics that target ECM stiffness, cell contractility or solid stress.

Inhibition of Cell Contractility

Targeting cell tension, by using drugs that inhibit signaling downstream of focal adhesions to reduce actomyosin contractility, has shown promise in blocking tumor progression. In genetic mouse models of pancreatic cancer, treatment with ruxolitinib, an inhibitor of JAK, significantly reduced STAT3 activity, collagen fibrillogenesis, and ECM stiffening (Laklai et al., 2016). While ruxolitinib has been FDA approved for the treatment of myeloproliferative neoplasms (reviewed in Santos and Verstovsek, 2012), a phase 2 clinical trial of ruxolitinib demonstrated little efficacy in the treatment of human pancreatic tumors (Hurwitz et al., 2015). Several clinical trials of ruxolitinib in breast cancer and leukemia are currently under way (https://clinicaltrials.gov/).

Treatment of mice bearing patient-derived pancreatic tumors with a ROCK inhibitor (Fasudil) enhanced the effectiveness of standard chemotherapies (Gemcitabine/Abraxane) (Vennin et al., 2017). Pre-clinical studies with a FAK inhibitor (VS-4718) in a PDAC mouse model demonstrated diminished tumor fibrosis, inhibited tumor progression, increased effectiveness of chemotherapy (Gemcitabine), and an augmented responsiveness to immunotherapy (αPD1) attributed to higher levels of infiltrating $CD8^+$ cytotoxic T lymphocytes (Jiang et al., 2016). These studies show the promise of enhanced therapeutic efficacy when combining drugs that target cell contractility with chemotherapy and immunotherapies. FAK inhibition is also being tested in clinical trials as a therapeutic strategy to inhibit cell contractility in various types of solid tumors (reviewed in Golubovskaya, 2014). To date, ten clinical trials aimed at testing various FAK inhibitors (PF-00562271, Defactinib/VS-6063/PF-04554878, VS-4718, GSK-2256098) in cancer patients have been completed; however, the study results are pending for all but the first phase 1 trial which assessed the safety and tolerability of PF-00562271 (https://clinicaltrials.gov/ct2/show/NCT00666926) (Infante et al., 2012).

Interestingly, retinoic acid treatment of pancreatic (PDAC) tumors also decrease cell tension in response to strain (Chronopoulos et al., 2016), suggesting that this pathway may be of therapeutic utility. Alternatively, molecular mediators of invadopodia formation could also be targeted in order to reduce metastatic spread. Furthermore, given their newfound involvement in cancer, mechanically-activated ion channels (such as Piezo1) are also potential therapeutic targets. However, some caution is needed as some side effects of the chemotherapeutic drug cisplatin, have been attributed to its non-specific inhibition of mechanosensitive ion channels (Milosavljevic et al., 2010).

Targeting Solid Stress and Interstitial Fluid Pressure

Solid stress compresses the vasculature and leads to hypoxia, impaired drug delivery, and higher interstitial fluid pressure. Thus, approaches have been developed to target solid and fluid stresses within the tumor microenvironment. Examples include hyaluronidases and angiotensin inhibitors. Hyaluronidases (e.g., PEGPH20), degrade the ECM protein hyaluronan to release immobilized fluid and improve tissue compliance (Whatcott et al., 2011). Angiotensin inhibitors (e.g., losartan) cause dilation of the vasculature and blood pressure reduction, thereby decreasing interstitial fluid pressure and enabling better perfusion and therapeutic efficacy. In breast and pancreatic cancers, inhibiting angiotensin with losartan decompressed tumor blood vessels and reduced stromal collagen, which increased vascular perfusion and improved drug delivery (Chauhan et al., 2013). Furthermore, losartan was shown to inhibit mammary tumor development and progression, as well as increase in blood vessel diameter, by inhibiting AT1R activation (Coulson et al., 2017). Both PEGPH20 and losartan are currently in clinical trials for the treatment of solid tumors (https://clinicaltrials.gov/) (results of completed studies were not available at time of publication).

Modulation of ECM Stiffness

The desmoplastic stromal response is common to several types of solid tumors, including pancreatic and breast cancers, and is a major impediment to treatment as it increases tissue

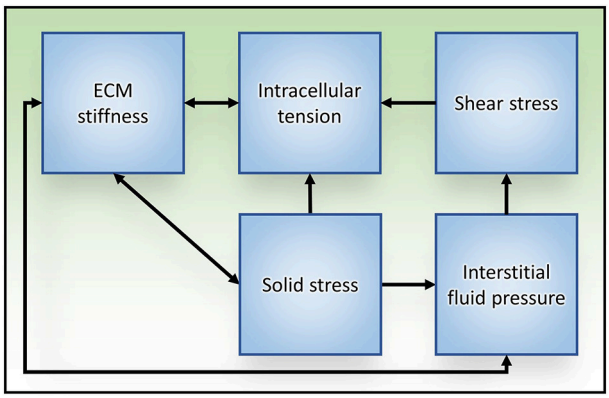

FIGURE 4 | Relationships between mechanical stress and tissue responses during tumor progression to metastasis. Increased intracellular tension is produced through actomyosin contraction in response to both biochemical and mechanical stimuli. ECM stiffening stimulates cells to generate higher intracellular tension to exert stronger traction forces on their surroundings, which subsequently exacerbates ECM stiffness. Solid stress is caused in part by unchecked proliferation of cancer cells that results in expansion of the tumor mass, compression of the tumor interior and distention of the surrounding stromal tissue. ECM stiffness can increase solid stress by augmenting the resistance to tumor expansion. Reciprocally, tumor expansion causes circumferential ECM tension and tissue stiffening. As a result of tissue compression and ECM stiffening, blood and lymphatic vascular function is impaired (due to vascular crushing) and interstitial fluid pressure is increased. Aberrant fluid flow throughout the interstitial spaces and within the obstructed tumor vasculature increases the shear stress experienced by tumor cells.

stresses and impairs drug delivery. Increased collagen deposition by stromal fibroblasts largely contributes to the fibrosis in the tumor microenvironment. Several drugs have emerged as potent stromal inhibitors. In a mouse model of breast cancer, targeting COX-2 with celecoxib significantly reduced αSMA-positive cancer-associated fibroblasts, inhibited immune cell recruitment and decreased collagen deposition, tumor growth and metastasis (Esbona et al., 2016). Celecoxib (Celebrex), which is clinically approved to treat inflammatory diseases, has been clinically demonstrated to improve cancer treatment outcomes but can cause serious adverse cardiovascular events, potentially reducing its utility as an anti-cancer agent (reviewed in Chen et al., 2014). Inhibitors of TGF-beta and hedgehog signaling (in combination with standard chemotherapy) are also attractive therapeutic targets to prevent desmoplasia, solid stress and tumor progression (Ko et al., 2016, reviewed in Neuzillet et al., 2015).

Disruption of ECM stiffening using LOX inhibition, (e.g., BAPN) is a potential therapeutic strategy that has been shown to greatly reduce tumor aggression in pre-clinical models of breast cancer (Levental et al., 2009; Pickup et al., 2013; Mouw et al., 2014). Another approach to targeting ECM remodeling is inhibition of matrix metalloproteinase; however, these inhibitors have shown little clinical promise (reviewed in Coussens et al., 2002). Lastly, given the recent success of immunotherapies in the treatment of cancer and the association between ECM stiffness, immune cell infiltration and tumor grade (Acerbi et al., 2015), it is conceivable that drugs which inhibit immune cell infiltration or modulate immune cell responses (e.g., simvastatin, metformin) may also improve cancer outcomes (Incio et al., 2015).

A potential limitation to all of these approaches is the high variability in tumor phenotype between tumors, and even within the same tumor, which could reduce therapeutic effectiveness. This is exemplified by heterogeneity in cellular response to stiffness between GBM tumors of same type (Grundy et al., 2016), likely owing to underlying genetic variability. Nevertheless, these approaches hold much promise for the treatment of cancers and the most effective therapeutic strategies will probably impact multiple sources of stress within the tumor microenvironment.

SUMMARY

Within the tumor microenvironment, tumor cells are exposed to a number of different mechanical stimuli including cell–cell and cell-ECM tension, compression stress from the expanding tumor mass, interstitial fluid pressure, and shear stress. Furthermore, positive feedback exists between these stimuli and the responses they elicit (**Figure 4**), such as increased cellular actomyosin contractility and ECM stiffening, which exacerbates tumor progression and aggression. While this review focused on solid tumors, these physical interactions are universally applicable to all types of malignancies. Thus, understanding the physical interactions between components of the tumor microenvironment and the molecular mechanisms regulating cellular responses to mechanical inputs will be key to developing effective therapeutics to treat all cancers.

AUTHOR CONTRIBUTIONS

JN and ID: drafted the article. JN, JM, and VW: revised and edited the article.

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MINI REVIEW
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Overcoming Barriers of Age to Enhance Efficacy of Cancer Immunotherapy: The Clout of the Extracellular Matrix

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There is a growing list of cancer immunotherapeutics approved for use in a population with an increasing number of aged individuals. Cancer immunotherapy (CIT) mediates tumor destruction by activating anti-tumor immune responses that have been silenced through the oncogenic process. However, in an aging individual, immune deregulation is positively correlated with age. In this context, it is vital to examine the age-related changes in the tumor microenvironment (TME) and specifically, those directly affecting critical players to ensure CIT efficacy. Effector T cells, regulatory T cells, myeloid-derived suppressor cells, tumor-associated macrophages, and tumor-associated neutrophils play important roles in promoting or inhibiting the inflammatory response, while cancer-associated fibroblasts are key mediators of the extracellular matrix (ECM). Immune checkpoint inhibitors function optimally in inflamed tumors heavily invaded by CD4 and CD8 T cells. However, immunosenescence curtails the effector T cell response within the TME and causes ECM deregulation, creating a biophysical barrier impeding both effective drug delivery and pro-inflammatory responses. The ability of the chimeric antigen receptor T (CAR-T) cell to artificially induce an adaptive immune response can be modified to degrade essential components of the ECM and alleviate the age-related changes to the TME. This review will focus on the age-related alterations in ECM and immune-stroma interactions within the TME. We will discuss strategies to overcome the barriers of immunosenescence and matrix deregulation to ameliorate the efficacy of CIT in aged subjects.

Keywords: aging, extracellular matrix, cancer immunotherapy, immunosenescence, tumor microenvironment, elderly

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INTRODUCTION

Statistics Showing Age-Related Increase in Incidence of Cancer

Cancer can be considered an age-related disease. In general, the incidence of cancer increases with age, up until the age of 75, with a 39% lifetime risk of being diagnosed with any type of cancer. The median age of any cancer diagnosis is 66 years (Howlader et al., 2017). **Figure 1** shows the age distribution of incidence of cancer diagnosis across various cancers. Moreover, the elderly

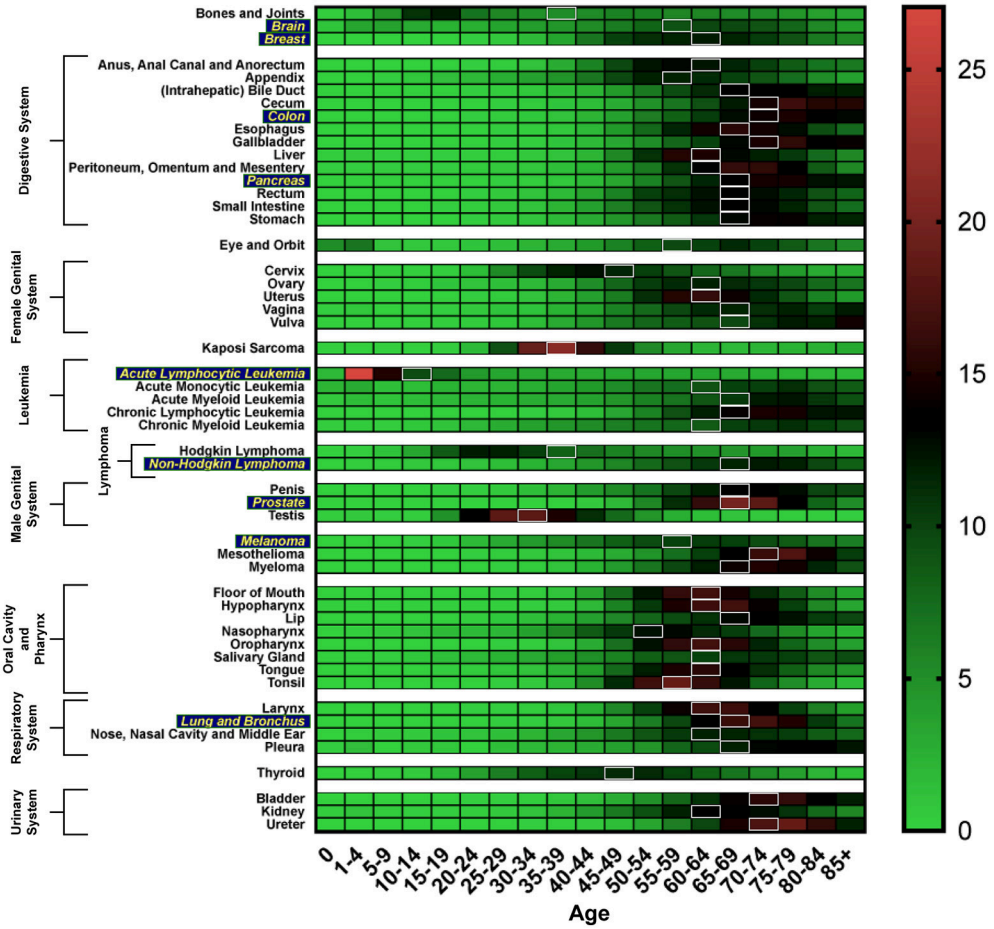

FIGURE 1 | Heat map depicting incidence of cancer diagnosis by age group, analyzed using the SEER database (Howlader et al., 2017). White boxes represent the median age range of cancer incidence. Cancer types more susceptible to the aging-related impact on efficacy of CIT are in yellow.

population in the United States is expected to grow to such a degree that by 2050, the estimated population aged 65 and over will double its size as compared to that in 2012 (White et al., 2014).

Age Biases in Cancer Immunotherapy Clinical Trials

Older patients are substantially underrepresented in cancer treatment studies, in particular cancer immunotherapy (CIT) clinical trials. The Southwest Oncology Group conducted a study to analyze the data on patient enrollment in clinical trials from 1993 to 1996 (Hutchins et al., 1999). They found that patients aged 65 or older made up only 25% of the enrolled population compared to 63% in the respective United States cancer population. This underrepresentation of elderly individuals was most notable in breast cancer where only 9% of elderly women, out of the 49% in the United States breast cancer patient population, were enrolled into clinical trials. Similar trends have subsequently been found in studies analyzing trials sponsored by the National Cancer Institute both here in the United States (Lewis et al., 2003) and in Canada (Yee et al., 2003), as well as in drug registration trials with the United States Food and Drug Administration (Talarico et al., 2004).

In this mini-review we provide a systematic overview of key age-related immune alternations that cause landscape remodeling of the tumor microenvironment (TME). Adding to other recent reviews detailing the impact of aging on CIT (Hurez et al., 2016; Daste et al., 2017; Elias et al., 2017; Yousefi et al., 2017), here we focus on how age-related changes to the extracellular matrix (ECM) affect immune and other stromal cells that are the chief targets for CIT.

EFFECTS OF AGE ON THE TUMOR MICROENVIRONMENT

Extracellular Matrix

Studies to date have largely overlooked the age-related changes to the ECM with respect to CIT efficacy (Quail and Joyce, 2013). Changes in cytokines, laminins, and collagens that alter the surrounding matrix (Sprenger et al., 2010), mitogens, and/or key enzymes [i.e., matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs)] (Ruhland et al., 2016),

all contribute to senescent cells creating a local inflammatory phenotype more permissive to tumor growth. The angiogenic stimulus acts as an inflammatory activator, causing inflammatory cells to produce growth factors, cytokines, chemokines, and MMPs contributing to ECM degradation or partial modification of ECM molecules that are all promoting tumor growth (Neve et al., 2014; Mongiat et al., 2016). Activation of the inflammasome (i.e., IL-1, IL-18, and NKκB) from mechanical stress on the aging ECM results in inflammation and immunodeficiency in aged subjects (Moreau et al., 2017). As a result, T cell mobility and apoptosis in elderly individuals is decreased. Stromal-derived factors also influence the ECM's pro-tumorigenic TME, which have been implicated in the regulation of senescence and malignant transformation (Acosta et al., 2008; Kuilman et al., 2008; Tchkonia et al., 2013).

Photoaging-related changes in collagen are associated with a reduction of fibrillar collagen mediated by secreted proteases, most notably MMP12 (Pittayapruek et al., 2016; Freitas-Rodríguez et al., 2017). Decreased collagen I levels and altered levels of laminins in the elderly negatively affect resident and infiltrating immune cells (Sprenger et al., 2010). On the other hand, MMPs' degradative potential in tumorigenesis tend to be dependent on the tumor type, rather than on age (Reed et al., 2000; Parikh et al., 2017). The lack of a specific and pervasive age-related effect on MMP regulation is of importance because the ability of solid tumors to express gelatinases (MMP2/9) is correlated with adverse pathological characteristics and clinical outcomes (Mancini and Di Battista, 2006). Studies on prostate tumors in aged mice further support the role of collagen I and gelatinase in vascular in-growth and tumor progression (Reed et al., 2007). It can be postulated that modification of the TME by modulating the ECM can reduce a blood vessel's efficiency; thus, impinging on drug delivery and efficacy. Taken together, ECM regulation is a therapeutic niche that can be exploited to decrease angiogenesis and subsequent tumor progression. Moreover, this and the upcoming sections below highlight the relevance of targeting MMPs and other ECM degrading proteins to specifically alter the immune landscape within the TME through ECM modulation to enhance CIT efficacy (Pickup et al., 2014; Bhome et al., 2016). This may have a direct impact on the aged TME.

The Predominant Immune and Stromal Cells Within the TME Relevant to CIT

Cancer Associated Fibroblasts

Fibroblasts are a stromal cell population within the ECM; activated fibroblasts are known as cancer associated fibroblasts (CAFs) due to their role in maintaining a permissive TME for cancer cell survival and proliferation (Tao et al., 2017). With an accumulation of senescent fibroblasts during aging, it has been shown that senescent fibroblasts co-cultured with pre-malignant epithelial cells caused a marked increase in proliferation and tumorigenicity *in-vitro* and *in-vivo* (Lawrenson et al., 2010). Senescent CAFs from aged humans produce growth-promoting chemokines, such as Ccl-5, that also cause enhanced angiogenesis (Eyman et al., 2009). In addition, breast cancer cell lines mixed with fibroblasts in a xenograft model demonstrated an increase in tumorigenicity mediated by MMPs (Lawrenson et al., 2010). Therefore, CAF accumulation enables a more permissive oncogenic TME to contribute to the rise of cancer incidence in the elderly and specifically impacts the immune landscape, as discussed below.

Effector T Cells—T Helper Cells and Cytotoxic T Cells

CIT has predominantly been focused on targeting effector T cells. However, the condensed ECM in elderly individuals serves as a biophysical barrier preventing T cells from invading the tumor and localizing around target tumor cells (Bhome et al., 2016). In aged mice, cytotoxic CD8 T cells have been shown to decrease in number with age (Lustgarten et al., 2004). The expansion of CD8 T cells is also impacted by the reduced expression of CD40 in older individuals (Elias et al., 2017). In addition, naïve CD4 T cells in aged mice proliferate less, produce significantly reduced levels of IL-2, and show poorer differentiation than those cells from young mice (Lustgarten et al., 2004). For effector T cells and natural killer (NK) cells to take action against solid tumors, they must leave the vasculature, enter the interstitium, and infiltrate the tumor mass. However, along the way they face many obstacles, most notably the impediment of the ECM (Edsparr et al., 2011). Degradation of the ECM adjacent to the tumor islets dictates T cell migration behavior and changes in T cell morphology, restricting its access to cancer cells (Salmon, 2012). Typically, TNFα and IL-6 bear anti-inflammatory roles in the late stages of disease and help mitigate the loss of function in CD4 T cells (Hurez et al., 2016), while PD-1/L1 increases proliferation of CD8 T cells (Francisco et al., 2009). With the role of the ECM as a barrier against effector T cells, immunosenescence plays a vital role in curtailing the effector T cell response within the TME and promotes a more permissive immunosuppressive microenvironment for tumorigenesis in elderly hosts.

Regulatory T Cells

Regulatory T cells (Tregs), a subset of CD4 T cells characterized by FoxP3 and CD25 expression, suppress the anti-tumor immune response and restricts the expansion and differentiation of effector T cells (Ha, 2009). The contribution of Tregs to the age-associated decline in immune response is widely contested by some studies. In general, an increase in Tregs is associated with a worse prognosis for cancer patients, most notably those with metastatic melanoma (Ha, 2009).

In mice, an age-related increase in Treg number and expression contributed to greater immune suppression compared to Tregs from young mice (Garg et al., 2014). Moreover, immune deficiency was noted in old vs. young mice due to elevated levels of effector Tregs; systemic depletion of Tregs may concurrently elicit deleterious autoimmunity (Tanaka and Sakaguchi, 2017). When using anti-CD25 to deplete Tregs, the elevation of IFNγ and IL-17 in aged mice restored the primary and memory anti-tumor T cell responses (Sharma et al., 2006; Hurez et al., 2017). Although Tregs from young and elderly individuals inhibited T cell proliferation similarly, the levels of IL-10 were lower in aged Tregs (Fessler et al., 2013). As our understanding of CIT evolves, more studies are required in

elderly subjects to support Treg mediation for attenuating tumor immune dysfunction and its subsequent effects on the ECM.

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of activated immature myeloid cells (Kusmartsev and Gabrilovich, 2006). During cancer, inflammation, and infection, the MDSC population expands and contributes to the negative regulation of immune responses (Gabrilovich and Nagaraj, 2009). Interestingly, Treg depletion increased MDSCs, but Treg depletion with MDSC depletion restored anti-tumor immunity and alleviated immune suppression in tumor-bearing aged mice (Hurez et al., 2012). This resulted in slowing tumor growth similar to only deleting Tregs in young hosts. Co-depletion of Tregs and MDSCs elevated cytotoxic CD8 T cells and IFNγ-producing CD4 and CD8 T cells in aged mice (Hurez et al., 2017). This potentially functions to modulate the TME's alterations of ECM components that affect signaling pathways for Arg and Nos levels. To our knowledge, co-depletion is the only immunotherapeutic approach that is primarily effective in aged, not young, hosts. Although the age effects of MDSCs on CIT in humans have not yet been reported, MDSCs are an attractive target to mitigate cancer-associated immune dysfunction in aged hosts.

Tumor-Associated Macrophages

Tumor-associated macrophages (TAMs) support tumorigenesis by promoting invasion and metastasis, tumor cell proliferation, and angiogenesis (Liu and Cao, 2014), while reducing the cytotoxicity and viability of T cells and NK cells (Edsparr et al., 2011). The pro-inflammatory (anti-tumorigenic) TAM, classically known as M1 polarized, develops in response to elevated levels of IFNγ/TNFα; the anti-inflammatory (pro-tumorigenic) TAM, classically known as M2 polarized, develops in response to elevated levels of IL-4/TGFβ (Owyong et al., 2017). Cytokines, produced through an inflammatory response, are used as predictive biomarkers for tumor development or regression (Martins et al., 2016). Interestingly, bone marrow (BM) derived and splenic TAMs, specifically M2 macrophages, increase in elderly mice and are hyper-responsive to tumor-derived factors (Jackaman et al., 2017). This was further validated in elderly patients, in which immunosuppressive M2 macrophages are elevated in lung, muscle, and lymphoid tissues (Jackaman et al., 2017). Consequentially, the age-related decrease in macrophages results in a reduction of major histocompatibility class II (MHCII) for antigen recognition alongside a reduction in IL-12 (Daste et al., 2017). We discuss the impact of decreased MHCII antigen presentation on CIT later in this mini-review.

Mice bearing different stages of Dalton's lymphoma showed that TAMs in old mice are inhibited with respect to cell binding cytotoxicity and expression of inducible nitric oxide synthase, a functional marker for M1 macrophages (Khare et al., 1999). Recent studies have illuminated a therapeutic potential utilizing anti-IL-10 to target tumor-associated M2 macrophages for a phenotypic change to M1 macrophages in young colon cancer-bearing mice (Guiducci et al., 2005). This was supported by another approach targeting TAMs with IL-12 to reprogram TAMs *in situ* within a young-aged model system (Watkins et al., 2007). However, whether this approach will be an effective treatment option in elderly individuals still requires further validation.

Tumor-Associated Neutrophils

Tumor-associated neutrophils (TANs) are phenotypically distinct from circulating neutrophils in their cytokine and chemokine profiles (Hurt et al., 2017). TANs are not well characterized throughout human tumor development or in young vs. elderly hosts.

Although studies of TANs are currently limited to young murine models, they have been shown to exhibit functional roles in either supporting cancer initiation through angiogenesis and metastasis or restricting cancer progression through expression of anti-tumor and cytotoxic mediators (Mantovani et al., 2011; Sionov et al., 2015). The most notable murine study examining TAN polarization (i.e., N1 vs. N2) indicates that anti-TGFβ can augment CIT by promoting a pro-inflammatory N1 polarization (similar to M1 macrophages) and subsequently promote the recruitment and activation of intra-tumoral CD8 T cells (Fridlender et al., 2009). In a pancreatic cancer mouse model, TANs were potent promoters of tumor angiogenesis through elevated expression of MMP9 (Nozawa et al., 2006). This was also seen in human hepatocellular carcinoma where there was a correlation between MMP9, neutrophils, and angiogenesis (Kuang et al., 2011). While it has been shown that TANs can be recruited to the tumor from splenic or BM-derived pools, further studies are required to determine if the elderly host's TME promotes an immunosuppressive N2 polarization (Cortez-Retamozo et al., 2012; Jackaman et al., 2017). With aging-associated deregulation of MMPs, N2-polarized TANs can enhance tumorigenesis in response to elevated levels of MMPs. These studies also reiterate the importance of targeting the ECM through MMP inhibition, gelatinases in particular, to specifically cause a differential immune response/recruitment to the TME.

CONTROL OF CANCER PROGRESSION THROUGH CIT

Advances in CIT have taken groundbreaking strides in treating primary and metastatic cancers. Of specific interest are the checkpoint inhibitors, which function by removing the "brakes" on the immune system, subsequently modulating the amplitude of immune responses (Yousefi et al., 2017). Another key aspect of CIT, chimeric antigen receptor T (CAR-T) cell therapy, uses an adoptive cellular therapy approach to enhance the adaptive immune response. **Figure 2** provides the tumor-stromal and tumor-immune cell dynamics within the TME for young vs. elderly individuals and the interactions with targets of currently approved CIT drugs. Although other checkpoint inhibitors and immunotherapeutic approaches in the preclinical and clinical space exist, below we discuss the current molecular targets of immune checkpoints and CAR-T that have been approved by the FDA for therapeutic use.

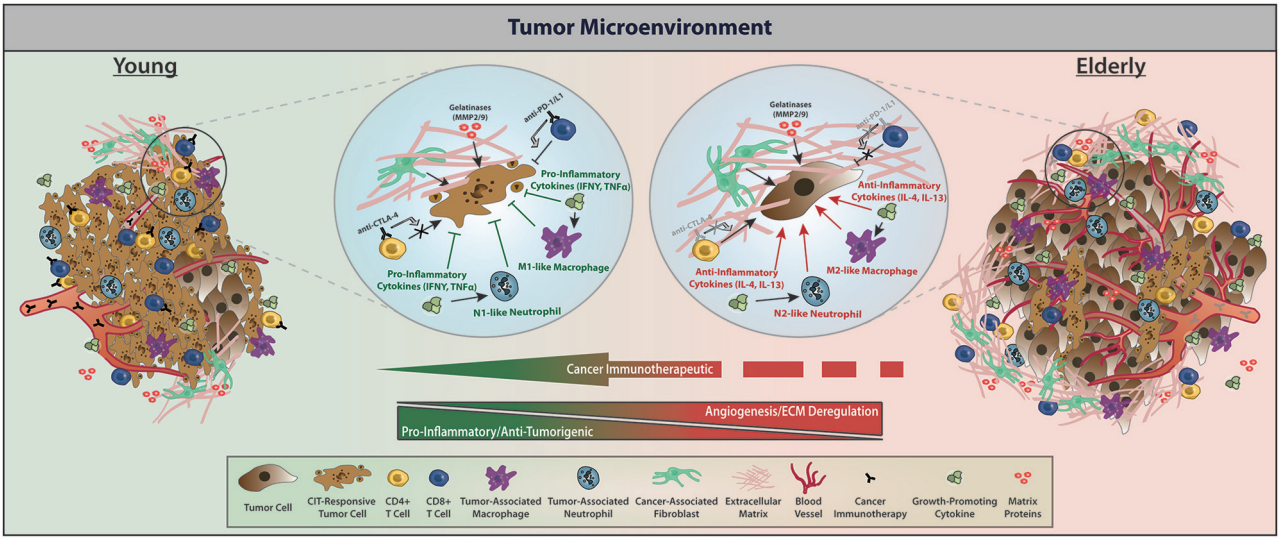

FIGURE 2 | With respect to factors affecting CIT, the TME of young vs. elderly hosts differs predominately in immune infiltration and cytokine profile, regulation of angiogenesis and the ECM, as well as interactions between tumor and stromal cells. Within the young TME, increased presence of CIT-responsive tumor cells, less angiogenesis and ECM deregulation, elevated immune infiltration, and elevated pro-inflammatory cytokines give rise to a relatively more CIT-responsive TME, subsequently contributing to apoptotic tumor cells with reduced proliferation. Within elderly TMEs, relatively bigger tumors but with fewer CIT-responsive tumor cells, more angiogenesis and ECM deregulation, decreased immune infiltration, and elevated anti-inflammatory cytokines contribute to reduced apoptosis and increased proliferation subsequently enabling tumor growth. Treatment with CIT in elderly individuals triggers a phenotypic landscape remodeling toward a TME with young characteristics through enhancing the function of effector T cells following CIT and promoting a pro-inflammatory TME. Within elderly hosts, the tumor stroma permits a deregulated ECM that creates a biophysical barrier preventing effective function of effector T cells, but CIT combinations may help alleviate these age-related TME dysfunctions and decrease tumor burden. Of note—while we acknowledge that there are dormant and/or tumor initiating cells, we only depict CIT responders vs. non-responders within the TME (Gonzalez et al., 2017).

Current Cancer Immunotherapeutic Molecules and Target Cell Types

Checkpoint Inhibitors

α*PD-1/L1*

Changes that affect the CD8 T cell compartment occur earlier than those affecting the CD4 T cell compartment. This results in only 30% of elderly patients retaining a CD8 population that is potentially efficient for CIT checkpoint inhibition (Daste et al., 2017). Programed cell death 1 (PD-1), predominantly functioning through CD8 immune modulation by binding its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC), reduces T cell proliferation and promotes an evasion of the immune response (Sgambato et al., 2017). In aging mice, $CD3^{+}CD8^{+}$ T cells up-regulate the expression of PD-1 (McClanahan et al., 2015). Nivolumab, an FDA approved anti-PD-1 inhibitor, showed similar toxicity levels between younger (<65 years old) and older (65+ years old) patients (Sgambato et al., 2017). Data examining 30 patients with thymic epithelial tumors demonstrated that chemotherapy could change the TME and induce PD-L1 expression and tumor-infiltrating immune cells. Taken together, inhibiting PD-1/L1 expression increases CD8 T cell activation and potentially restores CIT efficacy from aging-related immunosenescence (Maleki Vareki et al., 2017).

α*CTLA-4*

Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) delivers direct inhibitory signals to T cells, sequesters CD80 and CD86 from the surface of antigen-presenting cells, down-regulates T helper (Th) cells, and enhances Treg immunosuppressive activity (Elias et al., 2017). Additionally, the lack of CD28 expression from about 50% of the total CD4 T cell pool in adults over 65 years of age contributes to reduced T cell function in the elderly and less than optimal T cell activation (Czesnikiewicz-Guzik et al., 2008). Similarly, an increased expression of senescent CTLA-4 in the elderly contributes to inefficient activation of T cells when an antigen is presented (Daste et al., 2017).

Ipilimumab, an FDA approved anti-CTLA-4 inhibitor, has been shown to extend overall survival in elderly patients with metastatic melanoma (Chiarion Sileni et al., 2014). Although immunosenescence is a critical factor when assessing the efficacy of ipilimumab, treatment has provided a consistent survival benefit in patients (Chiarion Sileni et al., 2014). A clinical study in multiple myeloma patients showed that patients with higher expression of IFNγ, IFNγ-inducible genes, and Th1-associated markers achieved better clinical response to ipilimumab, which suggests that an activated immune microenvironment can be used as a biomarker of response (Maleki Vareki et al., 2017).

Combination with standard-of-care

Many studies on the effects of aging do not use the standard-of-care combination therapy that relies on radiation therapy (RT) or (neo)adjuvant chemotherapy followed by CIT. This is

attributable to our incomplete knowledge of how aging affects tumor-specific immunopathology to increase therapeutic efficacy and safety.

In the limited number of studies examining the efficacy of combination CIT in aged hosts, Treg depletion combination therapy has shown the most promise. A study in aged mice bearing B16 melanoma treated with anti-PD-L1 failed (Figueiredo et al., 2016). However, anti-PD-L1 treatment efficacy was partially restored in aged mice with lymphoma when combined with anti-CTLA-4 therapy (Mirza et al., 2010; Figueiredo et al., 2016). Most multifaceted CIT studies exhibited synergistic outcomes in young mice, but ineffective outcomes in elderly hosts. For instance, reducing immune suppression, while enhancing adjuvant effects using TLR agonists in a breast cancer mouse model worked in young, but not aged hosts (Pawelec et al., 2009). In addition, IL-2 combined with CD40 agonists to augment the immune system only showed efficacy in young mice bearing metastatic renal cell carcinoma (Murphy et al., 2003). However, treatment with combinatorial therapy exhibited toxic side effects and rapid deterioration in many organs leading to multi-organ toxic syndrome (MOTS) with marked increases in pro-inflammatory cytokines (Bouchlaka and Murphy, 2013). The severe cytokine snowball was alleviated through macrophage depletion, highlighting the critical role myeloid cells play within the TME as potential targets for CIT. More specifically, TNFα inhibition with etanercept, alongside an agonist for IL-2 and CD40, played a critical role in mediating a robust anti-tumorigenic effect in aged lung carcinoma-bearing mice with a significant increase in overall survival (Bouchlaka et al., 2013).

The limited number of studies using multifaceted immunotherapy highlights the lack of understanding of aging-related immune dysfunction to promote the development of novel CIT regimens for elderly hosts. With the field of CIT ever growing, efficacious combination therapy requires further specification with definitive biomarkers to stratify aged patients based on age-specific agents, doses, and schedules, while also assessing toxicity.

Chimeric Antigen Receptor T Cells

CAR-T cell immunotherapy is indicated predominately for hematological indications (Owyong et al., 2017), and relies on engineering T cells with a tumor-specific antigen receptor that interacts with tumor-associated antigens (TAAs), independent of human leukocyte antigen (HLA) expression (Yu et al., 2017). This is of clinical relevance because one of the major impediments of TCR-based immunotherapy is TCR's recognition of antigens in an HLA-dependent pathway. However, tumors tend to down-regulate HLA. Even more so, HLA-DR is down-regulated in aged individuals (Villanueva et al., 1990). This may explain the lack of clinical success and/or attempts of checkpoint inhibition in the elderly. Therefore, CAR-T cell immunotherapy could lead to new therapeutic options by bypassing an HLA-restricted pathway.

Although CAR-T cell CIT has thus far only been approved for hematological indications, pre-clinical trials for solid tumors targeting epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), and mesothelin (MSLN) have all shown immense promise (Yu et al., 2017). EGFR-target TAAs in glioblastoma (GBM), HER2-target TAAs in GBM and sarcoma, and MSLN-target TAAs in mesothelioma have all demonstrated an effective on-target therapeutic response with increased overall survival leading to the development of clinical trials (Yu et al., 2017). Limitations of CAR-T CIT include the lack of an ideal TAA, an inefficient delivery method for trafficking of CAR-T cells to the tumor site, and off-target toxicities associated with immunotherapy. To our knowledge, there are currently no published studies examining the effects of aged CAR-T cells and whether there is an age-related effect on the efficacy of CAR-T cells generated from elderly hosts. However, the tumor indications listed above for CAR-T CIT predominantly have a median age of incidence between 55 and 74 (**Figure 1**).

POST IMMUNOTHERAPY CHANGES TO IMMUNE AND STROMAL CELLS WITHIN THE TME AND ITS EFFECT ON THE ECM

With gaps in the clinical understanding of ECM alternations and its effects on the immune landscape in elderly individuals, our current knowledge relies heavily on pre-clinical models. Previous work has shown that anti-CTLA-4 may kill effector Treg cells or attenuate their suppressive activity. By combining Treg-cell targeting (i.e., by reducing Treg cells or attenuating their suppressive activity in tumor tissues) with the activation of tumor-specific effector T cells using immune checkpoint blockade, CIT has the potential to be more effective. One strategy for evoking effective tumor immunity without autoimmunity is to specifically target terminally differentiated effector Treg cells rather than all FoxP3 T cells. Additionally, one could hypothesize that if a cancer cell can be modified to secrete less collagen, then there will be decreased support for vascular in-growth and subsequent tumor progression. A recently developed CAR-T method using both CAR and IL-12 (referred to as armored CAR-T cells) has been shown to help T cells pass the biophysical barrier (Yeku et al., 2017). These armored CAR-T cells use heparanase, specific enzymes from T cells, to improve their ability in degrading the ECM, thereby promoting effector T cell infiltration into the tumor bed (Zhang and Xu, 2017).

Contrary to young hosts, aged individuals exhibit a toxic elevation in pro-inflammatory cytokines following a multifaceted immunotherapeutic approach (Bouchlaka and Murphy, 2013). Current studies using CIT in elderly pre-clinical and clinical studies need to be aware of the low efficacy potentially attributable to over-activation of the immune system. MOTS should also be considered in individuals receiving RT or chemotherapy and whether these effects can be ameliorated with examining novel pro-inflammatory cytokines, such as TNFα and IFNγ. Strategies to reduce immune dysfunction

must be tailored to account for age-related and tumor-related immune dysfunctions for optimal utility. Thus, improved and efficacious CIT for aged hosts, who are at the greatest risk for cancer, is a realistic goal that can be met with a better understanding of the specific effects of age on the stroma and tumor-related immune dysfunction. This will allow us to tailor novel therapeutic approaches in the aging population. Lastly, we advocate for the use of pre-clinical models encompassing a pertinent age spectrum to a specific disease indication; thus, meticulously discerning the therapeutic efficacy as well as pharmacodynamic and pharmacokinetic parameters of CIT in the intended patients.

AUTHOR CONTRIBUTIONS

MaO and VP conceptualized the ideas and designed the outline of the article. MaO, GE, MiO, and VP drafted the article with insight from AA and VS. All authors have approved the final article.

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The Bioelectric Code: Reprogramming Cancer and Aging From the Interface of Mechanical and Chemical Microenvironments

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Cancer is a complex, heterogeneous group of diseases that can develop through many routes. Broad treatments such as chemotherapy destroy healthy cells in addition to cancerous ones, but more refined strategies that target specific pathways are usually only effective for a limited number of cancer types. This is largely due to the multitude of physiological variables that differ between cells and their surroundings. It is therefore important to understand how nature coordinates these variables into concerted regulation of growth at the tissue scale. The cellular microenvironment might then be manipulated to drive cells toward a desired outcome at the tissue level. One unexpected parameter, cellular membrane voltage (Vm), has been documented to exert control over cellular behavior both in culture and *in vivo*. Manipulating this fundamental cellular property influences a remarkable array of organism-wide patterning events, producing striking outcomes in both tumorigenesis as well as regeneration. These studies suggest that Vm is not only a key intrinsic cellular property, but also an integral part of the microenvironment that acts in both space and time to guide cellular behavior. As a result, there is considerable interest in manipulating Vm both to treat cancer as well as to regenerate organs damaged or deteriorated during aging. However, such manipulations have produced conflicting outcomes experimentally, which poses a substantial barrier to understanding the fundamentals of bioelectrical reprogramming. Here, we summarize these inconsistencies and discuss how the mechanical microenvironment may impact bioelectric regulation.

Keywords: mechanotransduction, bioelectricity, morphodynamics, mechanical stress, morphogenesis

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INTRODUCTION

Membrane voltage (Vm) is defined as the electrical potential difference between the cytoplasm and extracellular space (Levin, 2007). This bioelectric field has been demonstrated to transmit extensive patterning information between cells at tissue-scale. For example, disrupting Vm gradients has been shown to impair regeneration and development, causing the growth of functioning ectopic organs such as eyes in *Xenopus* and head/brain structures in planaria (Beane et al., 2011; Pai et al., 2012). Excitingly, manipulating Vm has also been shown to induce limb regrowth in non-regenerative species (Tseng and Levin, 2013), prompting much interest in bioelectricity as a future

therapeutic tool to restore organs deteriorated during the aging process or accidentally damaged. In addition, manipulating Vm can prevent the formation of tumors (Chernet and Levin, 2014), suggesting promising future cancer treatments. However, it is unclear what Vm manipulation is needed to produce a desired outcome; separate studies report contradicting observations resulting from similar alterations of Vm. In particular, comparable Vm manipulations have been linked to both apoptosis and proliferation, which are seemingly opposite phenotypes (Bortner et al., 1997; Wang et al., 1999; Yu et al., 1999a,b; Thompson et al., 2001). Adding another level of complexity, cellular Vm varies significantly between cell types and with progression of the cell cycle. How then might this broad range of observations surrounding Vm be reconciled into a consistent theory for possible implementation in future medical therapies to combat aging and disease?

Although it is generally accepted that experiments in culture do not recapitulate the complexity of the cellular surroundings *in vivo,* a number of parameters altered by traditional culture methods are often not accounted for in experimental design and data interpretation. The ability of chemical components of the cellular microenvironment to impact phenotype is a classic topic of study. For example, factors such as hypoxia (Pang et al., 2016) and pH (Damaghi et al., 2013) have been demonstrated to drive cancer progression. However, it is becoming increasingly well recognized that physical signals also contribute to tumorigenesis: substratum stiffness and pressure are two key components of this *mechanical* microenvironment (Discher et al., 2005; Piotrowski-Daspit et al., 2016). Further studies regarding how mechanical parameters impact Vm are needed to more fully understand the processes that contribute to the emergence of bioelectric field gradients. Additional factors in the microenvironment such as the presence of multiple cell types and microbiota impact the transduction of bioelectric signals (Chernet and Levin, 2014). However, we lack a full understanding of how all of these regulatory cues function together to translate changes in Vm into physiological cellular states.

Not only do variables in the microenvironment change in studies of bioelectricity, but Vm itself may impact several variables within the microenvironment. The conflicting outcomes of previously published experiments then may have resulted from unintentionally altering different regulatory cues simultaneously. In addition, at the cellular level, the output of a given Vm input is often represented as a single biological state, such as growth or death. Apoptosis and proliferation are generally treated as bimodal opposites. However, this interpretation is incomplete at best. Rather than behaving as opposing cellular functions, division and programmed death occur in a coordinated fashion to sculpt growth and form, tuned by the complex web of surrounding microenvironmental signals. This review summarizes the seemingly conflicting reports of bioelectric signaling and discusses two topics that may help explain these inconsistencies: the mechanical microenvironment and the importance of cellular death.

Abbreviations: ECM, extracellular matrix; HDAC, histone deacetylase; Vm, membrane voltage.

BIOELECTRIC MANIPULATION: THE FUTURE OF CANCER AND GERIATRIC THERAPIES?

The idea that electricity could contain biological information was first demonstrated experimentally in the late 1700s, when Luigi Galvani electrically stimulated muscle contraction in an amputated frog leg (Verkhratsky et al., 2006). Although initially met with skepticism, the idea of "animal electricity" (Galvani, 1791) eventually led to our current understanding of how the brain and body are connected. Electrical properties are often only associated with excitable cells, such as neuronal tissue. However, all cells possess an electrical potential across the plasma membrane, and thus generate and receive bioelectric signals (Levin, 2010). One of the earliest indications that this electrical potential might serve as a growth-directing field came in 1903, when A.P. Mathews identified an electrical gradient in regenerating hydra (Mathews, 1903).

Modern experiments (Beane et al., 2011, 2013; Pai et al., 2012; Tseng and Levin, 2013; Adams et al., 2016) support the historical hypothesis (Mathews, 1903; Burr and Northrop, 1935) that electrical patterns regulate not only muscle movement and excitable cells as demonstrated by Galvani, but growth and form of the organism as a whole. In the 1970s and 1980s, separate studies measured Vm of different cell types (summarized in Binggeli and Weinstein, 1986). These data showed that cancerous and proliferative tissues were generally more positively charged than non-proliferative cells (Levin, 2012a; Adams and Levin, 2013). Consistent with this observation, it was found that experimentally causing cells to become more negatively charged reversibly blocked cellular proliferation (Cone and Tongier, 1971), which was thought to result from the blockage of ions such as Na^+ believed to be involved in DNA synthesis (Binggeli and Weinstein, 1986). When the cytoplasm is more positively charged than the extracellular space, Vm is referred to as "depolarized" (Adams and Levin, 2013). Conversely, when the cytoplasm is more negatively charged than the extracellular space, Vm is considered to be "hyperpolarized" (**Figure 1**). It was therefore hypothesized that a threshold Vm separates "normal" quiescent or resting cells from proliferative or cancerous tissues (Binggeli and Weinstein, 1986). However, this hypothesis did not account for several factors. First, although Vm was measured directly using patch clamping, these measurements were all conducted under different conditions in separate studies. Some measurements were taken directly *in vivo* under live dissection conditions (tumors), others were of single cells cultured on glass (fibroblasts), and some involved tissue slices, explanted organs (corneal epithelium), or intact 4–16 cell-stage embryos (Binggeli and Weinstein, 1986). Notably, it remains unclear how bioelectrical patterns are impacted by age both in cells and whole organisms, adding yet another uncontrolled variable to these data. Even during this early phase of Vm research, it was appreciated that Vm "deteriorates" rapidly under non-physiological conditions: an observation generally not accounted for in reports of that era (Binggeli and Weinstein, 1986). Although cancerous cells were observed to be more depolarized

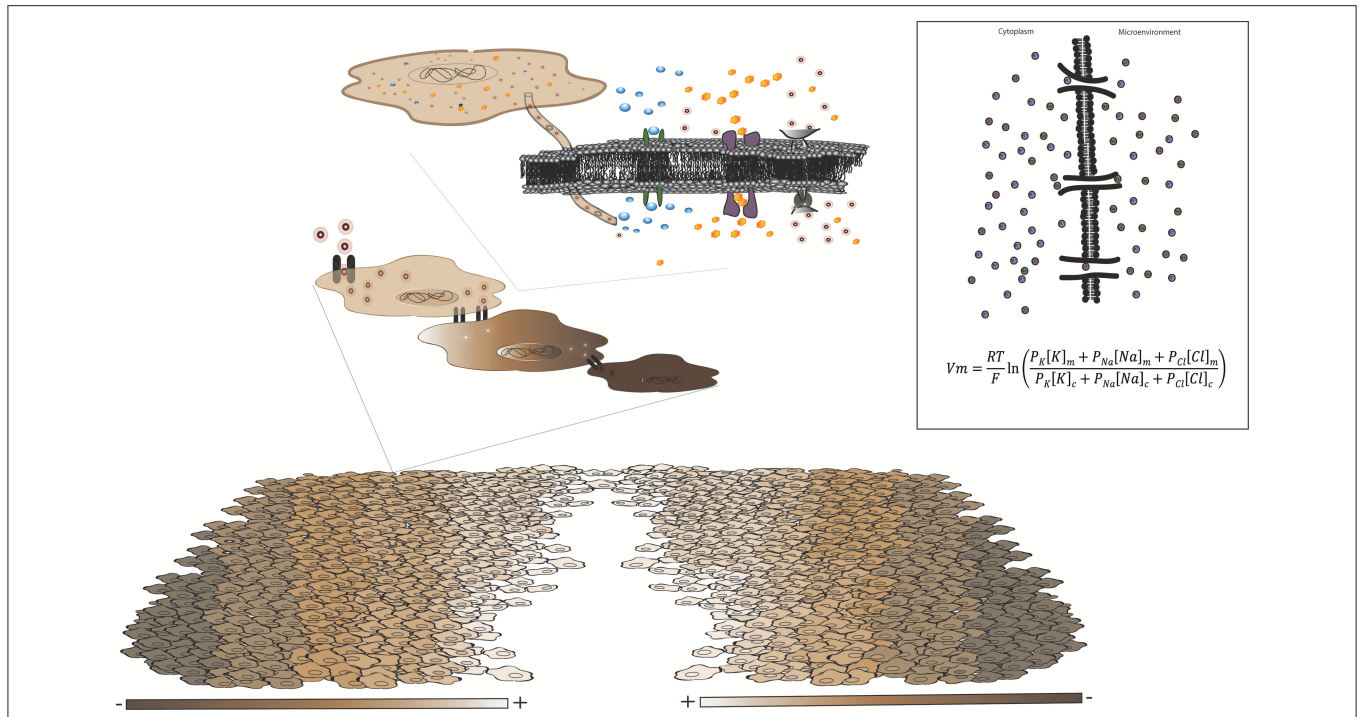

FIGURE 1 | The value of Vm in a given cell is determined by the intra- and extracellular concentrations of all ion species present (predominantly Na^+, K^+, and Cl^-) according to the Hodgkin-Katz-Goldman equation (Binggeli and Weinstein, 1986). Changes to Vm can propagate between neighboring cells via ion exchange through gap junctions, or local voltage gating of ion channels (Levin, 2014). This results in gradients of physiological electric charge within the tissue, referred to as the bioelectric field. For example, a wound creates a depolarized front of migrating cells (Chifflet et al., 2005), acting as a break in the tissue-wide bioelectric circuit.

than noncancerous cells (Binggeli and Cameron, 1980), this observation represented an average of the cell population rather than an absolute fixed value.

Today, we recognize that although cellular Vm usually ranges from approximately –90 to –10 millivolts (mV), these values can vary greatly depending on the cell type and physiological state of the cell (Yang and Brackenbury, 2013). Each cell regulates its resting Vm through a variety of mechanisms (Adams and Levin, 2006). ATP-dependent pumps allow the cell to push ions into or out of the cytoplasm, even against their electrochemical gradients. Cells also express a large variety of channels that allow the passage of ions across the plasma membrane. These ion channels may be gated in response to changes in extracellular ion concentrations, Vm, or mechanical stimuli (Coste et al., 2010; Pathak et al., 2014; Wu et al., 2016; Gudipaty et al., 2017). Thus, as might be anticipated, cellular Vm is neither spatially uniform throughout the cell nor static in time. This added level of complexity might carry additional biological signals. For instance, tumor cells confined in narrow microfabricated channels establish a polarized distribution of Na^+/H^+ pumps and aquaporins in the cell membrane, creating a net inflow of water and ions at the leading edge of the cell and an outflow at the trailing edge. This flux enables the migration of metastatic breast cancer cells through narrow channels in culture, independent of actomyosin contractility and integrin signaling (Stroka et al., 2014). Inhibiting the Na^+/H^+ exchanger involved in this process decreases the velocity of migration. In addition to spatial gradients, temporal variations in intracellular Vm play a significant role in cell cycle progression. Generally, cells are more hyperpolarized during S phase and more depolarized during mitosis, whereas G1 and G2 phases fluctuate partway between these extremes (Barghouth et al., 2015). This appears to be driven by changes in expression levels of ion channels for K^+, Na^+, and Cl^-, and gating of these channels in response to changes in cell volume or alterations in Vm (Ouadid-Ahidouch et al., 2001; Sundelacruz et al., 2009; Urrego et al., 2014; Barghouth et al., 2015). This cyclical variation in Vm observed during the cell cycle is believed to be required for a successful cell division (Barghouth et al., 2015). The regulation of Vm through time would appear then to be a critical part of bioelectrical signaling.

In addition, the role of mechanical factors in regulating both proliferation and Vm is becoming increasingly evident. During the early days of cell culture, an optimal cell density was known to be necessary for the growth of non-immortalized cells: not only did low cell density inhibit growth (few cell-cell contacts), but cells within an overly confluent monolayer also exhibited reduced proliferation (Todaro and Green, 1963). Intriguingly, it was later found that cells in a confluent monolayer are more hyperpolarized than individual cells (Blennerhassett et al., 1989)—an early indication that mechanical forces may help regulate bioelectric signaling. Despite the growing number of observations that Vm is a dynamic property influenced by features of the microenvironment, it is still generally accepted today that dividing or cancerous cells are more depolarized than

non-dividing tissue (Wang, 2004; Fraser et al., 2005; Ouadid-Ahidouch and Ahidouch, 2008; Sundelacruz et al., 2009; Yang and Brackenbury, 2013; Chernet and Levin, 2014) or quiescent cells (Barghouth et al., 2015). This concept is supported by recent experiments employing optogenetic control over ion channels, which have demonstrated that hyperpolarization decreases tumor incidence (Sundelacruz et al., 2009; Levin, 2012b; Chernet and Levin, 2014). Such studies generate excitement that bioelectric control could be implemented in medical strategies to combat cancer. This field also holds promise for restoring organs that are damaged or failing due to aging, potentially improving quality of life or even extending lifespan. However, will simply hyperpolarizing a tumor (using drug treatments or optogenetic methods) cause cancerous cells to enter a quiescent state or die? Would depolarizing an area of tissue cause a failing organ to regenerate, or would it cause a tumor to form? The observation that mechanical and chemical factors in the microenvironment interact with Vm signals both spatially and temporally has begun to change the bioelectric view of oncogenesis from a cellular switch in the Vm of healthy cells past a "threshold" value to an organism-wide defect in bioelectric patterning (Levin, 2012b).

THE BIOELECTRIC PARADOX: ONE INPUT, MULTIPLE OUTPUTS

Despite the growing attention being given to the promise of bioelectricity in medicine, a consistent theory that connects Vm to a desired phenotype remains largely elusive. Experiments aimed at understanding bioelectric regulation at both the cellular level and during global tissue patterning events such as regeneration have yielded conflicting results. Remarkably, at the cellular level, similar Vm manipulations can trigger both growth and death (Bortner et al., 1997; Wang et al., 1999; Yu et al., 1999a,b; Thompson et al., 2001). Specifically, separate studies in which depolarization was induced in culture using drug treatments or by adjusting ion concentration in the cellular medium reported increases in either proliferation or apoptosis (Magnis et al., 1991; Wang et al., 1999; Wang, 2004; Lang et al., 2005; Yang and Brackenbury, 2013; Leanza et al., 2014; Levin, 2014; **Table 1**). Further, one paper reports that hyperpolarization by potassium channels is responsible for inhibiting apoptosis of murine myeloblastic FDC-P1 cells, suggesting that increased depolarization is necessary for apoptosis to occur via the Mcl-1 pathway in this cell type (Wang et al., 1999). In contrast, another study reports that hyperpolarization is required for inducing apoptosis, which is thought to occur via an efflux of potassium ions, reducing cell size (Lang et al., 2005). Hyperpolarization drives an influx of calcium ions in some cases (Nilius and Wohlrab, 1992; Ouadid-Ahidouch and Ahidouch, 2008), which has notably been reported to contribute either to apoptosis or proliferation (Clapham, 2007). Although differing levels of calcium ion concentration amplified by Vm changes may seem like an attractive explanation for these disparate observations, it has alternatively been suggested that hyperpolarization does not propel, but inhibits influx of calcium ions by triggering the close of voltage-gated calcium channels (Wang, 2004). It is becoming more widely acknowledged that discrepancies exist regarding what types of ion flow (K^+, Ca^{2+}) contribute to either apoptosis or proliferation (Wang, 2004; Lang et al., 2005). Similar debates persist in studies of global patterning events such as regeneration of amputated limbs or reprograming of oncogenic tissues.

A fundamental question in regenerative medicine is how limbs and organs maintain consistent proportions. A study in planaria determined that depolarization by H,K- ATPase is required for proper head and pharynx scaling following amputation (Beane et al., 2013). Disrupting this Vm gradient resulted in shrunken heads and enlarged pharynxes in regenerated worms. It might be expected that such a phenotype would be caused by defects in proliferation compromising regenerative growth. Surprisingly, apoptotic remodeling of tissues, and not proliferation, was required for proper organ size. Disrupting either depolarization or apoptosis resulted in planaria with disproportionate head and pharynx size in response to amputation. These findings illustrate the link between Vm and apoptosis in regeneration. Although increased depolarization is generally correlated with dividing and cancerous cells (Levin, 2007), in this instance it induced apoptosis during regeneration in planaria.

Still, observations that hyperpolarizing treatments inhibit tumor formation (Chernet and Levin, 2014) might seem to suggest that depolarization is a disease phenotype that potential cancer therapies might seek to abolish. However, artificially creating areas of depolarization in bisected planaria did not generate tumors, but caused the formation of ectopic heads (Beane et al., 2011). Further, artificially hyperpolarizing bisected worms after amputation inhibited normal head regeneration (Beane et al., 2011). These studies indicate that depolarization relative to the surrounding tissue is a critical determinant of normal regenerative processes in planaria. Furthermore, depolarization is required for the regrowth of planarian head structures, including brain and eyes. However, separate studies in *Xenopus* revealed regions of *hyperpolarization* were critical for eye development (Pai et al., 2012). Expression of hyperpolarizing potassium channels induced ectopic eye formation in regions such as the gut and tail, while depolarization inhibited eye formation (Pai et al., 2012). One experiment that may shed light on these seeming discrepancies involved disrupting bioelectric signals by overexpression of hyperpolarizing channels in the frog embryo: widespread apoptosis or proliferation in the central nervous system of the tadpole was observed depending on which cells of the blastula were hyperpolarized, not simply on a specific Vm value (Pai et al., 2015a). This experiment adds an additional level of complexity to our understanding of bioelectricity, illustrating that Vm behaves like a morphogen field (Levin, 2010) rather than a cellular switch. Vm orchestrates behavior at both the cellular (Levin, 2010, 2012b, 2014) and tissue levels (Sundelacruz et al., 2009; Levin, 2010; Adams and Levin, 2013; Chernet and Levin, 2014) in the form of a bioelectric field gradient (**Figure 1**). In this way, Vm comprises a key component of the external cellular microenvironment. However, in contrast to a traditional chemical morphogen, Vm functions at the interface of chemical and mechanical signals by impacting the flow of biochemically important ions such as Ca^{2+} by creating

TABLE 1 | Apoptosis and proliferation in response to bioelectric field manipulations.

Experimental system	Experimental Vm manipulation	Resulting phenotype	Reference
Murine Myeloblastic FDC-P1 cell culture	Depolarization	Apoptosis increased	Wang et al., 1999
Mouse and human lymphoma cells; Jurkat T cells; Mouse cortical neurons	Depolarization	Apoptosis blocked	Bortner et al., 1997; Thompson et al., 2001; Yu et al., 1999a,b
NIH 3T3 fibroblasts	Depolarization	Proliferation blocked	Magnis et al., 1991
Xenopus laevis	Hyperpolarization	Inhibition of induced tumor-like structures	Chernet and Levin, 2014; Chernet et al., 2016
Xenopus laevis	Misexpression of hyperpolarizing ion channels	Induction of apoptosis or proliferation in the neural tube region, depending on whether dorsal or ventral blastomeres were hyperpolarized, respectively	Pai et al., 2015a
Planaria (*D. japonica*)	Depolarization	Disruption of regeneration: ectopic head formation following bisection;	Beane et al., 2011
Xenopus laevis	Hyperpolarization	Ectopic eye formation	Pai et al., 2012

an electrical gradient across cells. In turn, this electrochemical gradient gates voltage-sensitive ion channels, thus creating a tightly connected communication pathway between the cell and its microenvironment (Clapham, 2007; Ohkubo and Yamazaki, 2012; Rothberg and Rothberg, 2012; Martinac, 2014). In this way, cells can be described as charged points, creating an electrical field across the tissue, termed bioelectricity (Levin, 2007). This bioelectric field is formed by spatial differences in Vm both within and across individual cells. Bioelectricity also carries information temporally, by changing at the same time as growth or injury of the tissue (Levin, 2007, 2010, 2012b, 2014; Tseng and Levin, 2013; Barghouth et al., 2015). For instance, cells located at the edge of a wound become depolarized, then migrate and proliferate to close the lesion (Chifflet et al., 2005). Wounds can thus be described as breaks in the tissue-wide bioelectric circuit (Levin, 2007; **Figure 1**).

Not only have disparate outcomes been documented in response to bioelectric manipulations, but the postulated mechanism of Vm transduction also differs between studies. Both the planarian and *Xenopus* studies found that calcium signaling was critical for alterations in Vm to be transduced into a morphogenetic output. In planaria, depolarization proceeds via activation of L-type calcium channels, thus increasing the concentration of Ca^{2+} ions in the anterior region of the animal (Beane et al., 2011). Ca^{2+} is then thought to drive anterior gene expression through the activation of factors such as cAMP response element-binding protein (CREB). In *Xenopus,* hyperpolarization-induced ectopic eye formation is also calcium-dependent, as inhibiting voltage-gated calcium (Cav) channels represses this phenotype. *In vivo*, cells reside amongst different cell types as well as in the presence of a complex microbial network. Remarkably, bacteria can participate in propagating Vm signals (Chernet and Levin, 2014). This was discovered in experiments exploring the ability of hyperpolarization to inhibit tumor formation in *Xenopus.* Transduction of this bioelectrical signal into tumor repression proceeds via Vm-modulated transport of histone deacetylase 1 (HDAC1), a factor involved in control of the cell cycle, apoptosis, and differentiation (Chernet and Levin, 2014). Further, HDAC1 was found to be inhibited by butyrate, a by-product of native bacteria in *Xenopus*. In this case, the inhibition of tumor-like structures depends on a balance of both bioelectric cues and microbial (HDAC inhibition) signals, not calcium channels. Therefore, not only do cells internally transduce Vm signals into phenotypic changes, the surrounding microenvironment also plays a substantial role in the physiological outcome of a given Vm input. The key to resolving the differing observations associated with bioelectric manipulation may lie in the consideration of Vm not just as one morphogenetic property, but as a key parameter defined within a network of additional microenvironmental signals.

There are many examples in which disrupting the endogenous bioelectric field, by treating with ion channel inhibitors or overexpressing certain ion channels, has been demonstrated to control organ identity and placement in developing or regenerating organisms (Adams et al., 2007; Levin, 2007, 2010, 2012b; Tseng et al., 2007; Morokuma et al., 2008; Chernet and Levin, 2013; Tseng and Levin, 2013; Barghouth et al., 2015; Neuhof et al., 2016). For instance, depolarization was found to be a key step in the regeneration of the planarian head (Beane et al., 2011). Intriguingly, manipulation of the bioelectric gradient induces regrowth of amputated appendages in species such as *Xenopus* (Tseng and Levin, 2012, 2013), which lose regenerative capability with increasing age. Optogenetic hyperpolarization of amputated tail stumps in *Xenopus* tadpoles was found to induce regeneration of a complete tail structure containing a functioning spinal cord (Adams et al., 2013).

Because manipulating Vm or ion flux can alter phenotypes such as regeneration in aging organisms and correct tissue homeostasis defects such as cancer, much effort is currently being devoted to understanding the gene expression changes through which the bioelectric field regulates regeneration and

tissue homeostasis. Specifically, microarray analysis has revealed conserved gene networks regulated by Vm depolarization (Pai et al., 2015b) across three different processes and species (embryogenesis in *Xenopus*; spinal cord regeneration in axolotl; and human cells in culture). Common regulatory processes include cell cycle, cell death, and differentiation, as well as factors associated with cytoskeletal organization, cell interactions, and cell movement. However, these results represent a single time point: further analysis is needed to examine the genetic changes accompanying temporal fluctuations in Vm. An additional study in *Xenopus* revealed that genes associated with hyperpolarization varied temporally in expression (Langlois and Martyniuk, 2013). Specifically, voltage-gated potassium channels decreased at the earliest stages of embryogenesis, but increased in expression during later stages of development. Changes in the levels of these channels could contribute to changes in Vm during growth. This supports the observation that *Xenopus* embryogenesis is accompanied by bioelectric gradients that direct anatomical form (Vandenberg et al., 2011; Adams et al., 2016; Sullivan et al., 2016). Although this information provides valuable insight and confirms that Vm regulation is evolutionarily conserved, it is still unclear how an individual cell translates a given Vm into a fate decision. Furthermore, how is this information coordinated across the cells of a tissue to form the complex structure of an organ? The answers to such questions are collectively referred to as the "bioelectric code" (Tseng and Levin, 2013). Analogous to the way cracking the genetic code provided us with a deeper understanding of heritable illness, understanding the workings of bioelectricity is expected to provide exciting alternative therapies for both cancer and regeneration of organs lost to accidents or deteriorated due to aging, by allowing us to reprogram growth patterns (Levin, 2012b, 2014; Adams and Levin, 2013).

AGING VERSUS CANCER: OPPOSITE SIDES OF THE SAME PATHOLOGY?

There is a strong connection between age and regenerative capacity. For instance, there is an age-dependent decline in the ability of mice to regrow tissues including lung (Paxson et al., 2011) and muscle (Conboy and Rando, 2005). In addition, the proliferative ability of cell populations including β-cells (Tschen et al., 2009) and T-cells (Mackall and Gress, 1997) decreases with age. This decline in regenerative capacity is a large factor in onset of the disability and frailty often associated with aging, due to loss of muscle mass and inability to heal muscle tissue after injury. This is believed to be due to signaling downstream of members of the transforming growth factor beta (TGFβ) family, such as myostatin and growth differentiation factor 11 (GDF11) (Egerman et al., 2015), or Notch (Conboy et al., 2003). However, the mechanism of age-related muscle loss remains unclear. Much effort has focused on identifying molecular components that are impacted by aging: the idea being that artificially returning altered levels of signaling molecules in aged organisms to levels observed in youth might reverse the deleterious effects of aging (Egerman et al., 2015). However, conflicting results have been obtained regarding whether molecules, such as GDF11, are up- or down-regulated as a consequence of aging (Egerman et al., 2015). In addition, simply increasing/decreasing levels of one factor may not be an optimal strategy, as many other processes may be impacted. Tuning the activity of one molecule or pathway without a thorough understanding of all processes that might be impacted could produce unwanted effects. A simple example is that blocking cell death does not provide us with immortality, but instead potentiates the development of cancer (Reed et al., 1996). Bioelectric signaling appears to function as a global regulatory mechanism providing us with the capability of inducing the formation of an entire planarian head without a full understanding of every pathway that is involved in this complex regenerative process (Beane et al., 2011). The ability to regulate regeneration of muscle or deteriorated organs without harming other processes may thus be a potential use of Vm.

Whether changes in bioelectric gradients are involved in the age-related decline of regenerative ability is an open question. More research is needed to determine this. However, Vm may play a role in age-related ailments of neural tissue. In particular, Vm is involved in regulating intracellular Ca^{2+} levels (Clapham, 2007). Ca^{2+} influx occurs through ion channels such as Cav (Catterall, 2011) and transient receptor potential (TRP) channels (Clapham, 2003), while outflux primarily proceeds via uptake into mitochondria and endoplasmic reticulum (ER) (Bezprozvanny and Mattson, 2008). Perturbing this delicate balance can lead to diseases such as Alzheimer's. For example, blocking K^+ channels might cause a neuronal cell to become more hyperpolarized; this imparts a more overall negative charge to the cell, creating a more favorable electrochemical gradient for entry of positively charged ions (Clapham, 2007; Bezprozvanny and Mattson, 2008) such as Ca^{2+}. Elevated intracellular Ca^{2+} levels can lead to increased excitotoxicity and apoptosis (Bezprozvanny and Mattson, 2008), aiding the progression of neurodegenerative disease. It has long been known that a connection exists between Alzheimer's and age (Hardy, 2006). Age is also associated with increased neuronal intracellular Ca^{2+} levels (Thibault and Landfield, 1996). Although Vm and voltage-gated calcium channels are implicated in this process (Thibault and Landfield, 1996), many additional components are involved in heightened Ca^{2+} entry, such as the pores formed by amyloid β-peptide (Bezprozvanny and Mattson, 2008). More research is needed to determine how age impacts bioelectric gradients specifically in both neural and non-neural tissues.

It might be expected that animals with high regenerative capacity are more prone to cancer, because their cells are presumably highly prolific. However, the opposite is true, suggesting that highly regenerative species such as planaria and axolotls have tight regulation of morphogenetic patterning in both space and time (Levin, 2012b). The strong patterning regulation that enables organisms with high regenerative ability not only to maintain their form following injury but to ward off cancerous growth draws a close relationship between cancer and regeneration (Levin, 2012b). Intriguingly, an inverse relationship between the diagnosis of age-related diseases such as Alzheimer's and risk of developing cancer has been observed (Driver et al., 2012). This suggests that cancer, injury, and aging are not necessarily different ailments requiring specialized treatment

strategies, but variations of a common morphogenetic patterning defect.

THE APOPTOTIC CELL: IS INFORMATION DESTROYED OR DOES IT ONLY CHANGE FORM?

Although there are conflicting results and proposed mechanisms regarding bioelectric signaling, the studies discussed so far appear to agree that Vm transfers some form of information to the cell that is propagated as a stable physiological state (Neuhof et al., 2016). Logically, a change in Vm that triggers division, differentiation, or gene expression in one cell could impact surrounding cells, and thus propagate throughout the tissue. However, changes in Vm can also induce apoptosis. Can a dead cell communicate information to its surrounding tissue? Although counterintuitive, it has become clear that apoptosis is required for many growth and tissue maintenance processes including embryonic development (Haanen and Vermes, 1996), tumor prevention (Hanahan and Weinberg, 2000), and healthy maintenance of the epithelial cell barrier (Rosenblatt et al., 2001; Andrade and Rosenblatt, 2011; Gu et al., 2011; Slattum and Rosenblatt, 2014; Eisenhoffer et al., 2015). The expression of gene networks associated not only with proliferation but also with apoptosis is increased in the first stages of embryogenesis (Langlois and Martyniuk, 2013).

One of the earliest indications that cellular death is necessary for life processes came in 1842, when examinations of amphibian development revealed that both cellular proliferation and death occurred during embryonic growth (Vogt, 1842; Jacobson et al., 1997). Later on, it was recognized that apoptosis is an integral part of many processes during embryogenesis including the formation of the early structure of the brain, which is critical for proper brain function (Oppenheim, 1991; Kuida et al., 1996). Apoptosis plays key roles in sculpting appendages (Saunders et al., 1962; Milligan et al., 1995; Jacobsen et al., 1996), forming tubes and lumina (Glucksmann, 1951), metamorphosis (Lockshin, 1981), controlling cell numbers (Rosenblatt et al., 2001; Andrade and Rosenblatt, 2011), and deleting unwanted structures (Jacobson et al., 1997). Blocking apoptosis in the embryo was found to have deleterious or even fatal outcomes (Kuida et al., 1996). Apoptosis is not only necessary for embryonic development, but is required for regeneration in some species. For example, apoptosis controls the tissue remodeling essential for correct size ratios and cell lineage specification during planarian regeneration (Beane et al., 2013). In addition, studies in *Hydra* revealed that apoptosis was essential for head regeneration following amputation (Chera et al., 2009). Remarkably, a layer of apoptotic cells near the amputation site provided an increased source of Wnt3, functioning in the synchronized division of nearby stem cells. In this way, the dying cells propagated patterning information to the proliferating cells. Not only do apoptotic cells modify the chemical microenvironment of neighboring cells, but their elimination may change the geometry and density of a tissue. Disruption of the homeostatic balance between proliferation and death is proposed to be a primary driving force for both tumorigenesis (proliferation favored over apoptosis) and organ deterioration (apoptosis favored over proliferation) (Andrade and Rosenblatt, 2011; Slattum and Rosenblatt, 2014). The push to understand the disruption of this homeostasis may be behind the unfortunate categorization of apoptosis and proliferation into two opposing phenotypes. However, it is becoming increasingly accepted that apoptosis and proliferation work together to orchestrate growth, development, and maintenance of tissues. Furthermore, the cellular transition from growth to death does not appear to occur as the bimodal switch we often envision. Cells are capable of recovering from apoptosis even after apparently late stages, including caspase activation and DNA damage, a process termed "anastasis" (Tang et al., 2012). During early stages of apoptosis recovery, genes associated with proliferation and cell cycle are enriched. As anastasis progresses, cells take on a migratory phenotype and upregulate genes associated with focal adhesions and regulation of the actin cytoskeleton (Sun et al., 2017). Remarkably, scratch wounds of cell monolayers close faster when induced to undergo anastasis by treatment with and subsequent removal of ethanol, which induces apoptosis (Sun et al., 2017). It is therefore unclear at what point a cell can be considered fully dead, and recovery from apoptosis can even lead to enhanced healing and expression of proliferation-associated genes.

Current strategies for studying bioelectricity often involve manipulating Vm in a given tissue region then observing a particular phenotype, which functions as the output of the Vm input. However, proliferative and apoptotic phenotypes appear to have a complex co-dependence in many situations, and perhaps even cannot be absolutely characterized. The interconnection between apoptosis and proliferation as well as the disparities among bioelectric manipulation experiments imply that the difference between a regenerative signal and a tumor-initiating cue may be very subtle. A more comprehensive understanding of all the factors that contribute to bioelectrical signaling is needed to determine whether a Vm alteration will result in a beneficial or deleterious program.

BIOELECTRICAL SIGNALING WITHIN THE MECHANICAL MICROENVIRONMENT

Although much attention has traditionally been devoted to understanding how cell-intrinsic parameters (such as genetic alterations and changes in protein expression) drive phenotypes related to aging and cancer, it has become increasingly well recognized that the cellular microenvironment also plays a large role in cancer-related cellular behaviors as well as growth and form at the tissue-scale. The microenvironment functions not only to guide the cell in spatial dimensions, but directs tissue-scale growth through time (**Figure 2**). In addition, not only is Vm closely related to the microenvironment, the microenvironment is part of the Vm definition. Vm is the difference between electrical charge within the cytoplasm and the external medium (Levin, 2010). Notably, even if the cell did not have any way of controlling its internal charge via ion channels or pumps, Vm

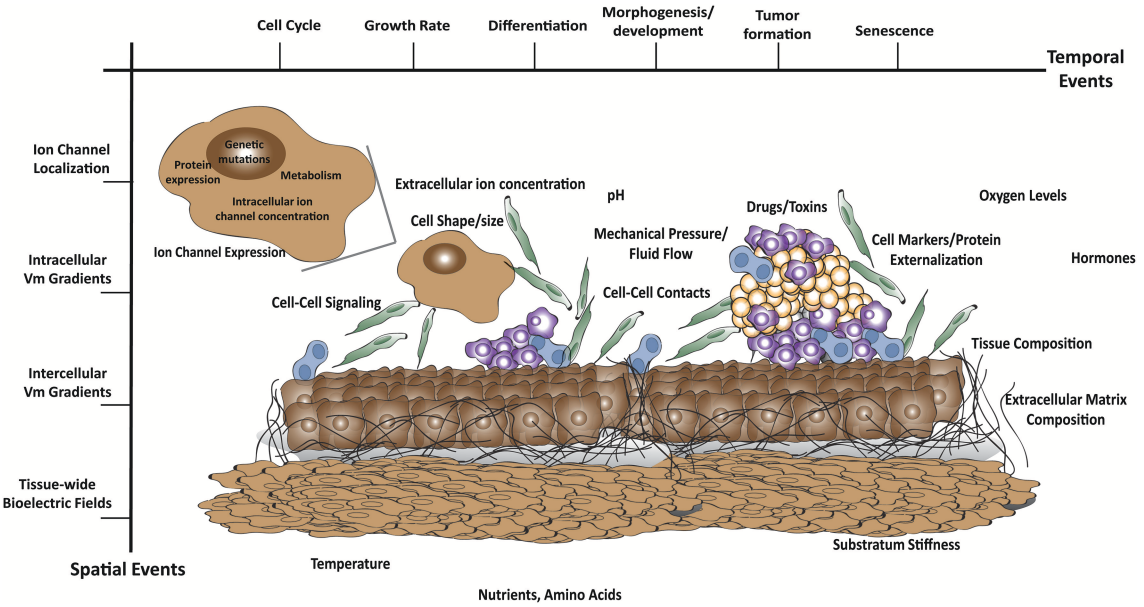

FIGURE 2 | A breadth of factors may impact Vm *in vivo*. All of these factors change not only throughout space (morphogen gradients, mechanical signals) but also over time during growth processes (development, cell cycle, organ regeneration, tumor formation, aging).

could still be changed by altering the charge of the extracellular medium alone.

The involvement of biologically active factors such as pH (Damaghi et al., 2013) in the chemical microenvironment is well recognized. However, it has become increasingly clear that cellular behavior is affected by the mechanical properties of the microenvironment. A number of key properties, including fluid and solid pressure, matrix stiffness (Engler et al., 2006; Kim et al., 2009; Kostic et al., 2009; Tilghman et al., 2010; Zhang et al., 2011; Lee et al., 2012; Pathak et al.), tissue geometry, and mechanical stress (Chen et al., 1997; Dike et al., 1999; Vogel and Sheetz, 2006) comprise the physical microenvironment in a process that depends in part on the mechanosensitive calcium channel Cav3.3 (Walsh et al., 2004; Basson et al., 2015). In addition, pressure activates the oncogenic factors p38, ERK, and c-Src (Walsh et al., 2004). Such findings are of interest because tumors are under higher pressure and also stiffer than the surrounding tissue, creating a microenvironment that promotes cellular proliferation (Basson et al., 2015). Additionally, increased pressure enhances the invasiveness of tumor cells (Piotrowski-Daspit et al., 2016). There are several connections between Vm and mechanical cues. For instance, bioelectric gradients influence osmotic pressure differences *in silico* (Pietak and Levin, 2016). Specifically, hyperpolarization is predicted to lead to lower osmotic pressure than depolarization, due to the outward flow of water predicted to occur along with K^+ flux out of the cell. Conversely, depolarization is predicted to occur by increased levels of Na^+ flowing into the cell, where the flow of water is directed from the extracellular space into the cytosol, increasing osmotic pressure.

Substratum stiffness is defined by the amount of force required to deform the surface to which a cell is adhered (Discher et al., 2005). Similar to the way we detect the rigidity of a surface by sensing the amount of force (applied through muscles) required to deform the material, it has been proposed that cells sense the stiffness of their substratum by applying force through actomyosin motors in stress fibers linked to focal adhesions (Kobayashi and Sokabe, 2010). This information is then transmitted to the cell in the form of biochemical signals that direct cellular activities. Varying the stiffness of cellular substrata has been demonstrated to dramatically influence cellular behaviors, including differentiation (Engler et al., 2006), apoptosis (Zhang et al., 2011), proliferation (Tilghman et al., 2010), gene expression (Provenzano et al., 2009; Bordeleau et al., 2015; Cunha et al., 2016), migration (Lo et al., 2000), cell stiffness (Tee et al., 2011), and epithelial-mesenchymal transition (EMT) (Lee et al., 2012). Many of these phenotypes are also regulated by Vm, drawing a tighter possible link between mechanical and bioelectric signaling. In addition, cytosolic Ca^{2+} concentrations play a role in important cancer-related processes including EMT (Davis et al., 2014), metastasis (Prevarskaya et al., 2011), and apoptosis (Orrenius et al., 2003; Zhang et al., 2011). Integrin signaling, a key communication pathway between cells and their ECM, regulates cytosolic Ca^{2+} levels in a manner that depends on both release from intracellular stores as well as influx of extracellular Ca^{2+} through L-type calcium channels (Kwon et al., 2000). This further strengthens the interplay between bioelectrical and mechanical signals. ECM stiffness also regulates mechanosensitive ion channels. For example, Piezo1/2 channels are activated by either stretch or compression (Wu et al., 2016), providing a means through which mechanical

signals can be translated into ion flow, which is possibly further propagated toward large-scale bioelectric changes. The importance of mechanosensitive channels is evidenced by their demonstrated role in some cancers (Kobayashi and Sokabe, 2010; Sachs, 2010; Martinac, 2014; Pathak et al., 2014; Li et al., 2015; Xu, 2016).

Epithelial cells within a tissue are not only subjected to microenvironments of differing rigidity, but also experience mechanical stress due to the dense packing of neighboring cells. It has been demonstrated that constricting cellular area activates apoptosis programs whereas permitting cellular spreading triggers proliferation (Chen et al., 1997). The physical microenvironment also impacts tissue-level patterning and self-assembly. For example, geometrically constraining endothelial cells on fibronectin-coated strips triggers formation of capillary-like tubes (Dike et al., 1999). During this process, single cells partially detach from the surface to form a hollow central lumen. We still largely do not know how cells sense their position in space and time to go from single cells to the complex multicellular machinery that makes up the body (Vogel and Sheetz, 2006), but the physical microenvironment likely plays a large role. Geometric confinement has also been observed to induce EMT in a manner dependent on ECM stiffness and cytoskeletal dynamics (Nasrollahi and Pathak, 2016). Tissue geometry may also contribute to cancer metastasis. Specifically, cancer cells in culture have been observed to migrate preferentially toward wider branches of microfabricated channels in a manner dependent on cytoskeletal contractility, integrin signaling, and cell alignment along the microchannel walls (Paul et al., 2016). Surprisingly, though, cells confined to narrow channels migrate faster than those in wide channels or on unconstrained surfaces due to increased alignment of stress fibers along the long axis of the channel (Pathak and Kumar, 2012). Mechanical forces are thus postulated to be critical for the prevention of tumor formation (Slattum and Rosenblatt, 2014; Eisenhoffer et al., 2015). This is supported by studies examining the phenomenon of cell extrusion (Eisenhoffer et al., 2015), where cells within a confluent layer are squeezed out in a process that depends on the mechanosensitive ion channel Piezo1. Cell density has been shown to directly influence Vm. Specifically, confluent cells are more hyperpolarized than single cells (Bossu et al., 1992). Cell-cell contacts are critical for propagating bioelectric signals via the transport of ions through gap junctions (Nogi and Levin, 2005; Chernet et al., 2015; Mathews and Levin, 2017). The integrity of cell-cell junctions is altered by mechanical factors including ECM stiffness and culture dimensionality as well as forces from actomyosin contractility, microtubule-based polarization, and integrin/cadherin-dependent adhesion dynamics. Specifically, epithelial cell clusters dissociate more readily on stiffer substrata or when confined to 3D settings (Pathak, 2016). The experimental connections established between chemical, mechanical, and bioelectrical cues place these factors within the same regulatory framework (**Figure 3**). However, the ways through which the bioelectric field may interact with the mechanical microenvironment and the consequent implications are still unclear.

Traditionally, cells are cultured in single layers on plastic dishes at low confluency. Although observing single cells spread on a plastic substratum may seem like an ideal opportunity to isolate and observe the intrinsic properties of a given cell, nature usually does not subject cells to the conditions recommended by conventional culture techniques. For example, the measured elastic modulus of mammary epithelial tissue is on the order of 150 Pa (Paszek et al., 2005) while that of polystyrene is on the order of 10^9 Pa (Paszek et al., 2005; Gilbert et al., 2010), over ten million times greater. This realization has led to the question of how closely experiments performed in culture and *ex vivo* can be compared to conditions *in vivo* (Paszek et al., 2005; Gilbert et al., 2010). This question is of great relevance because there are indications that as cells experience the passage of time, they are imprinted with a "memory" of their surroundings.

BIOELECTRICAL REGULATION: SENDING A SIGNAL OR RECALLING A MEMORY?

Of course, we are all familiar with the concept that our brain translates experiences in our environment into bioelectrical signals (action potentials) and changes in physical structure (connectivity) between neurons allowing us to preserve the memory of an event (Bailey and Kandel, 1993). The retention of this experiential information from one time point to the next guides future behavior. Although a discussion of memory (not to be confused with the concepts of higher-level thought or consciousness) is often restricted to neuroscience, analogous processes occur in many contexts within non-excitable cells. As with neuronal tissue, non-excitable cells also transduce cues from their surroundings into information (Neuhof et al., 2016). Several levels of information are encoded by cells in the form of stable physiological states that guide cellular behavior. These include genetic sequences, epigenetic factors (histone modifications, DNA methylation), metabolic differences, protein expression levels, and Vm (Neuhof et al., 2016). Cellular memory will be defined here simply as the transfer of such information from one time point to a future one, guiding subsequent cellular activities (Neuhof et al., 2016).

The Weismann barrier refers to a postulate that arose in the early stages of evolutionary science, which dictates that information can only be transferred from germ cells to somatic cells, not in reverse (Weismann, 1893). This would prevent a skin carcinoma that arose from a mutation in the DNA encoding for p53 in epidermal cells exposed to ultraviolet light (Brash et al., 1991) from being passed down to offspring. However, a germline mutation in p53 (Li-Fraumeni syndrome) resulting in increased cancer risk would be a heritable trait (Malkin et al., 1990). This theory was intended to explain why acquired traits did not appear to be transmissible. However, it is becoming recognized that some mechanisms may violate this postulate, such as epigenetic modifications. It has been noted that the incomplete erasure of DNA methylation patterns during germ cell development may result in the transfer of genetic modifications from the soma to germ cells (Hajkova et al., 2002). The mechanical microenvironment may provide another important route via

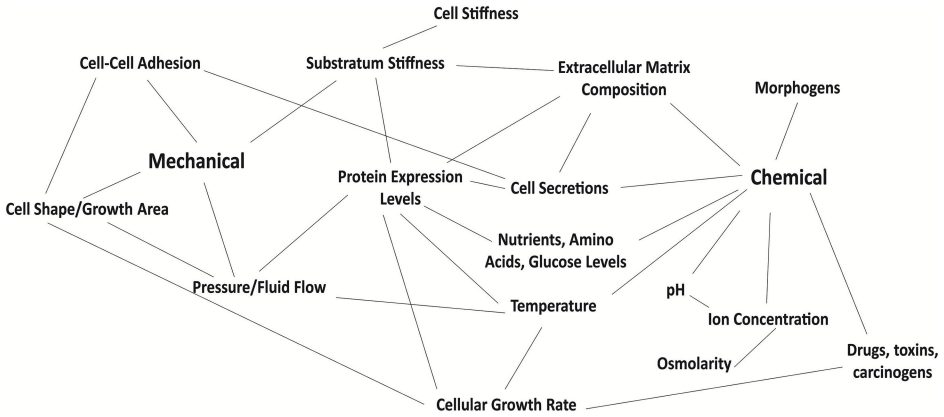

FIGURE 3 | Mechanical and chemical signals present in the cell and surrounding microenvironment are highly interconnected. Altering one variable within this picture could potentially change many factors impacting Vm.

which the Weismann barrier can be circumvented. Oocytes are derived from germ cells, providing half the nuclear genetic material as well as the majority of the membrane and cytoplasm required for reproduction (Li and Albertini, 2013). Intriguingly, the maturation and development of germ cells is controlled by somatic cells. Follicular somatic cells directly contact the oocyte throughout growth, maturation, and fertilization of the egg (Buccione et al., 1990). The somatic cells, therefore, not only transmit chemical signals, but also play a role in transforming the mechanical microenvironment of the germline cells. Mechanical factors within the cellular microenvironment are one means of information transfer between cells (Yang et al., 2014). For example, substratum stiffness directs lineage specification during the differentiation of mesenchymal stem cells (Engler et al., 2006; Yang et al., 2014). In this way, the stem cells preserve a "memory" of their previous ECM stiffness, in the form of a biological lineage. Migrating cells also preserve a memory of past ECM stiffness. Epithelial cells traveling from a stiff to a soft substratum migrate faster and form larger focal adhesions than cells traveling from soft to stiff, even 3 days after they arrive on the soft surface. This mechanical memory depends on nuclear localization of YAP (Nasrollahi et al., 2017). However, YAP is not the only mechanism of mechanical memory. MiRNA-21 levels gradually adjust to ECM stiffness, remaining stable for days after the cells transfer to the new substratum. This process was found to be responsible for stiffness-mediated regulation of fibrosis in mesenchymal stem cells. Either culturing cells on soft ECM or decreasing the levels of miRNA-21 to "erase" cellular memory of stiff ECM was found to protect against fibrosis, scarring, and pro-inflammatory responses in stem cell transplantation experiments (Li et al., 2017). This finding may increase the success of stem cell therapies for tissue repair in damaged or deteriorated organs. In addition to impacting tissue repair and cancer progression, the mechanical microenvironment plays a role in sculpting growth during embryogenesis. Fluid flow is involved in shaping branched tissues in the developing embryo such as vasculature and airways (Nelson and Gleghorn, 2012). In this way, mechanical information might also be transferred from the maternal microenvironment directly to the embryo, without necessarily being mediated by germ cells.

The ways that mechanical information from the microenvironment impact bioelectricity are not fully understood. Intriguingly, gap junctional communication between somatic and germ cells is essential for growth (Li and Albertini, 2013). Gap junctions are physical channels between two cells that allow the passage of small molecules and ions (Alexander and Goldberg, 2003). Gap junctional communication is therefore an important route to propagate bioelectrical signals (Nogi and Levin, 2005; Levin, 2014; Chernet et al., 2015; Mathews and Levin, 2017). Bioelectrical signaling patterns have been observed to be a key part of development and embryogenesis. For example, patterns of depolarization induced by H^+-V-ATPase were found to be necessary for proper left-right patterning in Xenopus embryos; disrupting the bioelectric field with drug treatments that increase depolarization causes heterotaxia (Adams et al., 2006). Another study found that optogenetically disrupting Vm in only the outermost ectodermal layers in the frog blastula was sufficient to induce craniofacial abnormalities (Adams et al., 2016). Proper patterns of bioelectricity are also required for correct development of the central nervous system in *Xenopus* (Pai et al., 2012). The importance of Vm in embryogenesis raises the question of which direction bioelectric information travels during development: is Vm an intrinsic signaling code emitted from cells during growth, or a physiological memory *imprinted* on cells by their surroundings? A better understanding of how the microenvironment contributes to Vm may enable us to more accurately recapitulate bioelectric patterns at will.

UNTANGLING THE DIRECTION OF INFORMATION FLOW: DECOUPLING THE MECHANICAL MICROENVIRONMENT AND BIOELECTRICAL SIGNALING

Computational network analysis is an increasingly necessary tool in biology (Ma'ayan, 2011) due to the vast number of

variables involved in physiological systems. Modeling tools from neuroscience applications may also be useful for understanding electrical dynamics in nonexcitable cells (Pezzulo and Levin, 2016). This will require that we know what parameters to model. Toward this end, a better understanding of how mechanical parameters in the microenvironment impact Vm is needed. One of the key concepts computational studies seek to illuminate is the idea of self-organization of a morphogenetic field, or "symmetry-breaking" of an initially homogeneous state (Pietak and Levin, 2016). Although often discussed in the context of embryonic development (Levin, 2005), mechanisms of asymmetry emergence are also important for understanding how patterning fields become disrupted during the onset of pathologies such as cancer. The generation of heterogeneous patterning cues is thought to occur largely through positive feedback mechanisms that amplify small variations from the realm of noise into measurable signals (Pietak and Levin, 2016). Therefore, even small interactions between the chemical and mechanical microenvironments with local Vm states may play a substantial role in the establishment of global bioelectric regulatory fields. Models such as BETSE (BioElectric Tissue Simulation Engine) examine computationally the emergence of Vm steady states (Pietak and Levin, 2016). The BETSE model considers parameters such as extra/intracellular ion concentration, membrane permeability, cell-cell junctions, and positive feedback between these factors. However, this system has several limitations: specifically, division/apoptosis, mobility including galvanotaxic movement, intracellular Vm components such as the mitochondria/ER, and control of ion channel gene expression are not considered. Furthermore, many additional feedback mechanisms may exist *in vivo*, where not only chemical properties such as ion concentrations and morphogens are at work, but also physical factors such as pressure, stiffness, and geometrical constraints.

Many of the experiments aimed at understanding bioelectric signaling employ input/output-based strategies, where endogenous Vm is altered, and the resulting phenotypic change observed. However, one single regulatory cue does not function in isolation: Vm responds and communicates with the cellular microenvironment via several feedback loops (Pietak and Levin, 2016). For example, many ion channels are themselves gated by changes in Vm; in turn, the ion influx or efflux alters Vm, and the voltage-gated ion channels continue responding to these fluctuations. Changes in expression levels of ion channels can theoretically impact the amount of ion flux occurring in response to physiological triggers such as Vm alterations. Since a number of ion channels are upregulated in tumorigenic cells, one possible treatment idea being explored is ion channel inhibition or knockdown (Li and Xiong, 2011; Stock and Schwab, 2015). However, simply targeting individual ion channels may not be an ideal strategy to combat cancer. First, not all tumors express the same ion channel targets (Schönherr, 2005). Second, Vm is established by ion channels that are gated posttranslationally. As a result, two cells that are in the exact same genetic and transcriptional states could theoretically be in very different bioelectrical states (Levin, 2014). Conversely, the identity of the ion channel is less important, as two cells with very different ion channel transcriptional profiles may be in the same bioelectric state. Bioelectrical signaling can thus be missed by conventional mRNA and genetic profiling. Third, there is substantial redundancy among ion channels: knocking down a single ion channel might not change Vm because other channels with similar function may be triggered to upregulate their activity in response to the knockdown (Levin, 2014). This phenomenon is referred to as ion channel compensation.

Overall, the major limitation of these strategies is that we cannot yet fully say what phenotypic changes, if any, will arise from simply blocking an ion channel or inducing hyperpolarization in a region of tissue. Influencing one variable such as Vm could have broad impacts on many aspects of the cellular microenvironment. For example, Vm depolarization decreases cellular stiffness (Callies et al., 2011). Therefore, a depolarizing treatment that reduces the stiffness of one cell could theoretically alter the mechanical stiffness experienced by a neighboring cell. It is therefore not surprising that bioelectric field manipulations have been documented to produce a wide variety of sometimes inconsistent phenotypes depending on the organism under study and the experimental setup. More studies are needed to fully decouple the outcomes of bioelectrical and mechanical signaling. Toward that end, further research examining the impact of specific changes in the physical microenvironment, including substratum stiffness, cellular stiffness, pressure, geometrical constraint, cell density, cell types, and dimensionality on bioelectrical signaling may help lead to an understanding of the events that cause symmetry breaking and self-generation of morphogenetic patterning events. In addition, although it is known that bioelectrical fields change dynamically during development, it is unclear how Vm signals are altered by the passage of time during aging. The ability of Vm manipulations to regenerate organs such as eyes and limbs has exciting implications for organ restoration in aging individuals, but a more thorough understanding of the long-term effects of Vm manipulations is critical to the success of such a procedure. Toward this end, additional studies monitoring the impact of Vm changes through time in adult organisms would be of great benefit.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Microenvironment-Cell Nucleus Relationship in the Context of Oxidative Stress

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The microenvironment is a source of reactive oxygen species (ROS) that influence cell phenotype and tissue homeostasis. The impact of ROS on redox pathways as well as directly on epigenetic mechanisms and the DNA illustrate communication with the cell nucleus. Changes in gene transcription related to redox conditions also influence the content and structure of the extracellular matrix. However, the importance of microenvironmental ROS for normal progression through life and disease development still needs to be thoroughly understood. We illustrate how different ROS concentration levels trigger various intracellular pathways linked to nuclear functions and determine processes necessary for the differentiation of stem cells. The abnormal predominance of ROS that leads to oxidative stress is emphasized in light of its impact on aging and diseases related to aging. These phenomena are discussed in the context of the possible contribution of extracellular ROS via direct diffusion into cells responsible for organ function, but also via an impact on stromal cells that triggers extracellular modifications and influences mechanotransduction. Finally, we argue that organs-on-a-chip with controlled microenvironmental conditions can help thoroughly grasp whether ROS production is readily a cause or a consequence of certain disorders, and better understand the concentration levels of extracellular ROS that are necessary to induce a switch in phenotype.

Keywords: reactive oxygen species, chromatin, epigenome, tissue stiffness, aging, cancer, neurodegenerative disorders, stem cell

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INTRODUCTION

The generation of reactive oxygen species (ROS) is part of normal physiology. Overproduction of ROS or insufficient enzymatic conversion of these molecules via antioxidant mechanisms results in oxidative stress that contributes to aging and disease. Oxidative phosphorylation, which provides cellular energy, is at the heart of ROS generation in the mitochondria, since it also results in the formation of superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$) and hydrogen peroxide (H_2O_2) (Murphy, 2009); normally, ROS-induced translocation of signal transduction proteins and

transcription factors to the nucleus promotes the expression of protective antioxidant enzymes (Poon and Jans, 2005; Kodiha et al., 2010). Other causes of intracellular ROS production include responses to infection, mental stress, physical exercise, and aging (Powers and Jackson, 2008; Bouayed et al., 2009; Romano et al., 2010; Ivanov et al., 2017). Interestingly, the generation of ROS in response to bacterial infections alters the host metabolism, triggering inflammatory signaling pathways that affect the transcription of proinflammatory and procarcinogenic genes such as cyclooxygenase 2, which may lead to metabolic diseases (Cassell, 1998; Mager, 2006; Ivanov et al., 2017), hence linking infectious disease to chronic disorders via ROS. The microenvironmental origin of ROS is due to extravasation and the activity of extracellular catalase, superoxide dismutase (SOD) and NADPH oxidases (NOX) in response to food and alcohol consumption and to pollutants such as heavy metals, cigarette smoke, and radiation (Limòn-Pocheco and Gonsebatt, 2009; Bauer et al., 2014). Importantly, although extracellular H_2O_2 easily enters in the cells (Ohno and Gallin, 1985; Limòn-Pocheco and Gonsebatt, 2009), thus potentially adding to intracellular burden in case of oxidative stress, its implication in health homeostasis remains poorly understood.

Theoretically, there are at least two means for microenvironmental ROS to affect cellular homeostasis via an impact on the cell nucleus. Direct diffusion of H_2O_2 into cells might contribute to high intracellular ROS that has been linked with alterations in chromatin organization and gene transcription (Rahman, 2002; Sundar et al., 2013; Kreuz and Fischle, 2016). Moreover, ROS-mediated activation of fibroblasts in the extracellular matrix (ECM) increases collagen I production (Tanaka et al., 1993), hence modifying tissue stiffness, which might influence gene expression in neighboring cells via mechanotransduction (Humphrey et al., 2014; Mouw et al., 2014; Handorf et al., 2015).

The nuclear compartment possesses an exquisite organization of chromatin necessary to maintain cellular homeostasis via its impact on the epigenome (Abad et al., 2007; Lelièvre, 2009; Grummt, 2013). Oxidative stress can be specifically sensed by cell nuclei (Provost et al., 2010), in part due to mitochondrial stress leading to signal transduction and the nuclear accumulation of respiratory enzymes like CDC like kinase 1 (CLK1). Specifically, mitochondria-nucleus cross talk controls the response to oxidative stress. For instance, the expression of DNA methyl transferase 1 (DNMT1) that mediates epigenetic changes in mitochondria is controlled by transcription factors responsive to oxidative stress (Shock et al., 2011), and nuclear CLK1 maintains mitochondrial homeostasis by regulating genes in the cell nucleus that deplete ROS (Monaghan et al., 2015). H_2O_2 entering cells can be metabolized into $OH^{\cdot}$ known to induce DNA lesions (Tsunoda et al., 2010); but the strong cellular influence of ROS might not involve such damage (Kirkland, 1991), suggesting that beneficial and deleterious effects of ROS likely involve transcriptional effects.

Here, we discuss how extracellular ROS might contribute to normal aging and diseases via a dual influence on the microenvironment, notably tissue stiffness, and on cellular homeostasis. Knowledge on chemical and physical consequences of incremental ROS on the cell nucleus is presented before proposing new *in vitro* models to help fill the gaps to understand the determining impact of ROS thresholds.

REACTIVE OXYGEN SPECIES AND CELLULAR HOMEOSTASIS

A Fine Line Between Normal and Abnormal Stem Cell Differentiation

High levels of ROS damage macromolecules, yet ROS is necessary for normal biological processes (Schieber and Chandel, 2014). Embryonic stem cell differentiation requires increased ROS and ATP production in mitochondria, as shown for the cardiovascular tissue (Schmelter et al., 2006). There is also upregulation of NOX within cells and the microenvironment. Yet, additional intracellular ROS, due to entry of environmental H_2O_2, might inhibit nuclear translocation of proteins responsible for the antioxidant response by binding to their cysteine motifs (Lennicke et al., 2015). Indeed, oxidative stress has been reported to impair the proliferation of embryonic stem cells (Brandl et al., 2011), but whether abnormally high microenvironmental ROS during embryogenesis alters organ development remains to be clearly determined.

The balance of self-renewal and cell-type specific differentiation, two functions controlled by low levels of ROS, is essential for the maintenance of a stem cell pool within adult organs (Maraldi et al., 2015; Cieślar-Pobuda et al., 2017), with a fine line between desired stimulation and unwanted damage. Adult stem cell differentiation in the central nervous system is directed by lens epithelial-derived growth factor (LEDGF), itself involved in the protective response to oxidative stress (Chylack et al., 2004; Basu et al., 2016). Stem cells have defective DNA repair capacity, which is further exacerbated by ROS (Cieślar-Pobuda et al., 2017). Prolonged exposure to ROS has been shown to result in cell senescence *in vitro* (Kuilman et al., 2010; Davalli et al., 2016) and has been proposed to contribute to pathologies associated with aging such as cancer and Alzheimer's disease in a dose-dependent manner (Sarsour et al., 2009; Zhu et al., 2013; Childs et al., 2015; Sikora et al., 2015; Qiu et al., 2017) (**Figure 1**).

For instance, stem cell self-renewal and resulting premature pool exhaustion occurs with a moderate increase of ROS concentration (Zhou et al., 2014; Maraldi et al., 2015). Understandably, detrimental exposure to ROS has to be chronic when at low dose, and, *in vitro*, it seems to preferably trigger the activation of the p38-p16 pathway that induces stem cell senescence (characterized by loss of replicative capability) (Shao et al., 2011); whereas acute and high ROS dose exposure activates the p53 pathway by accumulation of ataxia telangiectasia mutant (ATM) kinase in the cell nucleus, triggering not only stem cell aging (characterized by a diminished capacity to function and respond to the microenvironment), but also apoptosis (Mai et al., 2010; Liu and Xu, 2011).

The threshold at which an imbalance of ROS and thus, oxidative stress, leads to pathologies linked to an effect on stem cells might be low (Bigarella et al., 2014). The contributions to

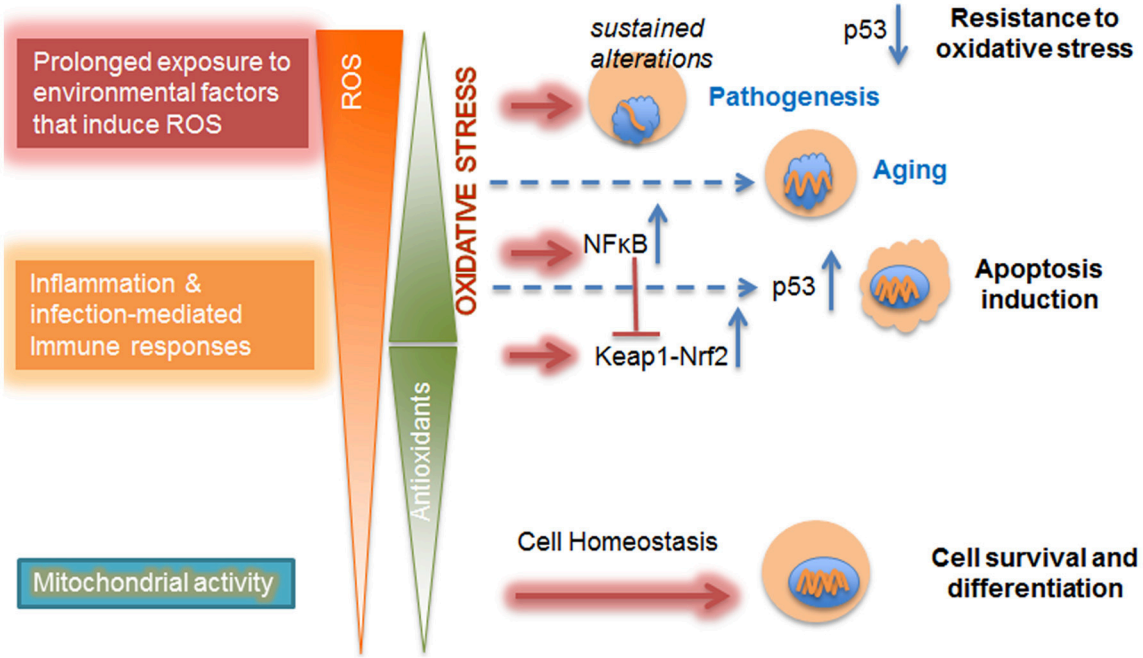

FIGURE 1 | Dose-dependent impact of ROS on cellular metabolism. Mitochondrial activities, such as oxidative phosphorylation, contribute to physiological levels of ROS that are counterbalanced and detoxified by antioxidant defense mechanisms. These ROS are produced as a response to increased cellular demand for energy and are essential for cell survival, differentiation, and tissue development. With the increase in imbalance between ROS and antioxidant levels due to inflammation or prolonged exposure to environmental factors, there is a shift in redox regulation pathways from Keap-Nrf2 to NFκB. At mild oxidative stress level p53-mediated cell death (apoptosis) is observed. Further increase in oxidative stress level in diseased cells inhibits p53-induced cell apoptosis and promotes resistance to oxidative stress. Furthermore, chronic oxidative stress leads to altered gene expression and changes in nuclear morphology already observed in aging; the level at which excess ROS might contribute to sustained alterations in the epigenome that trigger pathogenesis might depend on microenvironmental conditions (Chittiboyina et al., 2018). Nuclei are shown in blue and increasing alterations in the nucleus are displayed as shortening orange wiggles.

such threshold of extracellular ROS coming from the degradation of our environment and dietary habits remain unanswered questions.

Oxidative Stress in the Normal Process of Aging

Aging is the major cause of increased susceptibility to neurodegenerative diseases, cancer, and other metabolic disorders. It has been characterized as a progressive loss of tissue functions due to cumulative damages in cells and their microenvironment. The original free-radical theory of aging and derived mitochondrial theory of aging consider ROS as the main cause for these damages. Indeed, oxidative stress has been connected with all of the nine potential hallmarks of aging (López-Otín et al., 2013), including genomic instability (Hoeijmakers, 2009), telomere attrition (Sun et al., 2015), epigenetic alterations (Guillaumet-Adkins et al., 2017), loss of proteostasis (Bader and Grune, 2006), deregulated nutrient sensing (Luo et al., 2017), mitochondrial dysfunction (Harman, 1965), cellular senescence (Davalli et al., 2016), stem cell exhaustion (Cieślar-Pobuda et al., 2017), and altered intercellular communication (Poli et al., 2004). The unpaired electron of the active oxygen molecule of ROS can capture another electron from macromolecules such as DNA, lipids, and proteins resulting in changes in biological properties; however, the molecular alterations necessary for aging are still poorly understood. Furthermore, the free-radical theory of aging is challenged by the mixed results of gene knockout studies in animal models showing that on one hand, lifespan can be expanded by decreasing ROS level, and on the other hand, increasing ROS level has no effect or might even prolong the lifespan in individual mice (Hamilton et al., 2012). Among studies related to ROS and performed in mice, mutation and knock-out models have been developed that impair transcription factors TP53INP1, JunD, ATM, Forkhead box O (FOXO) and p53 normally involved in tumor suppression (Sablina et al., 2005; Reliene and Schiestl, 2006; Tothova et al., 2007; Laurent et al., 2008; Cano et al., 2009). As a result, there is an increased ROS level in mice suggesting that these transcription factors play a role in antioxidant defense. Moreover, an association between ROS accumulation and specific aging characteristics has been reported in multiple types of adult stem cells. For instance, ROS level increases in murine hematopoietic stem cells when FOXO is knocked down, which results in stem cell self-renewal exhaustion (Miyamoto et al., 2007; Tothova et al., 2007). It remains largely elusive whether and how deficiency of FOXO family members can lead to aging-related diseases; however, Genome Wide Association Studies conducted in human population samples

revealed a positive link between FOXO genes and extreme longevity (Martins et al., 2016). Among other examples of comparable murine and stem cell studies, a genetic knockout of deacetylase Sirtuin family members in mice (Mohrin et al., 2015) as well as in neural progenitor cells (Prozorovski et al., 2008) revealed the important function of sirtuins in ROS balance and stem cell aging.

The aging process might rely on repair ability, especially for DNA, more than the ROS level in the organism (Lewis et al., 2013; MacRae et al., 2015). Repair ability has been linked to epigenetic pathways (Dinant et al., 2008; Lahtz et al., 2013; Montenegro et al., 2016), and it is controlled by the ECM that influences proteins involved in higher order chromatin organization (Vidi et al., 2012). Microenvironmental ROS can damage the ECM and cell surface proteins, leading to altered cell adhesion and signaling like in atherosclerosis, a disease of aging (Kennett et al., 2011). Aging in general has been associated with an initial increase followed by a decrease in ECM stiffness (Achterberg, 2014), and tissue stiffness depends on the impact of ROS on fibroblasts (Tanaka et al., 1993; Siwik et al., 2001; Lijnen et al., 2012). Therefore, understanding the relative contribution of ROS to aging in different organs should take into account the microenvironment.

Oxidative Stress in Disease Development and Progression

In cells with high energy requirement the microenvironment contributes to oxidative stress. In normal cells, this is a concern with embryonic development as discussed above. In diseases, concerns are with cells associated with a metabolic syndrome (e.g., cardiovascular disease, diabetes) and other cells with high metabolic demands like in cancers and neurodegenerative disorders. Cell-required increased energy production, and thus ROS, by mitochondria is fulfilled by extracellular growth factors and hormones (Turpaev, 2002; Ward and Thompson, 2012).

The microenvironment also participates in ROS-mediated impact in disease via stromal cells. Under oxidative stress these cells secrete lactate and pyruvate, providing an alternate source of energy called "aerobic glycolysis" (or Warburg effect) in rapidly proliferating cancer cells (Gatenby and Gillies, 2004; Liberti and Locasale, 2016). Extracellular ROS activate stromal cells (Lijnen et al., 2012). Activated fibroblasts lose caveolin 1, which has been associated with poor survival in patients with triple negative breast cancer and with early breast cancer recurrence in general (Witkiewicz et al., 2009, 2010; Popovska et al., 2014). They also increase collagen I secretion, resulting in tissue stiffness (Karsdal et al., 2013; Eble and de Rezende, 2014), a condition associated with the aggressiveness of certain cancers (Hoyt et al., 2008; Acerbi et al., 2015; Northey et al., 2017). Whether changes in tissue stiffness associated with cancer progression truly result from oxidative stress remains to be confirmed.

Brain susceptibility to oxidative stress is in part linked to low levels of antioxidant mechanisms in the microenvironment leading to high amounts of remaining ROS (Uttara et al., 2009). An oxidative microenvironment is a feature of Alzheimer's disease that promotes the production and aggregation of extracellular amyloid β plaques by influencing the activity of α- and β-secretases (Behl, 2005; Mosconi et al., 2008). Moreover, cytoplasmic plaque accumulation triggers the overproduction of intraneuronal ROS by disturbing mitochondrial activity (Xie et al., 2013). In Parkinson's disease microenvironmental oxidative stress is due to the production of O_2^- triggered by microglial cells, with immediate conversion to H_2O_2 species that attack the surrounding neurons, eventually leading to neurodegeneration (Dias et al., 2013).

Chronic diseases like cancer and neurodegenerative disorders, with a microenvironmental component to their onset and progression, are linked to alterations in the epigenome, but deciphering the contribution of oxidative stress to epigenetic alterations is a difficult task.

INCREMENTAL IMPACT OF ROS ON THE CELL NUCLEUS

A major recipient of ECM signaling, the cell nucleus reflects cell phenotypes (Bissell, 1981; Lelièvre, 2010). Changes in the epigenome (i.e., the collection of epigenetic marks that control transcription profiles) and morphometry (notably size and shape) of the nucleus accompany differentiation disorders, including cancer and neurodegeneration (Zink et al., 2004; Lelièvre, 2009; Lattanzi et al., 2012). Beyond oxidative DNA damage induced by hydroxyl radicals that is not necessarily associated with disease development (Evans et al., 2004; Silva et al., 2014; Pereira et al., 2016), ROS might influence transcription depending on their concentration and origin.

Impact of ROS on Protein Activation and Translocation to the Cell Nucleus

Transcription regulation mediated by ROS occurs already in the nanomolar range of H_2O_2 (Schieber and Chandel, 2014) acting as redox signaling, mainly through reversible thiol modifications on phosphatase and kinase cysteine residues (Janssen-Heininger et al., 2008; Collins et al., 2012). Orchestrated enhancement of tyrosine kinase (Paulsen et al., 2011; Heppner et al., 2018) and inhibition of tyrosine phosphatases (Sundaresan et al., 1995; Bae et al., 1997; Denu and Dixon, 1998; Lee and Esselman, 2002) by H_2O_2 amplify the activation of PI3K/AKT and transcription mediators (e.g., STAT), that favor cell proliferation and survival. Cysteine modifications by low H_2O_2 concentrations are likely to maintain the expression of stress-responsive genes at basal level under normal conditions. This pathway involves Kelch-like ECH-associated protein 1 (Keap1) in which H_2O_2 modifies cysteines leading to the release and translocation of nuclear E2-factor-related factor 2 (Nrf2) into the nucleus, where it binds antioxidant response elements in the promoter of detoxification genes (McMahon et al., 2003), notably in response to inflammation (Suzuki and Yamamoto, 2017). This pathway seems attenuated in neurodegenerative diseases (Gan and Johnson, 2014; Buendia et al., 2016), whereas constitutive activation is commonly observed in cancers and linked to cell survival and resistance to ROS production-based therapies (Leinonen et al., 2014).

Concentration dependent regulation of signaling pathways by ROS is essential for tissue survival. Physiological levels of ROS are known to upregulate and activate proinflammatory cytokines such as IL-6 and IL-4 (Frossi et al., 2003), whereas elevated ROS can lead to activation of IL-6 that mediates Nrf2 translocation to the nucleus and subsequent upregulation of antioxidant mechanisms (Theodore et al., 2008; Hsieh et al., 2009). Cytokines are also linked to the NF-κB/p53 pathway under ROS stimulation. Tumor suppressor and transcription factor p53 participates in redox-responsive control of the cellular stress response (Budanov, 2014). Although NF-κB and p53 have opposing effects on cellular apoptosis, both are activated and translocate to the cell nucleus under ROS, and regulate the transcription of IL-6 (Lowe et al., 2014). Interestingly, p53 is shown to suppress Nrf2-mediated antioxidant response, but not the expression or activation of Nrf2 itself in mouse hepatocarcinoma cells (Faraonio et al., 2006), hence these two proteins seem independent from each other regarding their involvement with IL-6 pathway. Low levels of ROS do not induce NF-κB activation and nuclear translocation. Yet, if ROS levels lead to oxidative stress, NF-κB becomes activated and promotes cytokine-mediated inflammatory pathways. However, tissue insults from proinflammatory cytokines are countered by high levels of ROS that upregulate and activate anti-inflammatory cytokines such as IL-10 (Kelly et al., 2010; Latorre et al., 2014). If further strengthened, oxidative stress may trigger p53-mediated apoptosis and even inhibit p53 activity via oxidation of certain cysteine residues, which prevents the antioxidant response in cells, leading to further accumulation of ROS (Cobbs et al., 2001; Bensaad and Vousden, 2005; Halliwell, 2007).

At least part of H_2O_2 involved in low level activation is transiently produced by the oxidation of cell membrane-bound NOXs that are recruited by receptor binding of extracellular growth factors and hormones (Sundaresan et al., 1995). The involvement of such microenvironment-mediated cytoplasmic production of ROS in detrimental levels of oxidative stress remains to be determined.

Impact of ROS on Epigenetic Mechanisms

In cancer, diabetes and Alzheimer's disease, alterations in epigenetic pathways have been linked to oxidative stress, although the concentrations of ROS that matter are unknown. Epigenetic pathways encompass DNA methylation and several posttranslational modifications (methylation, acetylation, phosphorylation, ubiquitination) on various histone residues (Kreuz and Fischle, 2016; Guillaumet-Adkins et al., 2017). The combination of some of these epigenetic traits determines the level of transcription of a particular gene. As shown in the selected examples below, all of these types of epigenetic modifications can be affected by ROS.

ROS-induced global heterochromatin loss may follow DNA damage that, in turn, promotes chromatin relaxation (Pal and Tyler, 2016), and might be linked to reduced S-Adenosyl Methionine synthesis, caused by oxidized methyl adenosine transferase (Towbin et al., 2012). Direct oxidation of 5 methyl cytosine (5-mC) by ROS might also inhibit DNMT1, contributing to demethylation of CpG sites on the DNA. In contrast, DNA hypermethylation might be due to oxidative stress-mediated inhibition of methyltransferase-related TET proteins leading to an increase in 5-mC level (Chia et al., 2011; Wu and Zhang, 2017). Pericentromeric heterochromatin stimulated by oxidative stress is associated with increased expression of SIRT1 that stabilizes SUV39H1, leading to increased histone H3 trimethylated on lysine 9 (H3K9me3) (Bosch-Presegué et al., 2011).

Under oxidative stress histone demethylases are inhibited leading to H3K9me2/3 and H3K27me3 increase (Chervona and Costa, 2012; Niu et al., 2015; Kreuz and Fischle, 2016), which may repress transcription. Acetylation of histone lysine residues associated with chromatin relaxation and transcriptional activation might also occur following inhibition of histone deacetylases (Ropero and Esteller, 2007). Notably, ROS increases acetylated histone 4 (Tomita et al., 2003) and histone 3 (Choudhury et al., 2010). Several studies have shown histone phosphorylaton under oxidative stress, for example H2AX and histone 3 (Katsube et al., 2014; Marwick et al., 2015). Chronic ROS leads to H2AX poly-ubiquitination due to increased interaction between H2AX and E3 ubiquitin ligase RNF168, which results in reduced level of H2AX and increased sensitivity of cancer cells to chemotherapy (Gruosso et al., 2016). Thus, depending on the posttranslational modifications the resulting impact might be on the regulation of gene expression, DNA replication and DNA repair, making ROS a key regulator of all these processes via epigenetic influence.

Most interestingly, extracellular ROS alter nuclear morphometry (e.g., size and shape) (Barascu et al., 2012), in part by disrupting lamins involved in the structural organization of the nucleus (Shimi and Goldman, 2014). Peripheral heterochromatin is organized into lamina-associated domains that directly participate in the control of heterochromatin linked with the degree of tissue differentiation (Gonsalvez-Sandoval et al., 2013; Solovei et al., 2013). Impaired interaction between nuclear envelope and heterochromatin proteins, like HP1, leads to the mislocalization of heterochromatin (Eskeland et al., 2007; Schneider and Grosschedi, 2007), which could affect transcriptional regulation. We have shown that nuclear morphometry responds to ROS in a dose dependent manner (Chittiboyina et al., 2018). The impact of oxidative stress-mediated alterations of nuclear morphometry on chromatin organization and gene transcription remains to be studied.

Epigenetic changes might be protective against DNA damage and mediate ROS resistance in cancer. Many contributing experiments included extracellular H_2O_2, which calls for clarifications regarding the contribution of microenvironmental ROS in these nuclear events.

IMPROVING KNOWLEDGE ON THE ROLE OF OXIDATIVE STRESS IN CELLULAR HOMEOSTASIS

The antioxidant cellular mechanisms activated in response to elevated levels of ROS are fairly known. Glutathione (GSH) can directly scavenge super oxide anions ($O2^{-\cdot}$), one of the most reactive free radicals, while GSH peroxidases, peroxiredoxins

and thioredoxins all target H_2O_2 (Hanschmann et al., 2013). The expression of these antioxidant enzymes is due to the activation and resulting cytoplasmic to nuclear translocation of transcriptional coactivators Nrf2 and LEDGF upon ROS generation (Sharma et al., 2000; McMahon et al., 2003). However, the mechanisms by which these transcriptional coactivators sense altered ROS homeostasis and may themselves be compromised, leading for instance to oxidative stress resistance in cancer (Freitas et al., 2012; Balvan et al., 2015), remain to be understood.

Elevated ROS levels have been reported in various pathologies that result from epigenetic alterations and for which microenvironmental modifications are instrumental. For instance, oxidative stress leads to increased ECM stiffness (Eble and de Rezende, 2014). Enhanced microenvironmental stiffness triggers mechanotransduction via stimulation of the cytoplasmic form of transcriptional activator Yes-associated protein (YAP) (Dupont et al., 2011), leading to its relocalization to the cell nucleus. Upon reaching chromatin YAP may activate the transcription of genes such as those coding for β-catenin, ErbB4, FoxO1 that are involved in apoptosis, cancer progression and stem cell self-renewal (Dupont et al., 2011; Zhu et al., 2015; Moleirinho et al., 2017). Interestingly, ECM stiffness may not only activate YAP, but also regulate its transcription (Low et al., 2014). The modulation of mechanical strength affects the actin cytoskeleton, which in turn, influences YAP phosphorylation and thus, its nuclear localization (Das et al., 2016). Hence, mechanotransduction generated by changes in ECM stiffness has posttranslational and transcriptional effects. How the combined actions of microenvironmental ROS on ECM composition (via an effect on fibroblasts) and directly on epithelial cells (via chromatin regulation, as discussed in previous sections), ultimately transforms the cell phenotype is difficult to study in simple cell culture as well as in complex whole organisms like in mice. Thus, the mechanisms of microenvironmental ROS-induced differentiation and pathogenesis remain to be clarified. Moreover, the dual impact of ROS, either beneficial or detrimental, underlines the need to identify thresholds for action within the cell nucleus.

Microenvironmental impact on tissues is best studied with controlled 3D cell culture models (Lelièvre et al., 2017). Using a microfluidic system in which a gradient of H_2O_2 was delivered inside the ECM, we showed that there were thresholds for phenotypic response measured by nuclear morphometry depending not only on the tumor grade, but also on matrix stiffness (Chittiboyina et al., 2018). Microscale optics was used to assess H_2O_2 concentrations delivered in different regions of the culture chamber. However, this method is cumbersome and cannot be used in real time. Assessment of ROS impact on the cell nucleus was measured by indirect means, such as the expression of antioxidant genes, but there was no direct measurement of ROS concentration inside cells.

To better understand the relationship between cell nucleus and microenvironmental ROS production extracellular H_2O_2 should be measured. ROS concentration is not uniform, but rather a dynamic gradient as seen *in vivo* (Ogasawara and Zhang, 2009; Zorov et al., 2014). Measurements might be accomplished by placing biosensors within the ECM (Hynes et al., 2014). Once H_2O_2 enters inside cells, it remains there due to differences in diffusion radii (Huang et al., 2017). Intracellular ROS assessment relies on indirect and low precision methods such as antioxidant capacity or redox potential (Barzegar and Moosavi-Movahedi, 2011), using fluorescent probes that react with free radicals. To determine the contribution of microenvironmental ROS to intracellular ROS, it might be possible to use differential assessment techniques where mitochondrial ROS concentrations (primarily H_2O_2), detected using Mitotracker, are deducted from the total intracellular ROS. Noticeably, the nucleus is in a higher oxidizing state than mitochondria (Go and Jones, 2008), and maintenance of nuclear redox homeostasis is essential for proper transcription of antioxidants in response to oxidative

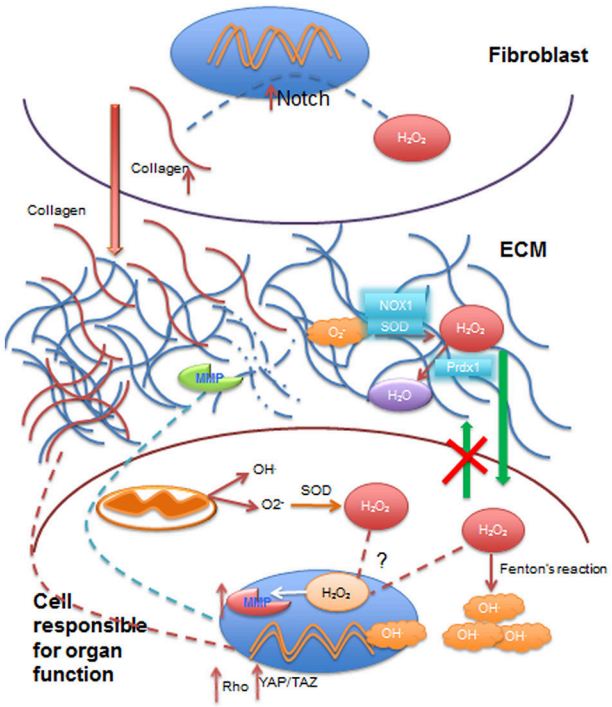

FIGURE 2 | Summary of some effects of ROS on extracellular matrix, cytoplasm and cell nucleus involved in dynamic reciprocity. Reactive oxygen species (ROS), such as superoxide anion ($O2^-$), transported from the vasculature to the extracellular matrix (ECM) are converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) and NADPH oxidases (NOX1) in the ECM. H_2O_2 may be reduced to water (H_2O) by reductases such as peroxireductase (Prdx1) in the ECM. Extracellular H_2O_2 can diffuse through cell membrane into the cytoplasmic compartment, but it cannot exit cells (green arrows), where it contributes to the increase in intracellular ROS levels by production of hydroxyl radicals ($OH^·$) by Fenton's reaction, or it can be transported to the nucleus to activate the transcription of matrix metalloproteases (MMP). Collagen can be broken down by MMP activity in the ECM. Besides extracellular ROS, mitochondrial activity also contributes to intracellular ROS, which can further add to H_2O_2 going to the cell nucleus. Stromal cells such as fibroblasts are activated by ROS (primarily H_2O_2), which increases collagen production via Notch signaling activation. Increased collagen deposited in the ECM (red arrow) contributes to increased stiffness of the ECM that, in turn, activates mechanotransduction pathways such as Rho and YAP/TAZ signaling with an impact on gene transcription. Nuclei are depicted in blue and mitochondria in dark orange.

stress (Provost et al., 2010; Espinosa-Diez et al., 2015). The nuclear accumulation of ROS may be measured by detection of Mn-Superoxide dismutase, but this enzyme is not influenced only by H_2O_2 (Miao and St. Clair, 2009; Wedgwood et al., 2011; Candas and Li, 2014). Nanoprobes might enable direct measurement of ROS in the nucleus, which would require removing interference from mitochondrial ROS possibly using Mitotracker (Puleston, 2015). Systems that track the production of ROS in the microenvironment and their transport to the nucleus through the cytoplasm are awaited since methods used to sense ROS in lower organisms with heavy metal and electrochemical probes or semi-stable paramagnetic compounds (Suárez et al., 2013; Koman et al., 2016) are considered too invasive and potentially toxic for mammalian cells.

CONCLUSION

Due to their dual effect as a benefactor and also a damage inducer, ROS are re-emerging as therapeutic targets. Most ROS inducing therapeutics are effective only under a highly oxidative microenvironment, further supporting the role of the microenvironment in disease and therapy. As an example, the tissue damage induced by ROS is considered to be an advantage in order to target tumor cells using agents such as procarbazine that releases azo compounds in the highly oxidative environment of cancers (Vallejo et al., 2017). These compounds further generate ROS that target tumor cells. Unfortunately, such an increase in ROS has been linked to differentiation of cancer stem cells and rapid recurrence of cancer (Ding et al., 2015). Improved cancer therapy based on ROS might require a combination of ROS inducers and inhibitors of ROS-mediated stem cell differentiation pathways, requiring further understanding of the epigenetic mechanisms influenced by ROS.

There is little doubt that microenvironmental ROS contributes to aging and disease. The concentration threshold necessary to induce lasting effects (via an impact on the epigenome) is essential to determine. Thorough investigations of the microenvironment are paramount in light of the influence of ROS on stromal cells and on the ECM. Physical alterations of the ECM nourish the concept of dynamic reciprocity between nucleus and microenvironment (Bissell, 1981), by possibly influencing the epigenome via mechanotransduction, which would consequently alter the impact of ROS on genes. Nuclear reciprocity targeting the ECM is illustrated by the fact that certain ROS-responsive pathways may control the expression of metalloproteases (Daugaard et al., 2007; Sims et al., 2011) that contribute to aging and cancer progression (**Figure 2**). Another example of reciprocity are the (epi)genetic alterations in cancer cell genes that regulate energy producing pathways, requiring more absorption of soluble factors from the microenvironment. Interestingly, tissue heterogeneity is emerging as an engine for cancer progression (Lelièvre et al., 2014; Mohanty et al., 2014; Kang et al., 2015). The microenvironment is likely a source of heterogeneity (Kim and Zhang, 2016; Natrajan et al., 2016) depending on variable local concentrations of oxygen, hormones, and growth factors. The contribution of extracellular ROS to such heterogeneity is an interesting avenue of investigation.

AUTHOR CONTRIBUTIONS

SC wrote several portions of the manuscript, designed the figures and participated in edits. YB wrote parts of the manuscript and participated in edits. SL wrote parts of the manuscript and edited the entire manuscript.

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Aging Mouse Models Reveal Complex Tumor-Microenvironment Interactions in Cancer Progression

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Mouse models and genetically engineered mouse models (GEMM) are essential experimental tools for the understanding molecular mechanisms within complex biological systems. GEMM are especially useful for inferencing phenocopy information to genetic human diseases such as breast cancer. Human breast cancer modeling in mice most commonly employs mammary epithelial-specific promoters to investigate gene function(s) and, in particular, putative oncogenes. Models are specifically useful in the mammary epithelial cell in the context of the complete mammary gland environment. Gene targeted knockout mice including conditional targeting to specific mammary cells can reveal developmental defects in mammary organogenesis and demonstrate the importance of putative tumor suppressor genes. Some of these models demonstrate a non-traditional type of tumor suppression which involves interplay between the tumor susceptible cell and its host/environment. These GEMM help to reveal the processes of cancer progression beyond those intrinsic to cancer cells. Furthermore, the, analysis of mouse models requires appropriate consideration of mouse strain, background, and environmental factors. In this review, we compare aging-related factors in mouse models for breast cancer. We introduce databases of GEMM attributes and colony functional variations.

Keywords: genetically engineered mouse models (GEMMs), gene knockout mice, aging, mammary tumorigenesis, mouse strain

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PREAMBLE

Since the introduction of gland cleared mammary fat pad by DeOme and associates (Deome et al., 1959), mammary gland and mammary cell transplantation has been used to study aging in the mammary gland. One of the initial observations was that normal mammary epithelium had a limited capacity to fill the mammary fat pad and the original mammary epithelium would not grow after five or so serial transplants (Daniel et al., 1968). Serial transplant senescence defines a property of normal mammary epithelium, with "immortalization" or non-senescence proof of a neoplastic transformation (Daniel et al., 1968, 1975). Subsequently, this "test-by-transplantation" became the basis for the early descriptions of mammary gland stem cells their "committed" intermediates (Smith and Medina, 1988, 2008). This experiment laid the foundation for modern stem cell research. Although endocrine influence on the mammary gland was well-understood at the time, additional environmental factors were somewhat ignored (Young et al., 1971; Daniel et al., 1983) and not widely applied to problems in neoplasia.

Genetic engineering technology brought a new scientific generation into the study of experimental mammary tumorigenesis. The literature produced deals only indirectly with the problems of aging and mouse mammary tumorigenesis. Our objective is to organize and discuss this abundant data set specifically in the context of aging-related cancer.

BACKGROUND

Breast cancer is an aging-related disease. According to American Cancer Society (www.cancer.org), multiple factors have been identified as risk factors. These factors include *aging*, gene mutations, ethnicity, breast tissue density, early menarche, menopause after age 55, exposure to certain chemicals and life style related factors (e.g., parity, breast feeding, birth control, hormone therapy, drinking alcohol, and obese). Each of the factors indicated above are also linking to aging. For example, first birth at an older age results in a higher risk (MacMahon et al., 1970) and the risk after parity is sustained in a woman who has her first pregnancy at an older age (Albrektsen et al., 2005; Meier-Abt and Bentires-Alj, 2014). Like the famous "nun study" suggesting that parity was protective, more recent studies comparing nulliparous and parous Norwegian women demonstrated that parity has a slight protective effect that increases with age (Albrektsen et al., 2005). The mechanism of increased cancer risk related to these proven epidemiological factors has been extensively investigated and debated. Clearly, endocrine changes with time affects incidence, but also raises a central conundrum of breast cancer risk—why does cancer risk increase with advancing age *even as endogenous estrogen levels drop after menopause?* And, for our purposes in this review, *do mice model this change?* Mice do not go through a "menopause" *per se*, but their reproductive capacity and hormonal levels, including estrogen, decline around 1 year of age (Nelson et al., 1982; Finch et al., 1984). Perhaps the better question is—*do mice live long enough to get mammary cancer?* This is where genetic engineering can help. Originally construed as a simple, cell intrinsic, proof of oncogene function, like a modern Koch's postulate for oncogenes, these genetic alterations, in many cases, instead result in a mild acceleration of the risk for developing mammary tumors. These genotypes are the important GEMM for studying breast cancer risk, risk modifiers, and aging.

A wide variety of GEMM have been generated to study beast and other cancers. Strong mammary epithelial specific-promoters driving potent oncogenes are most commonly used, with the obvious practical benefit of rapid and highly penetrant cancer development—a clear practical benefit to experimental design and execution (Borowsky, 2011). Mouse mammary tumor virus long terminal repeat (*MMTV-LTR*) (note: this construct does not include other viral elements, such as T antigens, and is not transforming by itself) and milk protein promoters such as whey acidic protein (*Wap*) and beta lactoglobulin (*Blg)* promoters are the most extensively used for transducing breast cancer-related genes in mouse mammary epithelial cells. However, the target molecule under *MMTV-LTR* promotion is transduced even in early developmental stages (Wagner et al., 2001), and most tumors with the *MMTV-LTR* as a promoter induce strong growth signals at a relatively early age in the mouse life span. On the other hand, *Wap* RNA expression is up-regulated by lactogenic hormones and is induced during the late stages of parturition and lactation (Pittius et al., 1988). This trait makes the *Wap* promoter a useful tool for analyzing the pregnancy-dependent effects. However, the transgene under *Wap* promoter activity is also activated in nulliparous mice and in early pre-lactation (Pittius et al., 1988) as well as during the early stages of involution (Wagner et al., 1997). The promoter activity is, however, the highest during pre-lactation/lactation.

Tumors arising in GEMM with transgenes constructed from strong epithelial promoters can be difficult to interpret. The promoters themselves are hormonally regulated, so that studying hormone blockers or depletion in these models can yield the trivial mechanistic result of down-regulation of the transgene. There is also the problem of simultaneous development and progression of multiple primary tumors, sometimes making the progression of a specific, single tumor impossible to study.

Gene knockout (GKO) mice also have been generated for breast cancer research and exhibit differences in mammary gland development and/or tumorigenesis. The GKO mice are generally designed to test for tumor suppressor type of genes. Therefore, the GKO does not usually result in the neoplastic alteration by itself. However, tumor suppressor GKOs often result in dramatic changes in tumor kinetics when crossed with an oncogenic transgene GEMM. Conditional gene knockouts using Cre-LOX technology and using mammary luminal specific (*MMTV-LTR-Cre*; *Wap-Cre*; *Blg-Cre*) or basal specific (*K14-* or *K5-Cre*) targeting are useful to focus on the molecular involvement in epithelial cells but may not reflect the importance of changes in the microenvironment during breast cancer progression.

The analysis of aging effects in breast cancer progression requires an understanding of senescence of any cell in the microenvironment as well as the cancer cell. Therefore, such phenomena as the immune cell activities on clearing damaged senescent cells must be considered. However, very little is known about how these microenvironment and immune cell activities are involved in GEMM. Variations in phenotypes depending on the mouse strain's background which also limits comparisons with other models. Further, the "environmental" factors, such as mouse housing, cycles of circadian rhythm, diet, viral—and bacterial-infections, can also affect the phenotype. However, environmental variables are almost completely ignored when models are compared. These environmental factors are also known as modifiers for immune cell activities and are important for immune cell-related aging. In this report, we introduce GEMM for breast cancer research which might be useful tools for analyzing the effects of aging and microenvironment.

AGING PARAMETERS IN GEMM

Aging in the mouse has been extensively described and discussed in literature and in monographic compendiums about pathobiology (Carlton et al., 2001) and physiology (Flurkey et al.,

2007). The pathobiology has been updated for application to GEMM (Brayton et al., 2012). Numerous biomarkers for mouse aging and senescence have been described and applied. For example, the use of T-cell populations and IGF-1 levels have been widely used but other markers are also quite useful (Flurkey et al., 2007).

Endocrine factors have also been analyzed for aging related study in mouse (Flurkey et al., 2007). The status of ovarian hormone production is key to understanding aging-related hormone receptor positive neoplasms in mouse and human mammary neoplasms. Although estrogen receptor (ER) positive mammary tumors in GEMM have been analyzed in prior studies (reviewed in Dabydeen and Furth, 2014), not all models have been tested for hormone dependent- and independent-tumors actions using ovariectomy or hormone disrupting chemicals. Ovarian ablation experiments are important in GEMM because aged mice are not anovulatory as in humans (see the section below: "Aging in the human breast and mouse mammary gland and microenvironment").

In the absence of biomarker data, life span is used for comparing potential aging effects on tumorigenesis in GEMM. Since the standard measurement of biomarkers have not been applied to GEMM of breast cancer and few, if any, laboratories keep colonies with sufficient populations to have accurate length of life data. We have arbitrarily adapted Harrison's life stages graph to estimate Mature (3–6 months), Middle Age (10–14 months), and Old (18–24 months) (Flurkey et al., 2007). Further, we have converted the numbers from months to weeks or days. The only data available, in most tumor related papers, is the T50 of tumorigenesis. These data can be misleading because of the chimeric populations used in some studies. However, the T50 is a useful metric for comparison.

MAMMARY TUMORS IN *MMTV-LTR* AND *WAP* PROMOTER DRIVEN MOUSE MODELS

MMTV-LTR was first used to generate Tg(*MMTV-Myc*) in 1984 (Stewart et al., 1984). The activity of this promoter is sensitive to progesterone and dihydrotestosterone but not to estrogen (Otten et al., 1988). It can also be affected by the combinations of other factors, such as cell density, nutrition, growth factor, and hormones (Young et al., 1975; Cardiff et al., 1976). Currently, 114 GEMM lines targeting 45 genes (except for *tTA*, *rtTA,* and *luc*) have been registered in Mouse Genome Informatics (MGI) (**Figure 1**) (Bult et al., 2015; Blake et al., 2017; Finger et al., 2017). Of these 45 genes, human or mouse derived, 27 genes promoted by the *MMTV-LTR* developed mammary tumors. Whereas the median tumor latency time (a.k.a. median tumor-free latency; T50) is not always indicated or reported and the estimated T50 is variable ranging from 4 weeks to more than a year (52 weeks). Since *MMTV-LTR* is also active in embryonic tissues, the gene induction can be higher in developmental stages of mammary gland growth (Wagner et al., 2001). Transgenes with strong signaling inducing activity (i.e., PyMT, Erbb2, Catnb, Nras, Haras) form palpable tumors at relatively young ages. However, interestingly, the T50 in several *MMTV-LTR* driven GEMM are longer than a year. Remember that according to Flurkey et al. mice aged 10–14 months are equivalent to 38–47 years middle-aged human, and 18–24months are equivalent to 56–69 years older human (Flurkey et al., 2007).

In *MMTV-LTR* driven tumor-forming GEMM (**Figure 1**), mice with T50 longer than 10 months (43 weeks) or older number 13 including: *Myc* (Cardiff et al., 1991), *Espl1* (Mukherjee et al., 2014), constitutively active *SRC* (Y527F) (Webster et al., 1995), auto-phosphorylation mutant *Erbb2* (NYPD) (Dankort et al., 2001), *Catnb* (Imbert et al., 2001), *DMP1β* (Maglic et al., 2015), *CCND1* (Wang et al., 1994), activated rat *neu* oncogene tagged with CD4 and CD8 epitopes (OT-I/OT-II) (Wall et al., 2007), *Cdc37* (Stepanova et al., 2000), *Myc* mutant insensitive to KRas signal (T58A) (Andrechek et al., 2009), *AURKA* (Wang et al., 2006) and dominant negative type II TGFb receptor (*TGFBR2*) (Gorska et al., 1998).

Some models, when analyzed, demonstrate that parity facilitates tumorigenesis. *MMTV-Catnb* (Imbert et al., 2001), *MMTV-Myc* (Stewart et al., 1984; Cardiff et al., 1991), and *MMTV-DMTF1* (Maglic et al., 2015) in parous mice have shorter T50 than nulliparous females. The *MMTV-Espl* GEMM forms tumor only with pregnancy (Mukherjee et al., 2014). Similarly, *MMTV-PTGS2* nulliparous female mice do not develop tumors in, but 85% of multiparous female mice develop tumors (Liu et al., 2001).

Even GEMMs without tumor, crossbred to a GEMM with mammary tumors often results in different tumor kinetics. For example, the following crosses all resulted in accelerated tumor development: *MMTV-MAT* × *MMTV-neu* (Rudolph-Owen et al., 1998), *MMTV-Vegfa* × *MMTV-neuYD* (Oshima et al., 2004), *MMTV-Akt1*T308D*S473D* × *MMTV-PyMT* (Hutchinson et al., 2001). In contrast, *MMTV-DTX1* × *MMTV-HRas* (or *MMTV-cMyc*) bigenic mice had better survival (Kiaris et al., 2004). Some GEMM publications which do not have sufficient information to estimate T50, are indicated in **Supplementary Data Sheet 1**.

Wap-promoter driven mouse model was first used to make *Wap-HRAS* (Andres et al., 1987; Nielsen et al., 1991). In 2017, 25 GEMM lines targeting 16 genes are registered in MGI. *Wap* promoter activity has been used to transactivate target cDNA in mammary epithelial cells during pre-lactation and lactation. These models might be useful for the study of parity-dependent tumorigenesis. In these GEMM, 13 genes transduced with *Wap*-promoter formed mammary tumor (**Figure 2**). Although the pregnancy usually occurs in younger, reproductive age mice, the T50s in *Wap* promoter driven GEMM vary widely from younger (21 weeks) to older (81 weeks) females. Examples include *Wap* promoter driving the expression of the intracellular domain of Notch4 with a longer T50 (43 weeks<) (nulliparous) (Gallahan et al., 1996), a truncated form of the SV40 T-antigen (*T121*) (Simin et al., 2004), SV40 large T (*Tag*) (Tzeng et al., 1993; Goetz et al., 2001), *TDGF1* (Sun et al., 2005), SV40 small T (*tAg*) (Tzeng et al., 1993; Goetz et al., 2001) and constitutively active rat *Mmp3* (Sympson et al., 1994).

Parity enhances tumorigenesis in the *Wap-Notch4* (Gallahan et al., 1996). *Wap-Shh* is a *Wap* promoter driven GEMM which

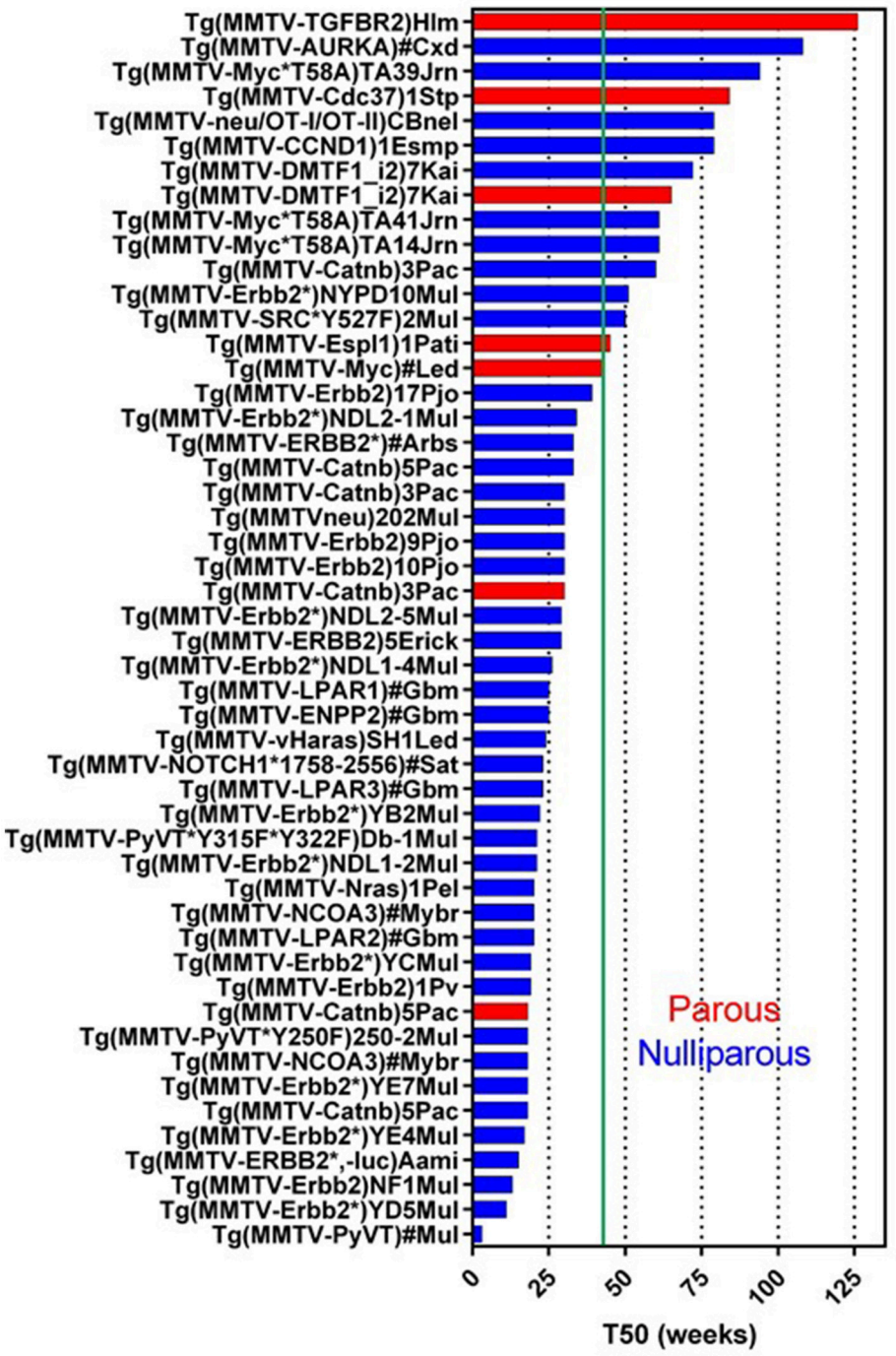

FIGURE 1 | Tumor latency in *MMTV-LTR* driven GEMM. A graph indicates a tumor latency (T50, weeks) in each *MMTV-LTR* driven GEMM. GEMM in Mouse Gemone Informatics (http://www.informatics.jax.org/) are shown. Blue and red bars indicate nulliparous and multiparous, respectively. Green line indicates 43 weeks (10 months) when the mouse is in age equivalent in mid-age human (Flurkey et al., 2007). If each publication described T50 or a graph for percentage of tumor free, T50 is indicated as weeks. If the publication does not indicate these parameters but has enough information to predict T50, indicated T50 were calculated. *MMTV-LTR* driven GEMM, which does not have sufficient information to calculate T50 or have other information (e.g., non-palpable tumor but microscopic observation for hyperplastic region), are summarized in additional **Supplementary Data Sheet 1**.

does not develop parity-dependent tumors other than in *Cre* expressing GEMM (García-Zaragoza et al., 2012). Interestingly, *Wap-Myc* showed the shortest T50 (21 weeks) (Schoenenberger et al., 1988) amongst the *Wap*-promoter driven GEMM. In contrast, *MMTV-Myc* showed longer latency (T50 = 43 weeks) (Stewart et al., 1984; Cardiff et al., 1991). This difference

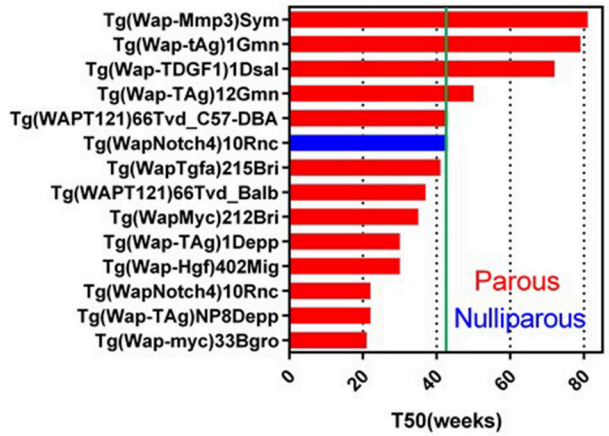

FIGURE 2 | Tumor latency in *Wap*-promoter driven GEMM. A graph indicates a tumor latency (T50, weeks) in each *Wap*-promoter driven GEMM. Blue and red bars indicate nulliparous and multiparous, respectively. Green line indicates 43 weeks (10 months). As same as *MMTV-LTR* driven GEMM, if each publication does not indicate T50, the T50 is estimated from the information. Additional information for GEMM not listed on this graph is available in **Supplementary Data Sheet 1**.

could reflect the transient *Myc* expression in *Wap-Myc* versus the sustained expression of *Myc* in *MMTV-Myc*. These *Wap*-promoted GEMM with longer T50s may provide useful clues for comparison of parity-related human breast cancer (Albrektsen et al., 2005; Meier-Abt and Bentires-Alj, 2014) and the effects of lactation in reducing human breast cancer risk (Ursin et al., 2004). For example, *Wap-Myc* (or other *Wap*-promoter driven) mice can have induced pregnancy at different ages and analyzed for tumorigenesis to identify the mechanism of higher risk in the first pregnancy in older age. The experiment can also be expanded to identify the mechanisms of multiparity dependent-or breast feeding dependent-risk reduction for breast cancer.

GKO MOUSE MODELS AND MAMMARY TUMORS

GKO mice have also been used to analyze the molecular mechanisms in breast cancer. The *Trp53* (*p53*) deletion is best known GKO mouse that develops mammary tumors. It is based on the study of heterozygous deletions found in *Li-Fraumeni syndrome* (Donehower, 1996). *p53* heterozygous and homozygous knockout mice on mixed genetic backgrounds have tumor-free survivals (T50) of 78 and 18 weeks, respectively (Donehower, 1996). In contrast, on a pure Balb/c background *p53* heterozygote and homozygous knockout mice have tumor-free survivals (T50) at 54 and 15.4 weeks respectively (Kuperwasser et al., 2000). Although these *p53* GKO mice develop various types of tumor, palpable mammary tumors are rare on the mixed backgrounds. The Balb/c model had "mammary tumors" at the rate of 78%. However, microscopic examination of the masses revealed that 68% of "tumors" consisted of palpable masses were stromal abnormalities and sarcomas rather than epithelial tumors (Kuperwasser et al., 2000). Other studies with p53 deletions targeted to the mammary gland resulted in forming heterogeneous types of mammary tumors, suggesting a second event with different oncogenic drivers (Cardiff et al., 2006).

We reported that a signal transducer and activator of transcription 1 (*Stat1*) GKO in 129S6/SvEvTac background has a reduced branching morphogenesis during mammary gland development (Chen J. Q. et al., 2015). Most important, this GKO developed ER positive luminal-subtype mammary carcinomas with T50 at 91 weeks in nulliparous mice and 78 weeks in parous mice (Mori et al., 2017). This is a rare GKO which forms a morphologically uniform type of mammary tumor without crossing with the other GEMM or *p53* GKO. This unique GKO will be discussed in more detail below.

Some studies using GKO crossed with tumorigenic GEMM targeting mammary epithelium have been instructive. Cell-cell association is also a microenvironmental signal to epithelial cells and provides important signals to polarize epithelial cells. *Cdh1 f/f; Trp53 f/f* is a Cre-dependent conditional deletion of *E-cadherin* (*Cdh1*) and *p53* resulting in double GKO only in cells (and their progeny) that express the bacterial (exogenous) Cre recombinase. When crossed with *K14-Cre* (Derksen et al., 2006) or *Wap-Cre (Derksen et al., 2011)* to conditionally knockout *Cdh1* and *p53* in mammary basal epithelial cells or epithelial cells, respectively, the resulting *K14-Cre; Cdh1 f/f; Trp53 f/+* developed mammary and skin tumors with T50 of 71 weeks. However, the homozygous deletions developed tumors with shorter T50s: *K14-Cre; Cdh1 f/f; Trp53 f/f* (T50 = 31 weeks) and *K14-Cre; Cdh1 f/+* (or *+/+*); *Trp53 f/f* (T50 = 48 weeks) (Derksen et al., 2006). In contrast, the *Wap* promoter versions of similar crosses, *Wap-Cre; Cdh1 f/f; Trp53 f/+*, formed mammary tumor with T50 of 83 weeks (calculated from Kaplan-Meier tumor free survival) which was much longer latency than *Wap-Cre; Cdh1 f/f; Trp53 f/f* (T50 = 28 weeks), *Wap-Cre; Cdh1 f/+; Trp53 f/f (T50 = 45 weeks), and Wap-Cre; Cdh1*$^{+/+}$*; Trp53 f/f* (T50 = 42 weeks) (Derksen et al., 2011). In both *K14-Cre* and *Wap-Cre*, genotype with *Cdh1 f/f; Trp53 f/+* exhibited prolonged latency, suggesting that these GEMM might be useful for the study of aging-related mammary tumorigenesis. *Cdh1* GKO model as another example of a homozygous GKO that is not sufficient for tumorigenesis but requires a cross with other GEMM to induce tumors.

Defects in *BRCA1 and BRCA2* genes have been recognized as risk factors for human breast cancer and ovarian cancer (Narod and Foulkes, 2004). *Brca1KO and Brca2KO* have been developed with deletions in different exons. Most of the homozygous *BrcaKO* (mutants of *Brca1KO* and *Brca2KO*) were embryonic lethal (Evers and Jonkers, 2006). Conditional KO models of exon11 of *Brca1* (*Brca1*$^{Ko/Co}$: mouse carrying a *Brca1*-null allele *Brca1*^{Ko22} and a conditional allele *Brca1*^{Co} crossed with *WAP-Cre* or *MMTV-Cre*) (Xu et al., 1999) have been analyzed for BRCA1 related studies. Females of these *Brca1* GEMM do not form mammary tumors in the first 10 months of life. Within 10–13 months they do develop a tumor with low incidences of 2 of 13 *Brca1*$^{Ko/Co}$*; WAP-Cre* and 3 of 10 *Brca1*$^{Ko/Co}$*; MMTV-Cre*. These lower rates of tumorigenesis are increased and accelerated in crosses with *Trp53* heterozygote: *Brca1*$^{Ko/Co}$*; MMTV-Cre; Trp53*$^{+/-}$ mice (8 out of 11 between 6 and 8 months

age) (Xu et al., 1999). The low incidence and prolonged latency suggest that these GEMMs could be useful for aging studies. However, like the majority of examples, to date these studies have not specifically emphasized "aging processes" in these models.

MOUSE STRAINS AND THEIR BASIC CHARACTERISTICS

Comparison of mouse models should be done in same background strain because of the intrinsic variation of phenotypes in each strain. Backcrossing GEMM to adjust the strain requires time, effort, and money. Any combination of these factors can be the reasons for avoiding breeding to homozygosity. But why this is important? Hunter's classical study of the *MMTV- PyMT* in 20 different background strains document the effect of genetic background a single transgene raised in the same colony (Lifsted et al., 1998). The F1 crosses exhibited different tumor latencies and different metastatic potentials. Studies from other facilities show that *MMTV-PyMT* had a T50 in C57BL/6: *MMTV-PyMT* of 92 days (14 weeks) as compared to the shorter T50 (43 days; 7 weeks) in FVB: *MMTV-PyMT* (Davie et al., 2007).

The normal life span in each colony is critical for the evaluation of any age-related study. Each inbred mouse strain has a different life span. Studies from the Jackson Laboratory have reported the comparison of survival rates from 12 to 20 months. They showed that the survival rate in C57BL/6, 129S1/SvlmJ, and FVB/NJ are almost identical (Sundberg et al., 2011). However, the survival rate at 20 months is lower in FVB/NJ (Sundberg et al., 2011). Therefore, the longevity is variable in different mouse strains. Comparison of life span in 32 inbred strains showed life spans that vary with gender and strain. For example, FVB/NJ (female = 760 days and male = 591 days), 129S1/SvlmJ (female = 819 days and male = 882 days) and C57BL/6J (female = 866 days and male 901 days) are life span in these mouse strains (Yuan et al., 2009).

The cause of the death is also variable in inbred mice. Age-related diseases and causes of death typical of some common strains have been documented (Brayton et al., 2012). Not surprisingly, their comparison of end of life neoplasms in three strains (B6, 129 and B6,129) in three institutions (UW, NTCR, NIH) also reveal differences incidence of neoplastic and non-neoplastic disease. These differences could be attributed to difference in mouse housing and maintenance.

Genetic drift in isolation is another factor. For example, some but not all FVB colonies have pituitary abnormalities. Shorter life spans in FVB might be partially related to the pituitary gland abnormalities (pituitary tumor 32%, pituitary hyperplasia 14%) in aging FVB/N mice. The study showed that about 30% of aged (>80 weeks) females also developed mammary gland tumors (Radaelli et al., 2009). Since FVB have been the most commonly used strain for transgenic targeting of the mammary epithelium, investigators using this strain need to collect pituitary at the end of the experiment.

The Brayton's study lists the factors which can affect mouse phenotype and experimental outcomes. Difference in diet (calories, its contents), enrichment, housing (cage type, density/cage, and pathogen status), ear tag, infectious agents, light cycle, and temperature can influence all phenotypes and experimental outcomes (Brayton et al., 2012). For example, studies with *MMTV-neu* show that diet alters the percent tumor free animals (Yang et al., 2003; Liu et al., 2005) and efficacy of tamoxifen (Liu et al., 2005).

THE MICROENVIRONMENT

The normal mammary microenvironment of the mouse has been extensively studied. However, most reviews emphasize the immune system or hypoxia (Rothschild and Banerjee, 2015). With the success of immunotherapy syngeneic tumor transplant models have become extremely useful. Without a doubt, all the other cell populations deserve mention. Whereas many factors play major roles in the epithelial-environmental, there is almost no information with the lack of mouse models of microenvironmental factor and aging. As examples of some of the molecules, we discuss herein the metalloproteinases and adhesion molecules.

Extracellular proteins, cell surface molecules and signaling molecules are the targets for the analysis of stromal-epithelial interactions. Some molecules involved in association between extracellular matrices (ECMs) and cell surface receptors have been analyzed in GKOs. These GKOs are often crossed with tumorigenic *MMTV-LTR* or *Wap*-promoter driven GEMM, because GKO of these molecules might not be sufficient to form palpable mammary tumors by themselves. As summarized in **Supplementary Data Sheet 1**, ECM molecule-related GKOs have been crossed mostly with *MMTV-PyMT* and *MMTV-neu.*

For example, metalloproteinases (matrix metalloproteinase: MMP; a disintegrin and metalloprotease: ADAM) are a family of zinc binding type extracellular proteinases which process ECM molecules and surface molecules (Wolfsberg et al., 1995; Nagase and Woessner, 1999). These molecules are known to be involved in various physiopathological activities including development, tissue remodeling and cancer cell invasion (Rudolph-Owen et al., 1998; Seiki et al., 2003; Fata et al., 2007; Reiss and Saftig, 2009; Mori et al., 2013). Some of these MMP genes (*Mmp-8*, and *-14*) and ADAMs (*Adam-12* and *-ts1*) have been used in studies of mammary tumor progression by crossing GKO with *MMTV-PyMT*. Deletion of *Adam* genes delay *PyMT* tumor growth and metastasis (Fröhlich et al., 2011; Ricciardelli et al., 2011). Deletion of the *Mmp* genes had a different effect in *PyMT* tumorigenesis. Interestingly, both *Mmp8*KO and *Mmp14*KO in bigenic crosses with *PyMT* developed tumors earlier than WT-*PyMT* control (Szabova et al., 2008; Decock et al., 2015). Lung metastasis of *PyMT* tumor was reduced in bigenic mice with *Mmp14*KO (Szabova et al., 2008). In contrast, *Mmp8* deletion enhanced *PyMT* pulmonary metastasis (Decock et al., 2015).

Tissue inhibitor for metalloproteinase 3 (*Timp3*) GKO when crossed with *MMTV-PyMT* and *MMTV-neu* inhibited tumor growth and lung metastasis (Jackson et al., 2015). The T50 in *MMTV-neu* in the absence of *Timp3* was ∼76 weeks compared with ∼31 weeks in $Timp3^{+/+}$; *MMTV-neu* (calculated from

Kaplan-Meier curve of the age at first detection of tumor) (Jackson et al., 2015), suggesting that *Timp3*$^{-/-}$ *MMTV-neu* might be another suitable model for studying aging-related breast cancer progression. Whereas *Timp3*$^{-/-}$; *MMTV-PyMT tumor* delayed T50 (∼13 weeks compared with control: 10 weeks), tumor growth was accelerated once tumor was formed (Jackson et al., 2015). Since TIMP3 has broad-spectral inhibition activity on both MMPs and ADAMs (Brew and Nagase, 2010; Murphy, 2011), TIMP3 target protease activities might be differentially involved in tumor growth and metastatic conversion. Once spatiotemporal distributions of MMPs, ADAMs, and TIMP3 were identified during mammary tumor progression are revealed, the TIMP3 effects on each target protease can be understood. Nonetheless, the delay in tumorigenesis in *Timp3*$^{-/-}$; *MMTV-neu* could be another useful model for studying age-related mammary tumorigenesis.

Other GKO mice crossed with *MMTV-PyMT* and *-neu* have also been used to study the functions of cell adhesion molecules' involvement during the neoplastic process. In particular, many of these molecules mediate the association between cells and microenvironment. Since *PyMT* tumors forms earlier than *neu* tumors, the *PyMT* tumor model may not be as good a model for the study of aging. However, accelerating or delaying tumorigenesis in *neu-* or *PyMT*-tumor might reveal the function of target molecules that may affect aging.

The models described here are GKO crossed with other mammary tumor models. To analyze the association between collagen and the receptor (integrin a2b1), *Itga2*KO was developed (Chen et al., 2002). This *Itga2*KO was then crossed with *MMTV-neu* (Ramirez et al., 2011). The bigenic cross showed the earlier T50 (31 weeks) with higher metastatic levels then the comparable *Itga2*$^{+/+}$; *MMTV-neu* control (33 weeks). CD151, a tetraspanin molecule associating with laminin binding integrins (Hemler, 2005), was knocked out and the mice crossed with *MMTV-neu* to study the molecular interactions with anErbB2 driven cancer (Deng et al., 2012). The T50 of the bigenic *CD151*$^{-/-}$; *MMTV-neu* was 48 weeks which indicates slower tumor growth compared with the control 38 weeks in the control *CD151*$^{+/+}$; *MMTV-neu* mice.

The GKO of *Hic5,* a focal adhesion scaffold/adaptor protein, was crossed with *MMTV-PyMT* and the T50 was 9 weeks longer than the heterozygous knockouts with *Hic5*$^{+/-}$; *MMTV-PyMT* (Goreczny et al., 2017). Protein-tyrosine phosphatase 1B (PTP1B), which is involved in regulating signals between cell and microenvironment, was knocked out and crossed with *MMTV-PyMT* and *-neu* (Bentires-Alj and Neel, 2007). Interestingly, *Ptp1b*$^{-/-}$*; MMTV-neu* exhibited a greatly reduced tumorigenesis of *neu* tumor (27% mice formed tumors at 69 weeks) as comparing with *Ptp1b*$^{+/-}$*; MMTV-neu and Ptp1b*$^{+/+}$*; MMTV-neu* (T50 = 61 weeks and 57 weeks respectively). *Ptp1b*$^{-/-}$*; MMTV-PyMT* did not, however, significantly delay PyMT tumor growth (Bentires-Alj and Neel, 2007). Some mammary tumor forming GEMM are also used to inhibit target molecule's activity (MMP9, Lysyl oxidase) or crossed with transgenic of mutant protein (e.g., collagenase resistant type I collagen alpha1 chain, Col1a1) are indicated in **Supplementary Data Sheet 1**.

These mouse models are useful for the study of various aspects of tumor progression. The mammary promoters (*MMTV-LTR, Wap-,* and *K14-*), however, are activated in other cells in different organs. *MMTV-Cre; Gt(ROSA)26Sor* transgenics demonstrate that the *MMTV-LTR* promoter actively transduces Cre in a variety of non-mammary tissues (e.g., salivary gland, skin, seminal vesicles, lymphocytes, megakaryocytes, erythroid cells, etc.) (Wagner et al., 2001). In addition, three *MMTV-Cre* promoted lines showed the defects in nursing pups; line A showed impairment in lactation, line F completely failed to nurse, and line D was normal.

Wap-cre; Tg(CMV-nlacZ)1Pgr showed Cre expression in brain, muscle and testes, and one line had Cre expression in mammary gland and the brain (Wagner et al., 1997). *K14-Cre* (Vasioukhin et al., 1999), *K5-Cre* (Tarutani et al., 1997), and *Lgr-CreERT2* (Kinzel et al., 2014) have also been used to conditionally knockout gene targets in mammary epithelial cells (Taddei et al., 2008; Koren et al., 2015; Liu et al., 2016). These promoters were originally developed to study skin tissue (Tarutani et al., 1997; Vasioukhin et al., 1999; Kinzel et al., 2014) and other organs (gut and kidney) (Kinzel et al., 2014). Thus, these promoter activities are not specific to mammary epithelial cells. This suggests that the effect on mammary gland in GKOs crossed with these Cre expressing GEMM might be more complex than mammary epithelial-specific promoters. Once aging dependent or independent inflammatory activity occurs in one organ, the activity of immune cells will be modified and can affect other organs in the same animal. In addition, crossing these models with different mouse background strains can cause the variations in phenotypes (e.g., immune cell activity, drug efficacy, tumorigenesis). In next section, we summarize these parameters.

AGING IN IMMUNE CELLS

The Jackson Laboratory's analysis of immune cell populations in peripheral blood in 6 months old inbred mice demonstrates that lymphocytes in female (F) and male (M) differ between strains and genders (Petkova et al., 2008). Examples include C57BL/6J which are F: 71.5% and M:78.2%, FVB/NJ are F:79.9% and M:52.5% and 129S1/SvlmJ are F:49.4% and M:60.5%(Petkova et al., 2008) Monocytes in C57BL/6J are F:4.47% and M:7.62%, FVB/NJ are F:5.79% and M:6.6% and 129S1/SvlmJ are F:8.3% and M:8.61% (Petkova et al., 2008). More importantly, these percentages of immune cells and the immune cell activities might also be different with age of the mouse strain. Studies of aging in immune cells, named "*inflammaging*" (Baylis et al., 2013; Salvioli et al., 2013; Franceschi and Campisi, 2014; Franceschi et al., 2017), indicate that each immune cell lineage and their activities change with age. These age-related changes can directly affect tumor initiation and progression. Since immune cells are a factor in "tumor microenvironment" and important in clearing damaged cells (such as senescence cells), immune cell activities in aged mice might also provide hints to decipher the link between aging and breast cancer.

LYMPHOCYTES

Decreased lymphoid progenitors and involution of the thymus occur during aging (Linton and Dorshkind, 2004; Palmer, 2013). This results in a decrease in T-cell output and shifts in cell types from naïve (CD62Lhigh CD44low) to memory T-cells (CD62Llow CD44high) (Shimatani et al., 2009; Sprent and Surh, 2011; Goreczny et al., 2017). These aging memory T-cells are PD1+ and CD153+CD44high CD4+ (Sato et al., 2017). They have the same phenotype as senescence associated T-cells found in lupus pathogenesis (Tahir et al., 2015), suggesting that the aging dependent increase in CD4+ T-cells might also be involved in increased risk of autoimmunity. Memory CD8+T cells are also increased in aged mice (Chiu et al., 2013). The memory CD8+ T cells in older mouse expand the numbers of effector CD8+ T cells in LCMV infection compared with younger mouse derived memory CD8+ T cells (Eberlein et al., 2016). This critical change in T cells could be an anti-pathogen defense system in aged mice with less naïve T-cells.

In aged humans, decreased memory B-cells and increased naïve B-cells are observed (Chong et al., 2005). In mice, B-cells numbers are decreased with aging because of decreased precursors in bone marrow (Riley et al., 1991). This is further defined as the defect in the process between pro-B and pre-B maturation in aged mice (Stephan et al., 1996). The defect in pro-B to pre-B maturation in aged mice is attributed to insufficient levels of IL-7 (Fleming and Paige, 2001). In *MMTV-PyMT* mice, splenic B-cells are increased, This might be considered as evidence of inhibitory factors secreted from the PyMT tumor cells that inhibit B-cell progenitor proliferation and maturation (Moreau et al., 2016).

Regulatory T cells (Treg: a.k.a. suppressor T cells) are also altered in aged mice. CD4+/CD25+/Foxp3+ and CD8+/CD25+/Foxp3+ Treg cells are almost two times higher in spleens and lymph nodes from aged mice, and prevents the activation of immune response (Sharma et al., 2006). Increased Treg are also observed in both peripheral blood and tumor microenvironment of invasive breast cancers (Liyanage et al., 2002). C57BL/6 with *Foxp3* knocked-out/knock-in human diphtheria toxin receptor (DTR; *Foxp3*^{DTR}) (Kim et al., 2007) is a GEMM for analyzing Treg activities in the presence or the absence of diphtheria toxin (DT). Orthotopic injection of a C57BL/6; *MMTV-PyMT* derived mammary tumor into *Foxp3*^{DTR} treated with DT results in reduction of PyMT tumor growth. Enhanced apoptosis was noted with proliferation of naïve CD4+ cells and CD8+ cells (Bos et al., 2013).

MACROPHAGES

Macrophage phagocytosis is involved in clearing apoptotic cells and is important for maintaining tissue homeostasis. However, aging reduces the macrophage capacity for clearing apoptotic cells (Aprahamian et al., 2008). Moreover, macrophage treated with tunicamycin, an inducer for endoplasmic reticulum stress, showed that macrophages isolated aged mice have more apoptosis compared with macrophages from younger mice (Song et al., 2013). Macrophage activation is also altered in aging. Cytokine induction to direct macrophage to M1 is increased in aged mice, but the M2 induction is decreased. Conversely, induction to M1 is weaker in younger mice, but induction to M2 is stronger (Lee et al., 2013).

Deletion of p53 in hepatic stellate cells (HSCs) induces fibrotic liver tumors which have proliferating HSCs and senescence of HSCs (Lujambio et al., 2013). Interestingly, conditioned media from proliferating HSCs induces M2 polarization, and contrary, conditioned media from senescent HSCs induces M1 polarization of macrophage (Lujambio et al., 2013). The macrophage polarization in two studies suggest that aging reduces macrophage induction to M2 status. This might decrease the anti-tumor activity of macrophages and facilitate tumorigenesis. This secretion from senescence cells is called senescence associated secretory phenotype (SASP). SASP causes pro- and anti-tumor activities through affecting to different types of immune cells (Rao and Jackson, 2016). Since secretion from cells have soluble factors (e.g., cytokines, processed proteins from cell surface) and extracellular vesicles (exosomes, microvesicles, and apoptotic bodies) (El Andaloussi et al., 2013), the identification of the contents within each fraction, function, levels, and distribution is now required to understand SASP as a part of aged microenvironment.

MYELOID DERIVED SUPPRESSOR CELLS (MDSCS)

MDSC is a key immune cell in the control of immune cell activities and reciprocally associates with Treg to regulate tumor progression (Hurez et al., 2012, 2017; Marvel and Gabrilovich, 2015). MDSCs are increased in aged mice (Grizzle et al., 2007; Enioutina et al., 2011) and human (Verschoor et al., 2013). This age dependent MDSC expansion is regulated by Nf-kB activity in aged mice (Flores et al., 2017). Aged mice have increased MDSCs and less T-cell cytotoxicity compared with young mice. The suppression of T-cell cytotoxicity is due to the high levels of arginase activity in aged MDSCs (Grizzle et al., 2007). Interestingly, the increase of MDSCs is also observed in bone marrow and spleen of tumor-bearing mice compared with naïve mice (Huang et al., 2006). These MDSCs from tumor-bearing mice induce the development of Treg (Huang et al., 2006) and T-cell tolerance (Kusmartsev et al., 2005; Huang et al., 2006). These age-related tumor permissive activities of MDSCs have been targeted for immunotherapy. Higher levels of tumor permissive activity in aged mice can be reduced with anti-GR1 targeting MDSCs, which reduces melanoma tumorigenesis (Hurez et al., 2012). Aged MDSCs have higher levels of B7-H1 (a.k.a. Programmed death ligand 1, PD-L1) induced by IL-10 activity, and the inhibition of B7-H1 reduced the tumorigenesis of Lewis lung cancer cells in aged mice (Chen S. et al., 2015).

DENDRITIC CELLS

Dendritic cells (DCs) are antigen-presenting cells and categorized by differentiation status into conventional DCs (cDCs), plasmacytoid DCs (pDCs), and monocyte DCs

(moDCs). The DCs are involved in a key function on T-cell activation (Gardner and Ruffell, 2016). Twenty six-month-old male C57BL/6 mice (from Jackson Laboratory and maintained at Oregon State Univ) had decreased splenic pDCs and CD8+ cDC (Wong et al., 2010). Another study with 18-month-old female C57BL/6 mice (from State Foundation for Production and Health Research and maintained at Instituto de Pesquisas Biomédicas, Brazil) found reduced numbers of CD11c+ cells but only cDCs were significantly reduced as compared with young mice (Pereira et al., 2011). However, C57BL/6 mice (from Animal Resources Center and maintained at The Centenary Institute for Cancer Medicine and Cell Biology, Australia) older than 18 months had increased cDCs in spleen and lung as compared to young mice. In contrast, Balb/c mice did not show the same difference between aged and young mice (Tan et al., 2012). This study also showed by comparison of T-cell activation of DCs from aged- and young- C57BL/6 mice no differences in co-stimulatory molecules (CD80, CD40, and CD70) except for CD86 (B7-2) which is reduced in aged cDCs with influenza infection (Tan et al., 2012). However, aging effect on CD86 levels are not differ in cDCs in above mentioned two other studies (Wong et al., 2010; Pereira et al., 2011) and without influenza infection (Tan et al., 2012). Thus, the difference in cDCs might be affected by variations in mouse strain background, gender, age, housing environment and pathogen activities. In a ovarian tumor model (C57BL6: *LSL-K-rasG12D/+p53*$^{loxP/loxP}$ generated *from Kras*^{tm4Tyj} and *Trp53*^{tm1Brn} *mice*), DCs changed the phenotype from immunosuppressive in the earlier stage of tumor expansion to immunogenic in the advanced stage of cancer (Scarlett et al., 2012), This switch suggests that the status of DCs might indicate the checkpoint for neoplasia as it become malignant.

NATURAL KILLER CELLS

Natural killer (NK) cell is a type of lymphocyte and an important cell for innate immunity.

A comparison of NK cells in healthy people from 4 to 106 years of age showed that NK cells were increased in older age, but NK activity in middle-aged subjects was weaker compared to young subjects and to centenarians (Sansoni et al., 1993). In mice, there were strain dependent and gender variations in a percentage of peripheral NK cells (Petkova et al., 2008). Whereas C57BL/6 and 129S1/SvlmJ mice have similar percentages of NK cells between the genders (C57 female: 3.74%, male: 4.02%; 129 female: 5.5%, male: 4.66%), FBV/NJ male mice have a significantly fewer NK cells (3.49%) than females (7.15%) (Petkova et al., 2008). NK cells are increased in bone marrow in aged Balb/c mice, resulting in decreased pro-B cells in aged animals (King et al., 2009). NK cells (with different levels of CD56 and CD16) were increased in the peripheral blood from breast cancer patients with advanced cancer but were mostly immature and non-cytotoxic subsets (Mamessier et al., 2013). Spleen tissues in FVB; *MMTV-neu* mice have almost similar number of NK compared with wild type (WT) FVB mice while other immune cells (T cells, DCs, MDSCs, and Tregs) are increased in FVB; *MMTV-neu* (Abe et al., 2010). In C57BL/6; *MMTV-PyMT,* tumor derived NK cells are immature compared with splenic NK cells. An adaptive transplantation of splenic NK cells into tumor resulted in decreased NK maturation, suggesting that tumor microenvironment in *MMTV-PyMT* tumor determines the NK cell maturation. The authors suggested that IL12 and TGFb are involved in NK maturation (Krneta et al., 2016). Thus, although NK cell numbers and activities differ in human and mouse, levels of immaturity in NK cells is similar in human breast cancers and mouse mammary tumors.

Immune cells' variations and activities different between mouse strains, genders, and age. The spatial density and activity of each type of immune cell within the tissue is critical to understanding associations between immune cells and immune targeted cells (e.g., tumor cells and cells in senescence). These associations are important for understanding age related tumorigenesis. They are equally critical for understanding the process of immunoediting from "elimination," "equilibrium" to "escape" (Schreiber et al., 2011; Mittal et al., 2014). The challenge is now to relate the two phenomena.

AGING IN THE HUMAN BREAST AND MOUSE MAMMARY GLAND AND MICROENVIRONMENT

Human breasts and mouse mammary glands are functionally similar. However, these tissues have distinct structural differences especially during the aging process (Cardiff et al., 2018). In puberty, human breast completes the formation of branching collecting ducts with terminal duct lobular unit (TDLU) in collagen fiber rich stroma. In contrast, mouse mammary glands form a ductal branching tree without TDLU structures and primarily embedded in adipose tissue. Mouse mammary gland develops lobulo-alveolar structures with pregnancy. The murine lobulo-alveolar units quickly involute within a week of weaning (Cardiff and Wellings, 1999). Human breast also expands lobulo-alveolar units with endocrine signals during pregnancy and parturition. In contrast to the mouse, the human involution is slow and the TDLU structures remain (Cardiff and Wellings, 1999) with some structural variations, which correlate with time, after the postpartum (Jindal et al., 2014). Age- related involution in human breast occurs during perimenopause (or surgical- or chemical induced menopause) with the atrophy of senescence, which results in major reduction in TDLU structures (Cardiff and Wellings, 1999; Cardiff et al., 2018). In contrast, aged mice continue to ovulate and have estrus cycles. This limits the use of mice to model human peri- and post-menopause conditions (Nelson et al., 1982; Finch et al., 1984). In order to analyze menopause dependent effects, the investigator can us surgery- and chemical treatment-dependent induced menopause in mice. One approach might be ovariectomy (Finch et al., 1984) which quickly shuts down the 17β-Estradiol levels, stops the cyclicity and quickly maximizes FSH levels (www.jax.org). Another is 4-vynylcyclohexene diepoxide (VCD) treatment, which slowly increases FSH levels and slows down cyclicity (Brooks et al., 2016).

STUDYING PARITY-RELATED BREAST CANCER AND AGING

Although multiple-pregnancies reduce the incidence rate of human breast cancer (Albrektsen et al., 2005), the risk reduction is sustained longer in women having a first pregnancy after 35 years old (Albrektsen et al., 2005; Meier-Abt and Bentires-Alj, 2014). Women having a first pregnancy after 30 years old also have higher risk for breast cancer and the risk is sustained higher until 65 years old compared with woman with nulliparous status (Albrektsen et al., 2005; Schedin, 2006; Meier-Abt and Bentires-Alj, 2014). The many factors (stem cells, hormone and hormone receptor status, growth factors, cytokines, ECM, and immune cell activities) which might be involved in these age- and parity-dependent breast cancer are discussed elsewhere (Schedin, 2006; Meier-Abt and Bentires-Alj, 2014).

The duration of breast-feeding is also an important factor. Prolonged nursing (lactation) reduces the risk of breast cancer even in aged cohort (50–64 years old)(Ursin et al., 2004). Analyzing involvement of each factor which functions as cancer promoting or suppressing will be a challenge for future research. The effects of parity have been well-documented in mouse models. Although some GEMM have prolonged tumor latency, factors relating to aging have not been recorded in many of the candidate models. But the current GEMM or newly generated GEMM combined with analysis indicated in this manuscript might give a hint for modeling the experiments. For example, the analysis of microenvironmental factors in parity facilitated, syngeneic orthotopic transplants in mice (Maglione et al., 2004; Namba et al., 2004, 2005; Borowsky et al., 2005) under different well-controlled conditions of age, parity status, parity cycles, and milk feeding duration. Since tumors from some GEMM grow too aggressively, tumors with slower tumor kinetics will be more suitable for analyzing aging and parity related tumorigenesis.

STAT1 GKO AS A MODEL FOR ANALYSIS FOR AGE RELATED MAMMARY TUMORIGENESIS

The focus on mammary cancer in mice has been, quite correctly, the mechanisms that make the neoplastic epithelial cell autonomous. The transgenic mouse has provided the "Koch's Postulate" for modern genomic science and cancer by defining the neoplastic potential of numerous genes and/or characterizing genes that modify neoplastic progression. In spite of a plethora of these mice, they have not been used to address the issue of aging in breast cancer. Although research on the cancer microenvironment has blossomed, the issue of aging in GEMM has not been effectively addressed.

We have studied in detail a GEMM that includes prolonged tumor latency, 129S6/SvEvTac: *Stat1*^{tm1Rds} (129: *Stat1*$^{-/-}$) mice (Meraz et al., 1996), which is a unique GKO that rarely develops mammary cancer before 1 year and has a T50 of 91 weeks (Mori et al., 2017). The *Stat1*$^{-/-}$ females were held as long as 120 weeks (840 days) which exceeded the expected 817-day life span of wild type 129 females. From this perspective, the T50 was reached at 78% of the total life span. The 129: *Stat1*$^{-/-}$ provides some insight into many of the factors considered in the review above and the opportunity to consider the complex issues in such models.

Other groups have demonstrated that deletion of *Stat1* leads to an increased susceptibility to mammary tumors (Klover et al., 2010; Raven et al., 2011; Schneckenleithner et al., 2011; Chan et al., 2012). The 129: *Stat1*$^{-/-}$ model forms mammary tumors with a T50 at 91 weeks in nulliparous and at 78 weeks in parous females, similar to the report from the colony in Washington University (Chan et al., 2012). Mice younger than 32 weeks old in our colony did not have detectable neoplastic histopathology. Mice older than 52 weeks nulliparous without tumors had microscopic MIN (mammary intraepithelial neoplasia; 3/32 cases), and 23/32 cases had preneoplastic MIN or tumors.

Tumor-bearing nulliparous (9/13 cases) showed lobulo-alveolar hyperplasia even without pregnancy or exposure to the pregnancy-associated hormones and these mice had dilated mammary duct filled with milky white fluid. The older tumor-bearing nulliparous, with stunted growth in mammary gland (4/32 cases) still had underdeveloped ductal network (Chen J. Q. et al., 2015).

The strain variation is also an important issue in *Stat1* GKO. Other *Stat1* GKO lines, *Stat1*^{tm1Dlv}, backcrossed into Balb/c strain also shows tumorigenesis in multiparous *Stat1-null* condition with mammary tumor and disease onset at 57 weeks which is shorter latency compared with *Stat1*$^{+/+}$ (69 weeks) (Durbin et al., 1996). Balb/c: *Stat1*^{tm1Dlv} crossed with Balb/c: *MMTV-Neu* shows shorter latency of *Neu* type tumors as compared with Balb/c: *MMTV-Neu* nulliparous mice (42 vs. 50 weeks) (Raven et al., 2011). Similarly, *Stat1f/f* was crossed with FVB: *Tg(MMTV-Erbb2*,-cre)1Mul* (Ursini-Siegel et al., 2008) to conditionally knock out *Stat1* gene and simultaneously transduce *neu* in mammary epithelial cells, which also results in an earlier T50 in the *Stat1-null* condition (50 vs. 63 weeks) (Klover et al., 2010). These two studies (Klover et al., 2010; Raven et al., 2011) suggest STAT1 as a tumor suppressing molecule, and the work of Klover et al. (2010) indicates that STAT1 function in mammary epithelium is anti-tumorigenic.

Interestingly, mammary gland development in *Stat1* GKO is only impaired in 129S6/SvEvTac (Chen J. Q. et al., 2015) but the defect has not been observed in GKO in the Balb/c background (Schneckenleithner et al., 2011). Mammary tumorigenesis is also different in these two strains: 129:*Stat1*$^{-/-}$ forms homogeneous mammary tumors with or without parity (Chan et al., 2012; Mori et al., 2017), but Balb/c: *Stat1*$^{-/-}$ formed heterogeneous tumors only with multiparity (Schneckenleithner et al., 2011). However, these animals may not have been maintained long enough to develop tumors. For example, in one study, the Balb/c: *Stat1*$^{-/-}$ females developed mammary tumors only after being force-bred seven times.

The interpretation of these observations is compounded by the use of different molecular constructs for the knockout of *Stat1*, which further could have influenced the outcome. The Meraz construct deletes the N-terminus of STAT1 in 129/Sv mice, whereas residual low expression level C-terminus

fragment remains (Meraz et al., 1996). The other construct deleting exon 18–20 in unknown background mouse strain does not have this fragment detected by anti-C-terminus STAT1 (Bailey et al., 2012). However, exon 18-20 deleted *Stat1*$^{-/-}$ mice still need to be investigated with anti-N-terminus STAT1 and analyzed for any defect. Thus, mechanisms leading to tumor development in 129: *Stat1*$^{-/-}$ remain unclear.

The initial study of normal mammary development in the 129: *Stat1*$^{-/-}$ females revealed a defect in branching morphogenesis accompanied by abnormal terminal end buds (Chen J. Q. et al., 2015). Given the type of molecular signaling, this could be expected. The DeOme's "Test-By-Transplantation" (Deome et al., 1959) with reciprocal transplants into gland-cleared mammary fat pads was used. The GKO epithelium developed normally in wild type mammary fat pad. Thus, the developmental phenotype was not intrinsic to the mammary epithelium.

Subsequent experiments proved that the GKO fat pad lacked cytokines needed for growth. Therefore, we concluded that the microenvironment of the fat pad was responsible for the growth defect. Interestingly, the GKO fat pad could be stimulated to produce adequate levels of cytokines using injections of a progesterone and prolactin. Unfortunately, we were unable to perform similar experiments using fat pads from older GKO females. These experiments, however, point to the importance of the microenvironment and the endocrine system in mammary development and demonstrate the consequences when they are defective.

The 129: *Stat1*$^{-/-}$ mammary tumors are morphologically and molecularly unique, ovarian dependent tumors. The tumors involve the neoplastic transformation of a stem cell population that is FoxA1+, ER+, PR+ with unique atypical cells with large, oval, moderately pleomorphic nuclei with an open chromatin and prominent nucleoli with pale cytoplasm, named large oval pale (LOP) cells. The characteristic of LOP cell is similar to "committed progenitor cells" in mouse mammary gland (Smith and Medina, 1988; Chepko and Smith, 1999) and suprabasal clear human breast cells (Toker, 1967; Stirling and Chandler, 1976, 1977; Smith et al., 1984). In addition, FoxA1 is known as a key factor ("pioneer transcription factor") (Zaret and Carroll, 2011; Jozwik and Carroll, 2012; Zhang et al., 2016) which regulates the expression of estrogen receptor alpha (Bernardo and Keri, 2012; Liu et al., 2016; Zhang et al., 2016) and is an essential factor for mammary branching morphogenesis (Liu et al., 2016).The cell of origin is readily identified as the LOP cell. In the early manifestations, the LOP cell can be found distributed along ducts. Later, the LOP cell forms large, invasive neoplasms. The epithelial intrinsic mechanisms have been described including activation of the JAK2 axis, deregulation of SOCS3 and mutation of the prolactin receptor (Chan et al., 2014; Griffith et al., 2016).

The differences between two GKO *Stat1*-null strains might be reflecting the differences in immune cells: percentages of CD4+ T cells, CD8+ T, cells and NK cells in peripheral blood are higher in Balb/cByJ, and granulocytes, eosinophils, monocytes and B-cells are higher in 129S1/SvlmJ (Brayton et al., 2012). Because of STAT1 involvement in interleukin 2 signal in T lymphocytes and NK cell lines (Frank et al., 1995), the *Stat1*$^{-/-}$ mice were originally generated to analyze the STAT1 involvement in NK cell activity via interferon (IFN) dependent signals and in other cytokines' signals (Meraz et al., 1996). The study showed that STAT1 deficiency leads to inactivation in IFNa and IFNg signals (Durbin et al., 1996; Meraz et al., 1996) and has a defect in responding to microbial pathogens (Meraz et al., 1996) and viruses (Durbin et al., 1996; Meraz et al., 1996), but the response for other signals (growth hormone, EGF and IL-10) were not impaired (Meraz et al., 1996). In later study, 129: *Stat1*$^{-/-}$ was shown to have a defect in cytolysis activity in NK cells (Lee et al., 2000).

Our pathological analyses of the three cohorts of older 129: wild type (WT), tumor free 129: *Stat1*$^{-/-}$ and tumor-bearing 129: *Stat1*$^{-/-}$ revealed characteristic lesions of aging: including. ovaries with luteinized stroma and fewer Graafian follicles. However, the hormone regulating glands (adrenal gland, thyroid gland, and pituitary gland) were all histologically normal. Evidence active estrus cycle was still found in 96 weeks old mice, indicating the ovarian hormones were still active. Although some 129: *Stat1*$^{-/-}$ showed the defects such as ovarian cysts, not all female with ovarian cysts developed mammary tumor, and one tumor-bearing female had histologically normal ovaries. These observations on ovaries and estrus cycle indicate that the tumorigenesis in 129: *Stat1*$^{-/-}$ is not due to the lack of ovarian hormones.

To understand the effect of the microenvironmental factors in *Stat1*-null tumorigenesis, syngeneic orthotopic transplants of *Stat1*-null tumor and MIN tissue into younger 129 WT and KO were performed. Tumors were palpable earlier in WT host. However, the tumor growth rates were almost the same in both hosts. This still resulted in 10-100 folds larger tumor in WT at the termination time point.

The analysis primary tumors in 129:*Stat1*$^{-/-}$ showed that stroma adjacent to the tumor had larger infiltrates of inflammatory cells (macrophage and T-lymphocytes) with fibrosis as compared to other GEMM (Cardiff et al., 2006). These observations of immune cell activities suggested that *Stat1*-null tumor makes an immune-attractive tumor-adjacent microenvironment and has an "excluded infiltrate" phenotype (Hegde et al., 2016). To investigate the possible mechanisms, we analyzed factors secreted from *Stat1*-null cell lines and found that soluble factors inhibit macrophage migration whereas the extracellular vesicle fraction stimulates macrophage migration. This indicates that SASP activity in *Stat1*-null tumors involves with cross talk with the microenvironment.

The point of introducing our study here is to provide an example of analyzing aging- related tumorigenesis in the mammary gland. First, as we discussed in above, environmental factors (housing, cycles of circadian rhythm controlled by light/dark cycles, diet, viral- and bacterial-infections and any other pathogens) for maintaining mouse colony can affect mouse phenotype. It is not easy to adjust these parameters in every institution to compare mouse models, at least checking viral- and bacterial-infection should be done to consider

if immune cell activity is different or not in each mouse colony. Our colony ismonitored using a serology profile (UC Davis mouse level2 serogenic profile: Mouse Hepatitis Virus, Sendai, Pneumonia Virus of Mice Reo-3, MPV, Minute Virus of Mice (parvovirus type) NS-1, M.pul and arth, Theiler's Murine Encephalomyelitis Virus part of GDVII strain, Reo-3, Lymphocytic Choriomeningitis Virus, Epidemic Diarrhea of Infant Mice Virus, Mouse Adeno DNA Virus 1 and 2, Mouse Noro Virus). In addition, bacterial pathogens are tested on cecum or nasopharynx and pinworms or fur mites are also routinely checked (Mori et al., 2015). In our case, although specific immune cell activities are not compared, 129: *Stat1*$^{-/-}$ at UC Davis and Washington University showed identical mammary tumorigenesis phenotypes, especially for T50 in nulliparous and parous dependent and tumor subtypes (Chan et al., 2012; Mori et al., 2017).

Secondly, wild type and KO were necropsied to find causative factors of mammary tumorigenesis and cause of death (COD) especially for evidence associated with aging. The guidelines of for determination for of COD contributing to COD (CCOD) or cause of morbidity are as suggested by The National Center for Toxicological Research (NCTR), which is the important for aging studies (Kodell et al., 1995; Brayton et al., 2012).

Third, syngeneic orthotropic transplantation was used to understand some microenvironmental effects. Since many of GEMM have targeted gene regulation for embryonic stages or hormone dependency or inducible gene expression system multiple cell types could be targeted in these GEMM. In our case, *Stat1* gene deletion could affect any *Stat1* expressing cells. Therefore, the tissue microenvironment developed abnormally, or the tumor cells generated a unique microenvironment, or gene modification affected the microenvironment in *Stat1* GKO. Some the non-epithelial effects can be eliminated using syngeneic orthotopic transplantation. This approach worked very well for the early experiments with younger host. Unfortunately, only young hosts were available for transplantation. Testing hypothesis in aged host mice will have to held for our future experiments. Syngeneic orthotopic transplantation is also useful for studying spatiotemporal factors in aging or cancer research.

Fourth, the understanding of targeted aging or microenvironmental factors in mammary tumorigenesis, can be analyzed applying these techniques. These can provide a broad-context picture of the biology of aging and cancer.

SUMMARY

GEMM have been generated for analyzing functions of a gene in physiopathological events and are often crossed with other GEMM to further investigate context dependent biological phenomena. In breast cancer research, GEMM made for analyzing the gene involvement for mammary tumorigenesis have been used to study tumor progression in mammary glands and metastasis. Although a longer tumor latency (T50 value) suggests involvement of the target molecule in aging-dependent mammary tumorigenesis, few studies have defined how aging and impacts tumor initiation or tumor progression. This lack research effort might be explained by multiple issues: difficulties in separating the aging effect from tumorigenesis; the commitment of time to complete a project; and the cost and the effort required to model aging are all confounding. Tumorigenesis in most GEMM is too rapid to permit aging related comparisons. Complex parameters must be considered to understand entire *in vivo* biological system. Finally, lack of good GEMM for studying aging and mammary tumorigenesis simultaneously has hindered progress in this area.

129: *Stat1*$^{-/-}$ is a model for the study of age-related mammary tumorigenesis. The frequency of tumorigenesis, T50 and tumor subtype of the model provides a model that is an age-related ER+ tumor model. The origin and the fate of LOP cells needs to be identified and coupled with immunoediting and microenvironment factors. One envisions that experiments with this model will provide a complex but informative picture of breast cancer. This will be major challenge for investigators including us but will lead to much sounder understanding. Fortunately, these issues are not limited to the 129: *Stat1*$^{-/-}$ model but also applicable to many other GEMM.

The challenge is to understand the complex biology in each GEMM. It very important to share experimental information, data, and materials in addition to each publication. This will facilitate collaborative research by reducing time, cost and mouse usage and lead to an improved understanding of the biology of cancer in aging.

SHARING GEMM DERIVED INFORMATION AND MATERIALS ON AGING

Databases available for sharing information:

Genome informatics (MGI: http://www.informatics.jax.org/), Mouse tumor biology database (http://tumor.informatics.jax.org/mtbwi/index.do), European mutant mouse pathology database (http://pathbase.net/), National Cancer Informatics Program (NCIP) hub for a collaboratory for cancer research (https://nciphub.org/). These databases give basic information of registered mouse models. However, each institution's environmental information for maintaining mouse colony and serogenic profile for each GEMM is not well-documented. Available tissue samples with detailed conditions have not been organized. There is an activity of "*Sharing Experimental Animal Recourses, Coordinating Holdings,*" which suggests sharing the materials from experimental animal models to reduce animal use and accelerate collaborative research. Especially for sharing aging research models, sharing hub website has been developed (www.sharmuk.org). Our website, Center for Genomic Pathology, additionally provides online courses for educating researchers (www.ctrgenpath.org). These are important to further understand each GEMM characteristics by comparing with a certain condition, which facilitate the cancer and aging related research.

AUTHOR CONTRIBUTIONS

HM and RC: Conceptualized this review article, preparing the manuscript; AB: Preparing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00035/full#supplementary-material

Supplementary Data Sheet 1 | Excel files are summarizing GEMM with following categories. Page1: a list of *MMTV-LTR* driven mouse models, page2: a list of *Wap*-promoter driven mouse models, and page3: a list of GKO and other models. Each mouse model is indicated as Symbol (registered name for mouse model), name (transgene information and registered person's name), background strain, mammary tumor, latency, and estimated T50. A list of GKO has PMID or PMCID as additional information.

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Targeting Tumor-Associated Macrophages as a Potential Strategy to Enhance the Response to Immune Checkpoint Inhibitors

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Inhibition of immune checkpoint pathways in $CD8^+$ T cell is a promising therapeutic strategy for the treatment of solid tumors that has shown significant anti-tumor effects and is now approved by the FDA to treat patients with melanoma and lung cancer. However the response to this therapy is limited to a certain fraction of patients and tumor types, for reasons still unknown. To ensure success of this treatment, $CD8^+$ T cells, the main target of the checkpoint inhibitors, should exert full cytotoxicity against tumor cells. However recent studies show that tumor-associated macrophages (TAM) can impede this process by different mechanisms. In this mini-review we will summarize recent studies showing the effect of TAM targeting on immune checkpoint inhibitors efficacy. We will also discuss on the limitations of the current strategies as well on the future scientific challenges for the progress of the tumor immunology field.

Keywords: tumor microenvironment, immunotherapy, checkpoint inhibitor, $CD8^+$ T cell, macrophage, TAM, tumor immunology

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INTRODUCTION

Solid tumors are "aberrantly developing organs" in the body initiated by oncogenic mutations, which causes infiltration of different population of immune cells. A recent study shows that high number of cytotoxic lymphocytes such as natural killer (NK) or $CD8^+$ T cells in the tumors correlate with favorable prognosis, whereas high infiltration of myeloid cells such as eosinophils, tumor-associated macrophages (TAM) and neutrophils is associated with poor prognosis in most solid tumors (Gentles et al., 2015). Since TAM is one of the most abundant cell types in tumor (Qian and Pollard, 2010), several metanalyses further evaluated the correlation of TAM infiltration with clinical stage, overall survival and recurrence free survival in different cancers, and indicated that high infiltration of TAM correlates with poor overall survival in breast, gastric, oral, ovarian, bladder and thyroid cancers, but not in colorectal cancer (Zhang et al., 2012; Guo et al., 2016; Mei et al., 2016; Yin et al., 2017; Zhao et al., 2017). These studies indicate that the poor prognostic outcome of a neoplastic lesion is determined not only by the type of mutation occurred but also by the tumor stromal composition especially immune cells, i.e., the recruitment and activation of cytotoxic lymphocytes (e.g., $CD8^+$ T cells) can suppress lethal tumor development whereas the infiltration of TAMs promotes it. Better understanding of these tumor suppressing and tumor promoting cells is thus essential to establish efficient cancer immunotherapies. Decades of dogged studies about the $CD8^+$ T cell functions have established several immunotherapeutic strategies including cancer vaccination, transfer of *ex vivo* activated

$CD8^+$ T cells, and administration of cytokines that activate $CD8^+$ T cells. Of special note is the success of checkpoint inhibitors that reboot $CD8^+$ T cells in the tumors (Farkona et al., 2016; Khalil et al., 2016). In contrast, studies about the effects of immune suppressor cells on these therapies are just started. In this review, we highlight the recent findings about suppressive effects of TAM on checkpoint immunotherapy, and discuss therapeutic potential of a novel immunotherapy combined checkpoint inhibition with TAM intervention.

THE ROLE OF MACROPHAGES IN THE IMMUNE RESPONSE IN SOLID TUMORS

During initiation and progression of solid tumors, mutant and thus potentially immunogenic cancer cells are exposed to and interact with a complex immune system, which will determine the fate of cancer cells.

Cytotoxic lymphocytes such as $CD8^+$ T cells and NK cells have potential to detect and eliminate cancer cells by inducing apoptosis. Macrophages are also potentially able to mount a robust anti-tumoral response as they can directly kill cancer cells if properly activated and support the adaptive immune response by presenting tumor antigens and by producing chemokines and cytokines that recruit and activate cytotoxic $CD8^+$ T cells and NK cells (Gifford et al., 1986; Brigati et al., 2002). So, if these immune reactions are dominant in the tumor microenvironment, the development of malignant tumors will be suppressed.

However, in many cases the tumor microenvironment alters macrophage functions from the pro-inflammatory (i.e., tumoricidal) to the trophic ones that resemble those of macrophages in the developing tissues (Pollard, 2009; Noy and Pollard, 2014). As a result, these tumor-educated macrophages promote malignant tumor development instead of suppressing it. For example, studies using different mouse models of solid tumors demonstrated that TAM promotes angiogenesis, cancer cell invasion and intravasation in the primary site, as well as extravasation and persistent growth in the secondary site (Qian and Pollard, 2010; Kitamura et al., 2015). Furthermore, several studies suggest that TAM is likely to protect cancer cells from the anti-tumor immune responses. For example, TAM expresses programmed cell death ligand 1 (PD-L1), PD-L2, CD80, and CD86 that restrict $CD8^+$ T cell activities upon binding to the immune-checkpoint receptors, programmed cell death protein 1 (PD1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (Noy and Pollard, 2014; Mantovani et al., 2017). It is also reported that macrophages isolated from the mouse and human tumors can directly suppress T cell responses *in vitro* (Ruffell and Coussens, 2015), and that depletion of TAM enhances $CD8^+$ T cell-mediated anti-tumor immunity in the mammary tumors in mice under treatment with chemotherapy (DeNardo et al., 2011). Therefore, TAM represents immune suppressor cells in the solid tumors that restrict anti-tumor immune reaction induced by $CD8^+$ T cells.

CHECKPOINT INHIBITORS AS A NOVEL ANTITUMOR THERAPEUTIC STRATEGY

One of the most successful approaches to mount $CD8^+$ T cell-mediated anti-tumor immune reaction is the administration of checkpoint inhibitors, i.e., blocking antibodies against inhibitory checkpoint receptors (e.g., PD-1 and CTLA4) or ligands (e.g., PD-L1) (Farkona et al., 2016; Khalil et al., 2016). Strikingly there have been successful clinical trials with the immune checkpoint inhibitors that have revealed a great potential of immunotherapies for the treatment of malignant tumors such as melanoma and lung cancers (Sharma and Allison, 2015). However, the majority of patients in most cancers do not fully respond to this type of immunotherapy for reasons still unknown. Although this lack of response could be due to the expression of checkpoint ligands in cancer cells and microbiota composition (Sharma et al., 2017; Gopalakrishnan et al., 2018; Routy et al., 2018), recent studies indicate that expression of PD-L1 in leukocytes rather than tumor cells is essential for PD-L1 blockade-mediated tumor regression (Lin et al., 2018; Tang et al., 2018), which emphasizes the contribution of tumor-infiltrating leukocytes to the insufficiency of checkpoint therapies. It has been reported that certain types of leukocytes such as regulatory T (T_{reg}) cell, myeloid-derived suppressor cell (MDSC), and TAM suppress T cell activities and promote tumor progression (Kitamura et al., 2015). Given their abundance in the tumor microenvironment, TAM is suggested as one of the important therapeutic targets to enhance the efficacy of immunotherapies utilizing checkpoint antagonists (Mantovani et al., 2017).

TARGETING TAM POTENTIATES THE EFFICACY OF CHECKPOINT INHIBITORS

One of the efficient strategies to target TAM is the blockade of colony-stimulating factor 1 receptor (CSF1R) that is essential for the recruitment, differentiation, and survival of TAM (Mantovani et al., 2017). In mouse models of solid tumors including colon cancer, breast cancer, and glioblastoma, monoclonal antibodies or small molecule inhibitors against CSF1R reduces the number of TAM and/or changes the phenotype of TAM, which impairs tumor development and progression (DeNardo et al., 2011; Pyonteck et al., 2013; Ries et al., 2014). For example, a CSF1R antagonist PLX397 inhibits the infiltration of TAM into the pancreatic tumor and alters phenotype of the remaining TAM, which results in the modest suppression of the tumor growth in mice that have received orthotopic injection of syngeneic pancreatic cancer cells (Zhu et al., 2014). In this model, a combined treatment of the tumor-bearing mice with anti-PD1 and anti-CTLA4 antibodies also limits the tumor outgrowth by ∼50% compared with a vehicle treatment. Importantly, the anti-PD1/anti-CTLA4 treatment in combination with PLX3397 completely blocks the tumor expansion and even regresses the established tumors by 15% (Zhu et al., 2014). These results provide a proof of concept that the TAM targeting improves efficacy of $CD8^+$ T cell-based immunotherapies using checkpoint antagonists.

In the pancreatic cancer model, blockade of CSF1R signaling significantly reduces the number of TAM in the tumor as well as mRNA expression of immunosuppressive molecules such as PD-L2, transforming growth factor-β (TGF-β), and arginase-1 (ARG1) in the remaining TAM (Zhu et al., 2014), suggesting that CSF1R inhibition improves checkpoint therapies not only by depletion of TAM but also by reducing their expression of suppressive molecules. It has been reported that TAM isolated from the subcutaneous tumor established by C3 fibrosarcoma express higher level of ARG1 compared with normal splenic macrophages, and suppress T cell proliferation via ARG1-mediated mechanisms (Kusmartsev and Gabrilovich, 2005). In mice that are subcutaneously injected with CT26 colon cancer cells, single treatment with a small molecule ARG1 inhibitor (CB-1158) or an anti-PD-L1 antibody suppresses the tumor growth, and their tumor suppressive effect is enhanced by combining these two inhibitors (Steggerda et al., 2017). Similarly, in mice that have received orthotopic injection of 4T1 mammary tumor cells, the anti-PD1/anti-CTLA4 treatment in combination with CB-1158 significantly reduces growth of the primary tumor and decreases the number of lung metastases (Steggerda et al., 2017). These results suggest that CSF1R signaling may yield immune suppressive phenotype to TAM by inducing ARG1 expression in addition to support TAM accumulation in the tumor, and that addition of antagonists for CSF1R and/or ARG1 to checkpoint therapies can be a promising strategy.

A recent study demonstrates that expression of *Arg1* and *Tgfb* mRNA in TAM is significantly reduced by genetic depletion or pharmacological inhibition of phosphoinositide 3-kinase γ (PI3Kγ) in the mammary tumors developed in Polyoma Middle T oncogene (PyMT) transgenic mice, as well as the subcutaneous tumors established by LLC lung cancer cells in syngeneic C57BL/6 mice (Kaneda et al., 2016). These results indicate that PI3Kγ is another important regulator of immune suppressive phenotype of TAM. Interestingly, T cells isolated from the LLC tumors in PI3Kγ deficient mice are more cytotoxic than those in wild type mice. Furthermore, treatment with a PI3Kγ inhibitor (TG100-15) significantly augments the tumor suppressive effects of anti-PD1 antibody in a mouse model of head and neck squamous carcinoma (Kaneda et al., 2016). These data suggest that PI3Kγ inhibitors promotes cytotoxic capacity of T cell responses by blocking immune suppressive functions of TAM, and thus is useful to enhance therapeutic effects of checkpoint antagonists.

The immune suppressive features of macrophages within the tumor can also be interfered by inhibition of class IIa histone deacetylase (HDAC), enzymes that regulate activity of many transcription factors (Di Giorgio et al., 2015). In the mammary tumors developed in PyMT transgenic mice, a selective class IIa HDAC inhibitor (TMP195) switches dominant macrophage populations in the tumor from TAM to highly phagocytic macrophages, which suppresses tumor growth (Guerriero et al., 2017). Importantly, the treatment with TMP195 in combination with anti-PD1 antibody further reduces tumor burden in this model, whereas a single treatment with anti-PD1 antibody is not sufficient to suppress tumor development. Therefore, the class IIa HDAC inhibitor has a potential to enhance checkpoint therapy by drawing anti-tumor functions from tumor-infiltrating macrophages (Guerriero et al., 2017).

It is reported that the macrophage polarization to an immunosuppressive phenotype is also regulated by cytokines such as IL-4, IL-10, and IL-13 (Sica and Bronte, 2007). In cultured human macrophages, IL-10 induces key macrophage receptors (Ley et al., 2016) including toll-like receptors, Fc receptors (e.g., FcγR), and macrophage receptor with collagenous domain (MARCO) (Park-Min et al., 2005). Although their contribution to the immunosuppressive phenotype of TAM is not known yet, recent studies suggest some of these receptors as targets for the improvement of checkpoint therapies. For example, in mice with melanoma established by subcutaneous injection of B16 cells, an anti-MARCO monoclonal antibody treatment enhances the efficacy of anti-CTLA4 antibody treatment in suppressing tumor growth (Georgoudaki et al., 2016). In the B16 tumors as well as human melanoma samples, MARCO is predominantly expressed by TAM. Furthermore, anti-MARCO antibody treatment reduces the percentage of a distinct TAM population (known as M2 macrophage) that is reported to express ARG1 and suppress *in vitro* T cell proliferation (Movahedi et al., 2010; Georgoudaki et al., 2016). These results suggest that anti-MARCO antibody can switch TAM phenotype from the immunosuppressive to immune activating one, and thereby promotes anti-tumor activities of cytotoxic T cells. However, precise mechanisms behind the synergistic effects of anti-MARCO on anti-CTLA4 antibody treatment need to be further elucidated.

Another potential target is Fc-gamma receptor (FcγR), a receptor of immunoglobulin. In mice that are subcutaneously injected with MC38 colon cancer, a single treatment with anti-PD1 antibody can suppress tumor growth whereas the response to this therapy typically varies among animals. In contrast, addition of FcγR blocking antibodies to the anti-PD1 treatment completely suppresses tumor growth in all mice (Arlauckas et al., 2017). Interestingly, the intravital microscopy of the tumor has identified that anti-PD1 antibodies that initially bind to T cells are transferred to TAM in the tumor by 24 h, and the treatment with FcγR blocking antibodies prolongs the binding of anti-PD1 antibodies to tumor-infiltrating T cells (Arlauckas et al., 2017). These data indicate that TAM in the tumor microenvironment limit the efficacy of checkpoint therapy by depriving the antibodies against checkpoint receptors/ligands, and that inhibition of the interaction between Fc region of checkpoint blocking antibodies and Fc receptors in TAM can be a therapeutic option to improve the therapy.

Accumulating evidences indicate that TAM is the one of the major immune suppressor cell types in the solid tumors and that pharmacological interventions of TAM accumulation and/or function are promising strategies to improve checkpoint therapies. Although therapeutic effects of the TAM intervention/checkpoint inhibition combination therapy is evident from the previous pre-clinical studies, further basic researches will be required to apply this novel strategy to the clinic.

CONCLUSION AND FUTURE PERSPECTIVES

Accumulating evidences indicate that TAM is one of the major components of the immune suppressive tumor microenvironment, and is an attractive target to improve responses to immunotherapies. Therefore, several TAM targeting strategies (e.g., TAM depletion, TAM reprogramming, and targeting functional molecules of TAM) have been proposed to enhance efficacy of the immune checkpoint inhibition, one of the most promising immunotherapy for the treatment of solid tumors (**Figure 1**). Preclinical studies have suggested that the combination of these strategies with checkpoint inhibitors can enhance the therapeutic responses at least in melanoma and tumors in the lung, colon, and breast. Despite the encouraging preliminary results, all these strategies need further investigation before being applied for clinic as a combination therapy with checkpoint inhibitors.

The total depletion of monocytes and macrophages by CSF1R inhibitors is a straightforward and efficient approach (Mantovani et al., 2017). However, this strategy is not TAM specific and may cause high toxicity if patients are treated for prolonged periods (Cannarile et al., 2017). Moreover recent studies suggest that normal monocytic cells are required for T cell-mediated immune reaction, and thus the full depletion of monocytes and macrophages may not be ideal to combine with checkpoint inhibitors. Namely, classical monocytes (precursors of recruited macrophages including TAM) seem to be required for better responses to anti-PD1 therapy (Krieg et al., 2018) and macrophage-mediated T_{reg} cell depletion can co-define the efficacy of anti-CTLA4 therapy (Simpson et al., 2013). A potential approach alternative to the "total" depletion would be the "pulsing" ablation of TAM followed by recovery periods during which monocytes can return into the tumors and promote initial anti-tumor immune reactions before turning to TAM. However, this attractive strategy requires more knowledge about a timing of pulsing and immune interactions ongoing in all phases of tumor formation (Gil Del Alcazar et al., 2017). Another alternative approach to overcome potential issues in monocyte/macrophage depletion would be the targeting of cancer-associated immature myeloid cells (or monocytic-MDSC) that possess intrinsic immunosuppressive functions *in vitro* and give rise to TAM in tumors (de Haas et al., 2016; Kitamura et al., 2018; Veglia et al., 2018). Since gene expression profile of these TAM progenitor cells is distinct from that of normal monocytes (Kitamura et al., 2018; Veglia et al., 2018), targeting the progenitors would block TAM-mediated immunosuppression without affecting normal monocyte functions and thus improve checkpoint inhibitor more efficiently. The challenge of this approach is to identify specific markers for the progenitors, which will allow the selective targeting of a source of immunosuppressive myeloid cells.

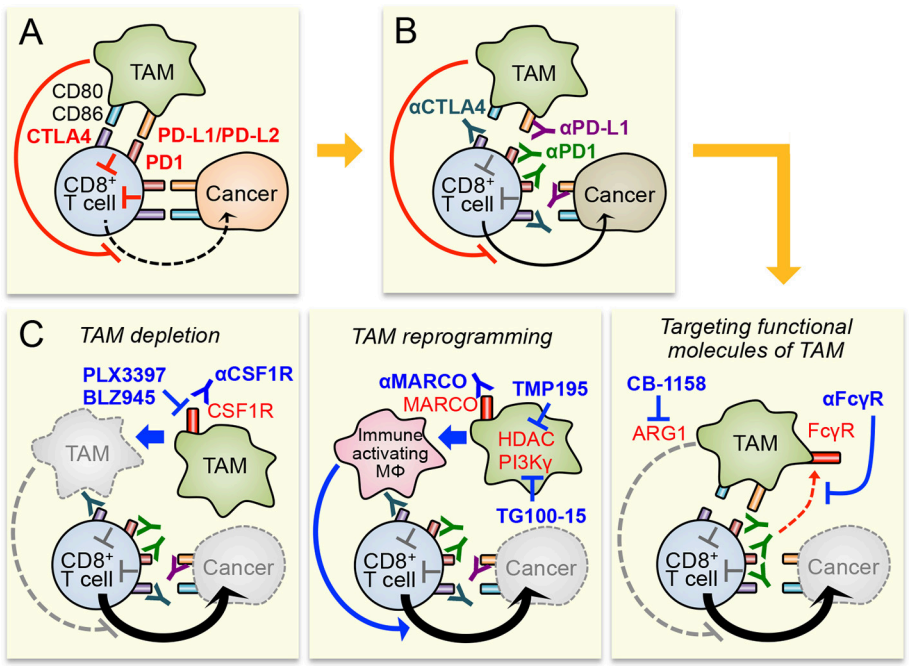

FIGURE 1 | Potential therapeutic strategies to enhance immune checkpoint inhibitors by targeting tumor-associated macrophages (TAM). **(A)** Cytotoxicity of $CD8^+$ T cell in the tumors is suppressed by immune checkpoint pathways activated by cancer cells and TAM. TAM also suppresses $CD8^+$ T cell functions via checkpoint pathway independent mechanisms that are still under investigation. **(B)** Blockade of immune checkpoint pathway by antibodies for CTLA4, PD1, and PD-L1 enhances $CD8^+$ T cell cytotoxicity. However, immune suppressive tumor microenvironment, especially TAM in it, will limit the anti-tumor efficacy of the checkpoint inhibitors. **(C)** Therapeutic efficacy of checkpoint inhibitors can be improved by TAM targeting through different strategies, i.e., TAM depletion (left), TAM reprogramming (central), and targeting functional molecules of TAM (right). MΦ means macrophage.

The second promising strategy for TAM targeting is the reprogramming of TAM from immune suppressive and trophic cell to immune activating and tumoricidal one. However the extreme level of macrophage plasticity (Sica and Mantovani, 2012) will cause a potential risk of this strategy, i.e., the macrophages existing in the tumors can switch back to pro-tumor TAM when the reprogramming treatment is interrupted. The majority of studies published so far did not fully investigate the long-term effects of the TAM reprogramming agents after the treatment interruption, and thus more studies that fully elucidate the phenotype of macrophages after reprogramming are required for clinical application of this strategy.

The third TAM intervention strategy is to target functional molecules of TAM. An encouraging example is the blockade of Fc receptors on TAM that prevents deprivation of anti-PD1 antibodies and thereby enhances the efficacy of the checkpoint therapy (Arlauckas et al., 2017). However, Fc receptor inhibition may negatively affect another type of immunotherapy since myeloid cells and cytotoxic lymphocytes also express Fc receptors and require the receptors for antibody-mediated phagocytosis or antibody-dependent cellular cytotoxicity. Indeed, it has been reported that anti-CTLA4 antibody exerts the therapeutic effect through FcγR dependent T_{reg} cell depletion by macrophages and loss of FcγR reduces therapeutic response to CTLA4 therapy in mice with B16 melanoma (Simpson et al., 2013). It will be thus fundamental to identify TAM specific targets (such as MARCO) to improve therapy specificity of this TAM targeting strategy.

These potential issues for each TAM targeting strategy suggest that the next challenges in the tumor immunology field will be to identify specific markers and tailor the targeting only of the tumor promoting macrophage subpopulations in different cancers and cancer subtypes. The extensive use of single cell RNA sequencing, multiplex immunohistochemistry and mass cytometry techniques will considerably enhance our knowledge on the heterogeneity of TAM in tumors and define the selection of novel TAM targets for the improvement of cancer immunotherapy.

AUTHOR CONTRIBUTIONS

LC and TK: conceptualized this review, decided on the content, and wrote the manuscript; TK: prepared the figure. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Microenvironment-Induced Non-sporadic Expression of the AXL and cKIT Receptors Are Related to Epithelial Plasticity and Drug Resistance

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The existence of rare cancer cells that sporadically acquire drug-tolerance through epigenetic mechanisms is proposed as one mechanism that drives cancer therapy failure. Here we provide evidence that specific microenvironments impose non-sporadic expression of proteins related to epithelial plasticity and drug resistance. Microarrays of robotically printed combinatorial microenvironments of known composition were used to make cell-based functional associations between microenvironments, which were design-inspired by normal and tumor-burdened breast tissues, and cell phenotypes. We hypothesized that specific combinations of microenvironment constituents non-sporadically impose the induction of the AXL and cKIT receptor tyrosine kinase proteins, which are known to be involved in epithelial plasticity and drug-tolerance, in an isogenic human mammary epithelial cell (HMEC) malignant progression series. Dimension reduction analysis reveals type I collagen as a dominant feature, inducing expression of both markers in pre-stasis finite lifespan HMECs, and transformed non-malignant and malignant immortal cell lines. Basement membrane-associated matrix proteins, laminin-111 and type IV collagen, suppress AXL and cKIT expression in pre-stasis and non-malignant cells. However, AXL and cKIT are not suppressed by laminin-111 in malignant cells. General linear models identified key factors, osteopontin, IL-8, and type VIα3 collagen, which significantly upregulated AXL and cKIT, as well as a plasticity-related gene expression program that is often observed in stem cells and in epithelial-to-mesenchymal-transition. These factors are co-located with AXL-expressing cells *in situ* in normal and breast cancer tissues, and associated with resistance to paclitaxel. A greater diversity of microenvironments induced AXL and cKIT expression consistent with plasticity and drug-tolerant phenotypes in tumorigenic cells compared to normal or immortal cells, suggesting a reduced perception of microenvironment specificity in malignant cells. Microenvironment-imposed reprogramming could explain why resistant

cells are seemingly persistent and rapidly adaptable to multiple classes of drugs. These results support the notion that specific microenvironments drive drug-tolerant cellular phenotypes and suggest a novel interventional avenue for preventing acquired therapy resistance.

Keywords: breast cancer, MEMA, microenvironment, epithelial plasticity, AXL, cKIT, drug resistance

INTRODUCTION

The confounding reality for anti-cancer treatments is the heterogeneity of tumors. Generated by genetic and adaptive epigenetic alterations in gene expression, tumor heterogeneity supports acquired resistance to anti-cancer treatments. Sporadic drug-tolerant states within subpopulations of cancer cells are rapidly achieved by activating drug-resistance genes, that are also implicated as stem cell-related genes, through chromatin modifications or transcriptional upregulation (Sharma et al., 2010; Shaffer et al., 2017). These epigenetic mechanisms provide rapidly acquired resistance and tumor cell persistence during treatment. Heterogeneity within the tumor microenvironment is a source of adaptive drug resistance that supports stem cell-like phenotypic plasticity in the tumor cells (Bissell and Labarge, 2005; LaBarge, 2010). However, the nature of these plasticity-inductive microenvironments remains elusive.

In normal tissues, stem cell-states are maintained in specialized microenvironments termed, niches. Epigenetic plasticity gene programs that are characteristic of regenerative stem cells responsive to tissue damage and inflammation are prominent in aggressive cancers with poor clinical outcome. These plasticity gene programs are triggered both by protective anti-tumor immune surveillance and inflammation, and the constant nutrient and oxygen deprivation characteristic of the chaotic tumor microenvironment that follows the breakdown of normal tissue architecture (Nieto, 2013). Tumor cells exploit these acquired stem cell traits to promote survival and enable flexibility to transition between different functional states such as epithelial-to-mesenchymal transition (EMT) (Bissell and Labarge, 2005; Thiery et al., 2009; LaBarge, 2010; Mora-Blanco et al., 2013). The connection between EMT and stem cell traits has been well studied in the epithelial cells of the mammary gland, an organ formed by branching morphogenesis, where epithelial plasticity is essential and where substantial cellular dynamics continue throughout adulthood (Petersen and Polyak, 2010). Regulators of EMT induce epithelial plasticity during mammary gland development and cancer progression (Mani et al., 2008; Guo et al., 2012). The importance of epithelial plasticity during the development of malignant breast cancer is evidenced by EMT gene signatures, which correlate with drug resistance, stem cell-like traits, basal breast cancer subtypes, metastasis and poor patient survival (Blick et al., 2010). The receptor tyrosine kinase (RTK), AXL, is a key driver of tumor cell EMT and is widely implicated in acquired drug-resistance to multiple cancer drug classes (Davidsen et al., 2017; Ferreira et al., 2017). Blockade of AXL inhibits the EMT program and reverses acquired drug resistance and metastasis (Gjerdrum et al., 2010; Kirane et al., 2015). AXL is an important therapeutic target currently being investigated in several cancer clinical trials (Antony and Huang, 2017). The RTK cKIT is enriched on mammary epithelial progenitor cells and increased expression was observed in high-risk breast tissue (Lim et al., 2009; Garbe et al., 2012). cKIT pathway activation is a driver in several cancers and it is related to acquired drug resistance (Javidi-Sharifi et al., 2015; Zhang et al., 2015; Lai et al., 2016; Pu et al., 2017). The ability of cells to modulate proteins related to stem cell-states such as these RTKs, is an example of epithelial plasticity, which can be useful for maintaining healthy tissue architecture in the normal context. Conversely, that same property is dangerous when coopted by cancer cells, as it promotes their survival and spread.

We hypothesized that sporadic stem cell-like states, which may be drug tolerant, are favored by specific microenvironment contexts. To address this, we functionally interrogated an isogenic human mammary epithelial cell (HMEC) progression series comprising primary normal (184, pre-stasis), immortal (184A1, non-malignant), and adenocarcinoma-forming (184AA3, tumorigenic) cells on combinatorial MicroEnvironment MicroArrays (MEMA) for induction of cKIT and AXL. The MEMA consisted of 228 distinct microenvironment features comprising different combinations of ECM, growth factors and cytokines. Hierarchical clustering, general linear modeling (GLM), and dimension reduction analyses were applied to identify plasticity-inductive microenvironments. Specific combinatorial microenvironments were shown to induce or maintain cKIT and AXL, activate an EMT-related gene expression program, and induce paclitaxel tolerance. The microenvironment components that were functionally predicted to induce AXL expression on MEMA, were found co-expressed by cells in breast tumor microenvironments adjacent to cells expressing AXL. We report evidence that sporadic drug-tolerance can result from phenotypic plasticity of carcinoma cells in response to different microenvironments.

RESULTS

The Normal and Neoplastic Mammary Microenvironment

Mammary epithelial ducts are encapsulated by a basement membrane that is enriched with laminins (LAM1 and LAM5) and type IV collagen (COL4). This matrix systematically regulates cell growth, induces lumen formation, and serves as a crucial polarity cue (Petersen et al., 1992). Examples of immunofluorescence staining in normal breast tissue demonstrate that epithelial cells are enveloped by basement membrane components COL4 (**Figures 1A,B**) pan laminin

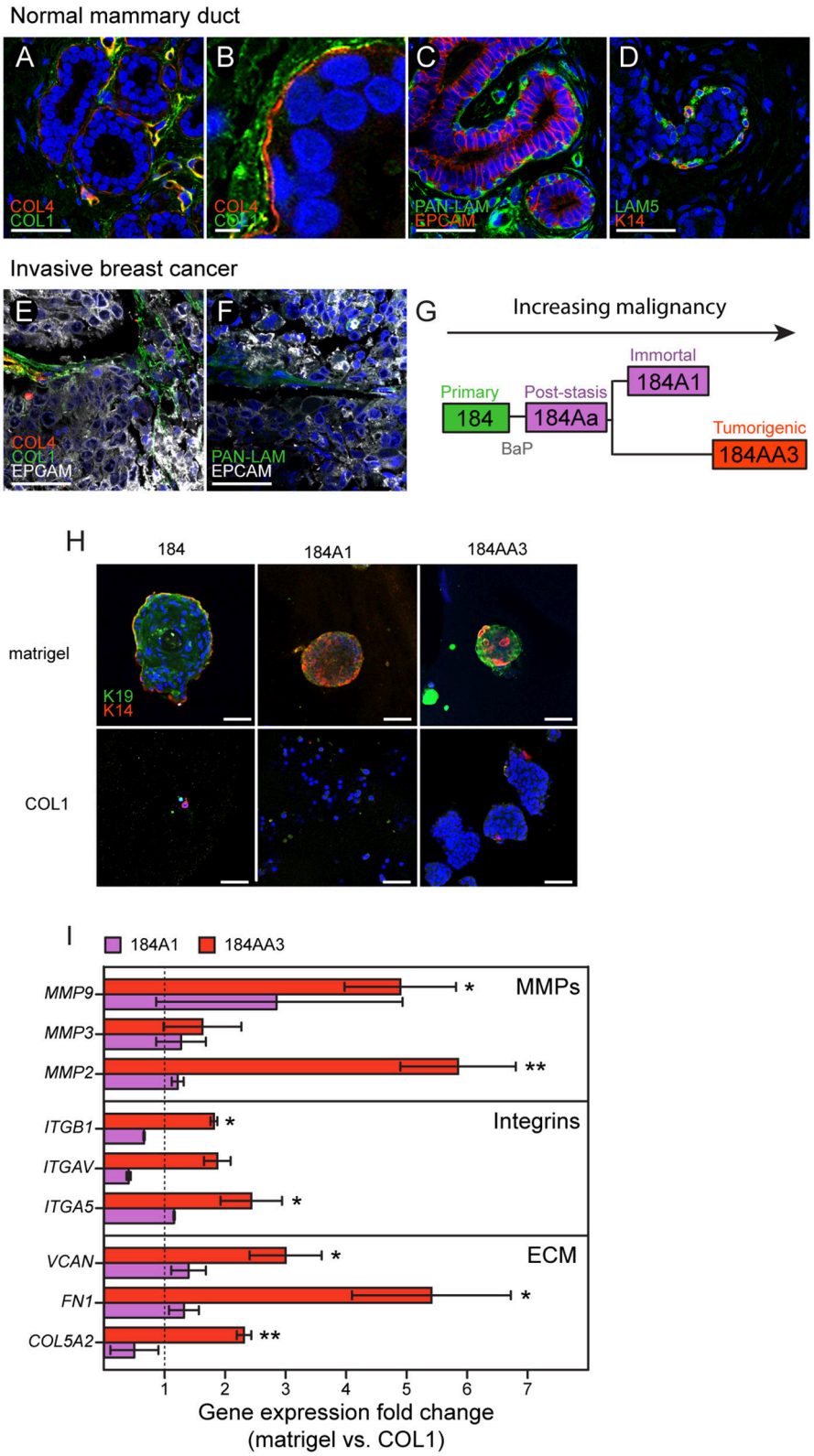

FIGURE 1 | Human mammary epithelial cells from different stages in a malignant progression series exhibit unique growth characteristics in normal- and tumor-like microenvironments. Immunofluorescence staining of **(A–D)** normal and **(E,F)** invasive breast cancer tissue sections. ECM components; **(A,B,E)** COL4 (red), COL1

(Continued)

FIGURE 1 | (green), **(C,F)** pan-laminin (LAM, green) and **(D)** LAM5 (green) in **(A–D)** normal mammary gland tissue and **(E,F)** invasive breast cancer, were stained with **(A–F)** nuclei marker Hoechst (blue), **(C,E,F)** epithelial cell marker (EPCAM) or with **(D)** myoepithelial cell marker (K14, red). **(G)** Diagram of the 184-progression series derivation. (H) Single cell suspensions of 184, 184A1, and 184AA3 cells were embedded in matrigel- and COL1-3D gels, after 12 days cells were fixed and stained with luminal cell marker (K19, green) and myoepithelial cell marker (K14, red). Images are representative of three individual experiments. **(A,C–F,H)** Bars represent 50 μm and **(B)** 5 μm. **(I)** Gene expression of microenvironment related genes (RT^{2}Profiler^{TM} PCR array, Human Epithelial to mesenchymal transition EMT, Qiagen) in 184A1 and 184AA3 cells cultured on matrigel (control = 1) or on COL1. Data represent mean ± SE, from two (184A1) or three (184AA3) individual experiments, statistical significance was calculated by using student *T*-test (*$p < 0.05$, **$p < 0.01$).

staining (PAN-LAM) and LAM5 (**Figures 1C,D**, respectively). Normal breast epithelia were clearly separated from type I collagen (COL1) that is most prevalent in the surrounding stroma (**Figures 1A,B**). The basement membrane is disrupted during breast cancer progression and local concentrations of COL4 and laminins decrease (Insua-Rodriguez and Oskarsson, 2016), while expression of matrix components characteristic of tissue remodeling increase (e.g., hyaluronan, HA, tenascin C, TNC, osteopontin, OPN, and fibronectin, FN1; Insua-Rodriguez and Oskarsson, 2016). COL1 accumulates and aligns at the epithelial-stromal borders of tumors (Provenzano et al., 2006). Immunofluorescence staining of invasive breast cancer tissues demonstrates that tumor cells are exposed to COL1 (**Figure 1E**), and only modest levels of PAN-LAM (**Figure 1F**). COL4 was absent from the tumor stroma, with only perivascular COL4 observed (**Figure 1E**). Accompanying these changes in ECM composition, heterogeneous breast cancer microenvironments are enriched with hormones (Garcia-Robles et al., 2013; Simões et al., 2015), growth factors (Mimeault et al., 2007; Ye et al., 2009; Zheng et al., 2014; Ho-Yen et al., 2015; Voudouri et al., 2015), cytokines (Esquivel-Velazquez et al., 2015; Weichhaus et al., 2015), chemokines (Palacios-Arreola et al., 2014) and cell adhesion proteins (Spivey et al., 2012; Beauchemin and Arabzadeh, 2013; Karousou et al., 2014; Yu and Elble, 2016).

HMEC Progression Series for Probing Responses to Normal- and Stromal-Like Microenvironments

The 184 HMEC progression series provides a model of cancer progression comprising normal, finite lifespan, pre-stasis cells and derivative cell lines that range from non-malignant immortal non-malignant to malignant immortal cells (**Figure 1G**; Stampfer et al., 2013). The pre-stasis HMEC 184 strain was derived from normal reduction mammoplasty tissue of a 21-year old female with no pathological changes. Pre-stasis HMEC strains grown as described are known to possess luminal and myoepithelial cells and cells with progenitor activity (Garbe et al., 2009, 2012; Labarge et al., 2013). Finite post-stasis 184Aa were derived following benzo-a-pyrene (BaP) exposure of pre-stasis 184, and lack expression of the CKI p16^{INK4a} (Stampfer and Bartley, 1985; Brenner et al., 1998). The non-malignant immortal non-malignant cell line 184A1, which is wild-type for p53 and retinoblastoma (RB) protein, emerged from 184Aa as it approached replicative senescence, and exhibits a low level of genomic instability (Stampfer and Bartley, 1985; Walen and Stampfer, 1989). The tumorigenic cell line 184AA3 emerged from 184Aa following insertional mutagenesis that inactivated p53 function (Stampfer et al., 2003). It exhibits increased genomic instability and forms clinically relevant ER+ luminal adenocarcinomas in the mouse xenograft model (Stampfer et al., 2003; Hines et al., 2016). To evaluate how the HMEC progression series responds to normal-like and stroma-like microenvironments, we cultured single cell suspensions in laminin-rich ECM [lrECM (matrigel)] and COL1 3D gels, respectively. Normal 184 cells enriched for cKIT expression gave rise to growth arrested acini that have a lumen, with (K)eratin 14+ myoepithelial cells that are basal relative to K19+ luminal cells (**Figure 1H**), whereas growth in COL1 was negligible (**Figure 1H**). 184A1 and 184AA3 form solid, multi-lineage spheres in lrECM (**Figure 1H**). 184A1 exhibits modest growth in COL1 gels resulting in small colonies. In contrast, 184AA3-derived spheroids were large and proliferative in COL1 gels (**Figure 1H**). Gene expression analysis after 24 h growth on COL1 gels showed that tumorigenic 184AA3 cells, as compared to 184A1, upregulated expression of matrix metalloproteinases (*MMP2, MMP3,* and *MMP9*), integrins (*ITGB1, ITGAV*, and *ITGA5*) and matrix components [Versican (*VCAN*), *FN1* and type Vα2 collagen (*COL5A2*)] (**Figure 1I**), indicative of enhanced microenvironment-adaptive activity in the malignant cells.

The Relationship Between Cancer Progression Stage and Plasticity Marker Expression in Combinatorial Microenvironment Contexts

We next asked whether induction of phenotypes associated with plasticity and drug-tolerant states is sporadic (equally likely to occur in all microenvironment contexts), or whether those states are associated with specific microenvironments (microenvironment-induced). MEMA were used previously to identify combinatorial microenvironments that induce and maintain stem- and differentiated-states in HMEC (LaBarge et al., 2009), and microenvironments that modulate lapatinib activity in HER2-amplified breast, lung, and prostate cancer (Lin et al., 2017). We applied this principle to determine whether cKIT and AXL were expressed in a microenvironment dependent manner. Individual microenvironment components were selected based on their enrichment in normal and cancer microenvironments. In order to recapitulate simplified normal- or tumor-like microenvironments purified COL1, Laminin-111 (LAM1), COL4, and LAM1+laminin-332 (LAM5) were mixed pairwise with OPN, HA, TNC, FN1, bone morphogenetic protein−2/7 (BMP-2/7), BMP-4, carcinoembryonic antigen related cell adhesion molecule 6 (CEACAM6), CEACAM8, CD44, type XXIIIα1 collagen (COL23A1), E-cadherin (ECAD), epidermal growth factor (EGF), fibroblast growth factor 2

(FGF-2), growth arrest-specific 6 (GAS-6), hepatocyte growth factor (HGF), insulin-like growth factor (IGF1), interferon-γ (IFN-γ), interleukin-1β (IL-1β), IL-6, IL-8, leptin, melanoma growth stimulating activity alpha (GRO1), nidogen1, lumican, osteoprotegerin (OPG), stem cell factor (SCF), stromal derived factor-1β (SDF-1β), and tumor growth factor β (TGFβ) to make a total of 228 unique combinations that were printed in 10-fold replicate features. RNA sequencing of the 184 progression series was performed to characterize the baseline levels of expression of the genes corresponding to the proteins printed on MEMA (**Figure 2A**) and to their known receptors (**Figure 2B**). Significant differences were not detected in expression of any of these genes. However, levels of *AXL* gene expression are 5 fold higher in 184A1 cells compared to the other cells and *cKIT* gene expression was detected only 184 cells (**Figure 2C**).

Cells have a dynamic and reciprocal relationship with their microenvironment, and one would expect cells to gradually modify their microenvironment following initial exposure. Thus, in order to measure the impact of the printed combinatorial microenvironments on the 184 HMEC progression series, they were cultured on MEMA for only 48 h. After fixing and staining for AXL and cKIT protein expression, every MEMA feature was imaged, and single cell data were obtained through use of marker-based watershed segmentation (**Figure 2D**). In addition to protein levels, cell-segmentation enabled assessment of eight morphological properties of cells that were evaluated independently. Unsupervised clustering of AXL expression Z-scores as a function of microenvironment revealed that AXL expression in 184 and 184A1 was mainly observed in COL1-rich microenvironments, with less expression in COL4-rich ones (**Figure 2E**). In contrast, AXL expression was high in COL1- and LAM1-rich microenvironments in malignant 184AA3 cells (**Figure 2E**). We show an example of AXL and cKIT expression in cells of the progression series, at the single cell level, on three single component microenvironments: COL1, COL4, and LAM 1 (**Figure 2F**). These data show that microenvironments that impose expression of the RTKs may not do so uniformly, that it is more a case of triggering a percentage of the cells to express those proteins instead of shifting the mean of the population, which underscores the importance of single cell analysis. Single cell data also revealed that AXL and cKIT expression in malignant 184AA3 cells was overall more heterogeneous than in 184 and 184A1 cells (**Figure 2F**). General linearized modeling (GLM) confirmed that the coefficient of variance describing the percentage of AXL and cKIT-expressing cells in a given microenvironment between 184 and 184A1 cells was low, while the variance between 184 and 184AA3, and 184A1 and 184AA3 was more than two-magnitudes greater (**Figure 3A**). Thus, whereas expression of these RTKs in normal and non-malignant HMEC is tightly regulated by microenvironment, the malignant cells are effected but not fully restricted by microenvironment.

Next, we applied tSNE to project all the dimensions in 2D and visualize the data, a method particularly sensitive to the types of non-linear relationships that are common in biological data to visualize the relationship between microenvironment and RTK expression (Amir et al., 2013). Microenvironments were readily clustered by the primary ECM component (**Figure 3B**).

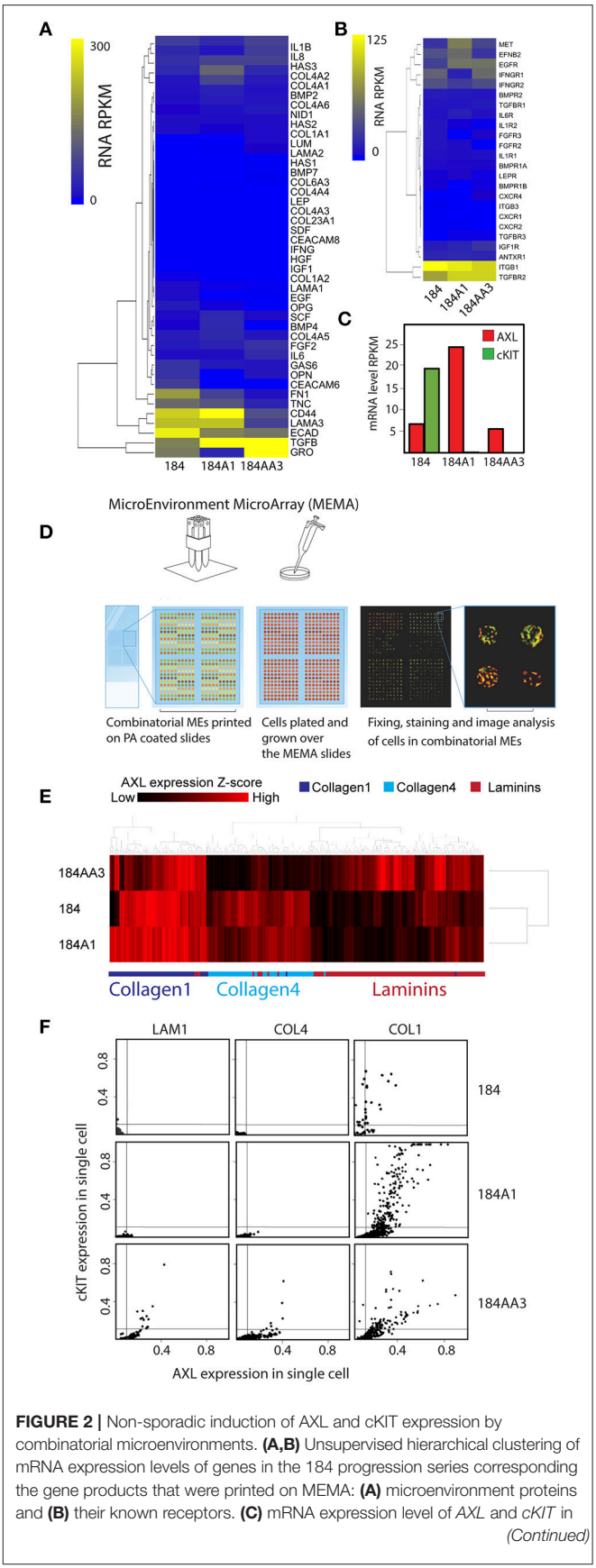

FIGURE 2 | Non-sporadic induction of AXL and cKIT expression by combinatorial microenvironments. **(A,B)** Unsupervised hierarchical clustering of mRNA expression levels of genes in the 184 progression series corresponding the gene products that were printed on MEMA: **(A)** microenvironment proteins and **(B)** their known receptors. **(C)** mRNA expression level of *AXL* and *cKIT* in

(Continued)

FIGURE 2 | the184 progression. **(D)** Diagram of the MicroEnvironment MicroArray (MEMA) experimental design. MEMAs are printed on microscope slides coated with polyacrylamide (PA) gel. 228 unique extracellular microenvironments with 5–20 replicate spots are printed on one slide. Cells are cultured on the arrays and grown 48 h before fixing. AXL and cKIT are stained for immunofluorescence imaging, and image analysis is used to obtain single cell expression data in discrete microenvironment contexts. **(E)** Unsupervised hierarchical clustering of AXL expression z-scores as a function of microenvironment in the 184 progression series. Non-sporadic clustering of AXL expression by major ECM component of microenvironment was detected. **(F)** Scatter plot representation of AXL and cKIT expression in single cells, as a function of ECM components (LAM1, COL4, and COL1) in the 184 progression series.

Whereas, there was no clear clustering driven by presence of soluble ligands (**Figure 3C**), nor by GAS6 (**Figure 3D**), which is the cognate ligand for AXL. After only 4 h, array features were fairly uniformly bound (with a potentially universal attachment preference for COL1) (data not shown), but differences in cell number per feature changed with time, revealing some matrix-type preferences that were progression stage dependent by 48 h. Normal and non-malignant HMEC preferentially increased in cell number/spot on LAM1 and COL4, and malignant cells on COL4 (**Figure 3E**). The majority of AXL and cKIT expression in normal and non-malignant cells was detected in cells cultured on COL1-containing microenvironments, with some weak enrichment also seen on COL4-containing (**Figures 3F–H**). In comparison, malignant cells expressed high amounts of AXL and cKIT, in COL1- and LAM1-containing microenvironments, but COL4 was the only ECM that was not associated with expression of those RTKs (**Figures 3F–H**). 184AA3 was more likely to have significantly greater proportions of AXL and cKIT expressing cells in microenvironments that included COL1 or LAM1 (**Figures 3F–H**), and GLM analysis showed significantly more variance (expression of AXL+/cKIT+ population) in COL1 and LAM1 including microenvironments compared to other ECMs (**Table 1**). Collectively this cell-based functional analysis of microenvironment-phenotype associations showed that COL1-rich stroma-like and LAM1-rich normal-like microenvironments enabled the induction of AXL and cKIT-expression in malignant cells, whereas their expression in normal and non-malignant cells was far more restricted. These data provide a functional rationale for normal epithelial cell segregation from the stromal microenvironment by the basement membrane, and reveal an inherent plasticity of epithelia that engages components of stem cell-related signaling pathways when exposed to stromal ECM, for example during trauma or disease. Malignant cells, by comparison, readily switch between stem- and resistance-related pathways in microenvironments that normally suppress plasticity, e.g., LAM1-rich contexts.

OPN, IL-8, and COL6A3 Promote States Consistent With Drug-Tolerance in Malignant Cells

GLM analysis of the 184AA3 MEMA showed that TGFβ, OPN, lumican, leptin, IL-8, HA and COL6A3 were significantly

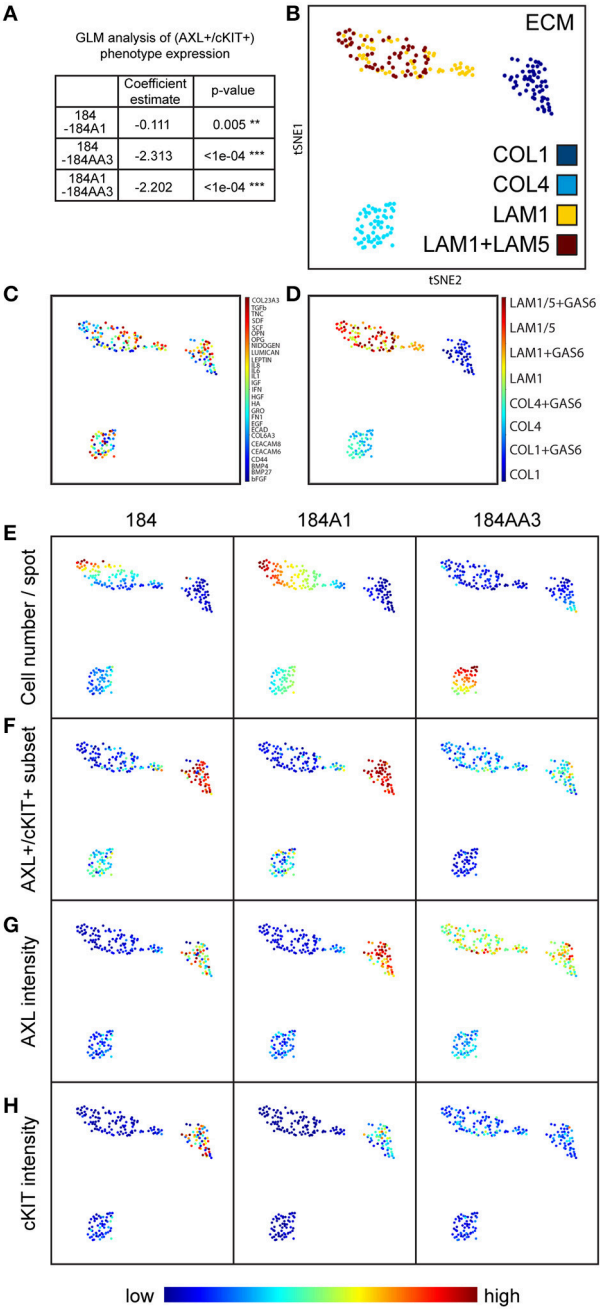

FIGURE 3 | Visualizing the impact of microenvironment on higher-order cell phenotypes, including AXL and cKIT expression. **(A)** Table representing GLM analysis of expression of the AXL+/cKIT+ phenotype in different microenvironments and significant differences was detected in patterns of microenvironment-phenotype associations between comparisons of all progression stages. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. **(B–H)** Dimension reduction and visualization of microenvironment-driven phenotypes with tSNE visualization. Each point represents a unique combinatorial microenvironment, and the distance between any two points reflects similarity of the cellular phenotype that is begotten by the microenvironment. The characteristics that were measured in cells to establish phenotype were: % of cells that are $AXL^+/cKIT^+$, mean AXL, and cKIT fluorescent intensity in subpopulation and in ungated population, cell count/spot, -eccentricity, -solidity. **(B–D)** Shows the *(Continued)*

FIGURE 3 | composition of each microenvironment, where **(B)** shows distribution of the major ECMs, **(C)** shows the distribution of the soluble factors, and **(D)** shows the distribution of GAS6 among the major ECM. The major ECM is a key driver of microenvironment-imposed phenotypes. Even GAS6, the cognate ligand of AXL does itself impact the tSNE distributions. **(E–H)** Show how specific aspects of cell phenotype distribute with microenvionment: **(E)** cell number per spot, **(F)** $AXL^+/cKIT^+$-subpopulation size, **(G)** mean AXL intensity, and **(H)** mean cKIT intensity.

TABLE 1 | Effect of ECM on expression of $AXL^+/cKIT^+$-phenotype in 184AA3.

ECM	Co-efficient estimate from GLM analysis	*P*-value
COL1-COL4	−1.64569	< 0.001***
COL1-LAM1	0.04385	0.0621
COL1-LAM1/LAM5	−0.37975	< 0.001***
COL4-LAM1	1.53714	< 0.001***
COL4-LAM1/LAM5	1.26594	< 0.001***
LAM1-LAM1/LAM5	−0.2712	< 0.001***

*GLM analysis with Tuckey's post-hoc test. ***$p \leq 0.001$.*

associated with increased frequency of the AXL and cKIT phenotype (**Figure 4A**). To further investigate this differential plasticity-inductive effect, we focused on OPN, IL-8, and COL6A3, which were associated with the largest proportion of AXL-expressing 184AA3 cells. We examined the expression of these plasticity-inductive factors in normal human mammary gland and in triple negative breast cancer (TNBC) tissue sections (**Figures 4B–G**). By RNA *in situ* hybridization the expression of *OPN* and *IL-8* were found to co-localize with the rare population of cells with high *AXL* expression in normal tissue (**Figures 4B,D**) and, as well, *OPN* and *IL-8* expressing cells were also found in the vicinity of the *AXL* positive tumor cells in the TNBC tissues (**Figures 4C,E**). Expression of COL6A3 was prominent in the normal epithelia (**Figure 4F**), and also in the malignant epithelium of TNBC (**Figure 4G**). This result contrast with the detection of mature type VI collagen (COL6) fibrils, which were detected strictly in the stromal compartments in normal mammary tissues as well as in TNBC specimens (data not shown), and might indicate a particular role of the α3 chain of COL6 in homeostasis of mammary epithelia. Thus, *in vivo* cells expressing *OPN*, *IL-8* and COL6A3 are co-located with cells expressing AXL in normal and tumor contexts.

AXL is associated with drug resistance and metastatic spread of breast cancer (Li et al., 2015; Antony and Huang, 2017; Davidsen et al., 2017). We evaluated expression of a panel of EMT- and stem cell-related genes (**Table 2**) in 184AA3 cells cultured on lrECM (matrigel), COL1, or COL1 supplemented with OPN, IL-8 or COL6A3. A number of EMT related genes were upregulated by the COL1 microenvironment, and the upregulation was enhanced when COL1 was supplemented with any of the three factors (**Figure 4H**). These results indicate that OPN, IL-8, and COL6A3 in COL1- and LAM1-rich contexts non-sporadically induce AXL and cKIT expression, and gene expression consistent with engagement of EMT-related programs in the COL1-rich context, in tumorigenic HMEC, which may induce drug-tolerant states. To determine if plasticity-inductive microenvironments were sufficient to increase drug-tolerance, 184AA3 cells were cultured on COL1-only, COL1+COL6A3, COL1+IL-8, or COL1+OPN microenvironments and treated with paclitaxel. These microenvironments significantly increased tolerance to the drug, consistent with the notion that epithelial plasticity enables non-sporadic induction of drug-tolerant states (**Figures 4I,J**).

DISCUSSION

Here we provide evidence in a breast cancer progression series, that specific tumor-associated microenvironments favor induction of two RTKs implicated in plasticity and drug-tolerant states, in a non-sporadic manner. COL1 is found extensively in tumor stroma, and when combined with certain other common tumor microenvironment proteins (i.e., COL6A3, OPN, and IL-8) the frequency of AXL-expressing cells significantly increased. We showed this functionally on MEMA, in follow up validation cell culture experiments, and using RNA *in situ* hybridization and immunohistochemistry, we demonstrated coordinated expression of these microenvironment factors with AXL expressing cells in breast tumors. Microenvironment proteins such as ECM molecules are not directly targeted by currently approved anti-cancer therapeutics, and they have long *in vivo* half-lives, e.g., the half-life of COL1 is estimated between 14 and 400 years depending on the tissue (Verzijl et al., 2000). Thus, microenvironment-imposed reprogramming could explain why resistant cells are seemingly persistent and rapidly adaptable to multiple drugs. Drug-induced sporadic transcription of a number of other gene markers that are implicated in drug-tolerance was shown, *AXL* among them, though they did not account for microenvironment context (Shaffer et al., 2017). We reported previously that the response of HER2-amplified cells to the HER2-targeted drug lapatinib is partly determined by combinatorial microenvironments (Lin et al., 2017). We speculate that microenvironment-induced drug-tolerance via induction of plasticity-related genes and proteins is a widespread mechanism. Because specific microenvironments are associated to enable certain cellular phenotypes (e.g., AXL and cKIT states) the mechanism is not sporadic, and it suggests an avenue forward for circumventing drug-tolerance.

AXL also is implicated as having a functional role in cells that possess cancer stem cell (CSC) activity. AXL expression is a strong negative prognostic factor for human breast cancer survival and its expression is associated with spread of metastatic breast cancer (Gjerdrum et al., 2010). AXL expression is upregulated during EMT, and the EMT gene program is associated with cells that exhibit normal stem cell and cancer stem cell (CSC) activity (Liu and Fan, 2015). It enhances migratory activity of pre-malignant breast epithelial cells and contributes to breast cancer cell extravasation into lungs (Vuoriluoto et al., 2011). cKIT also is associated with cells that possess mammary progenitor activity (Lim et al., 2009; Garbe et al., 2012), so it is not surprising that many

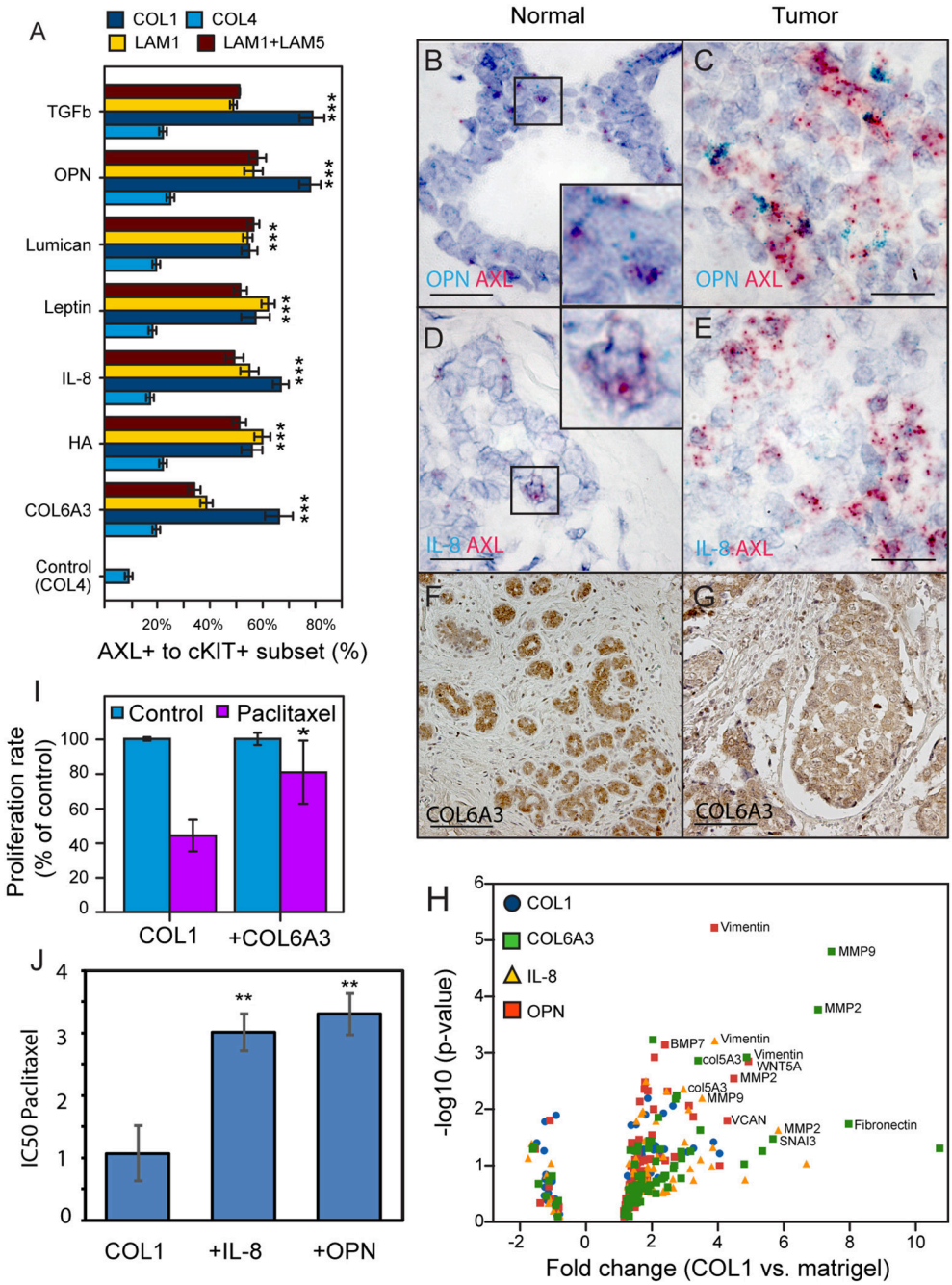

FIGURE 4 | Identification and *in vivo* validation of microenvironment factors that impose AXL and cKIT expression phenotypes in malignant breast cancer cells. **(A)** $AXL^{+}/cKIT^{+}$-184AA3 phenotype expression in MEMA experiments was analyzed by GLM, and the most significant (Tuckey's *post-hoc* test, $p < 2e\text{-}16^{***}$) microenvironment supplemental factors combined with different ECM are presented in bar graph format. **(B,C)** Co-expression of *OPN* (cyan), and *AXL* (red) were determined by RNA *in situ* hybridization of normal human mammary gland tissue **(B)**, and TNBC **(C)** specimens. Co-expression *IL-8* (cyan), and *AXL* (red) in normal human mammary gland tissue **(D)**, and TNBC (E) specimens. Scale bar represent 20 μm **(B–E)**. Expression of COL6A3 in normal human mammary gland tissue **(F)**, and TNBC **(G)** specimens were assayed by IHC-P. **(F,G)** Scale bar = 100 μm. Counterstaining by hematoxylin **(B–G)**. **(H)** Volcano plot represent EMT related gene expression ($RT^{2}Profiler^{TM}$ PCR array, Human Epithelial to mesenchymal transition EMT, Qiagen) in 184AA3 cell cultured (24 h) on COL1, with or without OPN, IL-8, or COL6A3 was compared to expression profile of 184AA3 cells on matrigel. Results represent mean of three individual experiments, and *p*-values are calculated by comparing each gene expression in each group with the matrigel group, $^{***} \leq 0.001$. **(I)** To study drug resistance, 184AA3 cells were cultured on COL1 coated dishes supplemented with or without COL6A3, and treated with 0.1 μM paclitaxel. Data represent EdU positive cells as a percentage of total cells compared to COL1 control culture. Results represent mean ± SD in 6 individual experiments, significance between COL1 and COL1+COL6A3, $^{*}p = 0.02$. **(J)** To study impact of OPN and IL-8 on paclitaxel IC50 values (μg/ml), 184AA3 cells were cultured on COL1 with or without OPN or IL-8. Cells were treated with Paclitaxel (ranging from 0.001 to 1 μg/ml). Results represent mean ± SD in 3 individual experiments, significance between IC50 values, $^{**}p < 0.01$.

TABLE 2 | EMT related gene expression profile ($RT^2Profiler^{TM}$ PCR array, Human Epithelial to mesenchymal transition EMT, Qiagen) of 184AA3 cell cultured (24 h).

Gene symbol	COL1	+OPN	+IL-8	+COL6A3
CAMK2N1	0.59	0.59	0.53	0.55
RGS2	0.68	0.57	0.71	0.56
MAP1B	0.68	0.63	0.74	1.53
GEMIN2	0.69	0.94	1.00	0.90
FGFBP1	0.69	0.79	0.77	1.18
TGFB2	0.69	0.76	0.51	1.05
ZEB2	0.70	0.96	1.38	1.45
ESR1	0.72	1.36	1.91	2.28
STEAP1	0.74	1.39	1.55	1.57
NUDT13	0.75	0.95	1.03	0.61
SNAI2	0.82	3.99	1.08	4.76
PTP4A1	0.82	1.06	0.88	0.81
EGFR	0.88	0.97	1.08	1.07
OCLN	0.88	1.13	0.91	1.37
SMAD2	0.89	1.09	1.23	1.03
RAC1	0.89	1.04	1.06	1.12
DSP	0.91	1.34	1.32	1.29
DESI1	0.91	0.98	1.01	0.97
KRT19	0.93	1.01	1.40	1.87
SPP1	0.93	1.08	0.84	0.70
SNAI1	0.93	2.31	1.47	1.27
PTK2	0.94	1.12	1.21	1.20
GSK3B	0.95	1.02	1.13	1.35
KRT7	0.95	0.77	0.79	0.75
NODAL	0.97	1.37	2.54	1.41
TGFB3	0.98	1.19	1.10	0.90
VPS13A	0.98	1.36	1.61	1.29
IL1RN	1.00	1.35	0.97	2.15
TIMP1	1.00	1.26	1.13	1.25
CAV2	1.00	1.08	1.16	1.16
TSPAN13	1.02	1.16	1.31	1.16
TMEFF1	1.02	1.15	1.30	1.78
TCF3	1.05	1.21	1.00	1.05
BMP7	1.11	2.29	1.73	1.40
FZD7	1.12	1.34	1.30	1.48
STAT3	1.12	1.26	1.10	1.20
TWIST1	1.14	1.28	1.12	1.15
CALD1	1.15	1.49	1.65	1.45
ERBB3	1.21	1.20	1.36	1.45
AHNAK	1.22	1.32	1.47	1.45
AKT1	1.23	1.06	1.17	0.96
F11R	1.24	1.19	1.22	1.19
DSC2	1.25	1.73	1.93	2.20
CTNNB1	1.25	1.40	1.38	1.41
NOTCH1	1.27	1.56	1.33	1.17
TCF4	1.28	1.57	2.90	2.08
ILK	1.29	1.24	1.62	1.57
ZEB1	1.34	1.36	1.91	2.56
CDH1	1.34	1.30	1.55	2.01
ITGB1	1.36	1.94	2.00	2.65
PLEK2	1.36	1.45	1.60	1.91
MSN	1.39	1.66	1.69	1.79
ITGAV	1.41	1.67	1.78	3.39
COL1A2	1.43	1.89	3.17	2.57
FOXC2	1.43	1.52	1.33	1.31
TFPI2	1.43	1.14	1.49	1.60
BMP2	1.45	1.11	1.85	2.37
MST1R	1.48	1.41	1.79	1.40
JAG1	1.57	1.65	2.24	2.81
BMP1	1.62	1.68	2.05	2.00
SERPINE1	1.64	1.47	2.23	2.01
TGFB1	1.69	1.52	1.76	1.57
IGFBP4	1.75	1.33	1.75	1.12
MMP3	1.79	1.74	3.39	3.08
KRT14	1.84	3.18	3.03	1.82
TMEM132A	1.93	1.26	1.41	2.03
COL5A2	2.00	2.38	2.87	3.32
SNAI3	2.03	2.59	3.78	5.66
VCAN	2.07	4.23	3.75	5.33
ITGA5	2.12	1.76	2.54	2.79
GNG11	2.24	1.97	2.33	2.62
SPARC	2.28	1.84	3.07	2.80
VIM	2.53	3.84	3.83	4.84
FN1	3.00	2.12	4.79	8.02
WNT5A	3.18	4.90	6.69	10.86
MMP9	3.80	3.04	3.44	7.49
MMP2	4.00	4.44	5.81	7.06

Fold expression presented in table were calculated using the formula $2^{(-\Delta\Delta Ct)}$, *where* $\Delta\Delta C_t$ *is* $\Delta C_{t(sample)} - \Delta C_{t(matrigel\ sample)}$, ΔC_t *is* $C_{t(gene\ of\ interest)} - C_{t(average\ from\ control\ gene\ setup)}$, *and* C_t *is the cycle at which the detection threshold is crossed.*

of the microenvironments that induced AXL also induced cKIT, as well as the EMT gene signature. Thus, prior to this study epithelial plasticity, and the underlying EMT-related gene programs, have been widely discussed in the context of metastatic spread. The MEMA platform is probably not the correct model for understanding processes related to metastasis, but we demonstrate here its utility in examining the roles of microenvironment in drug-tolerance. MEMA have proven useful in functionally defining putative normal stem cell niche components using the juxtaposition of lineage specific keratins as cell fate markers (LaBarge et al., 2009). From these new results, we speculate that CSC niche components also could be functionally identified using the MEMA platform.

In the mature mammary gland, the basement membrane is located between the epithelium and stroma, compartmentalizing breast tissue components. COL4, LAM1, and LAM5 are important basement membrane constituents that promote attachment of epithelial cells and maintenance of epithelial polarity (Kleinman et al., 1981), while stroma is rich in COL1. The MEMA approach revealed that normal HMEC express AXL and cKIT in the COL1-rich microenvironments. This suggests that breakdown of normal tissue compartmentalization and

exposure to COL1-rich stromal ECM is a plasticity signal for differentiated mammary epithelial cells. While stem cell traits are a common feature of malignant carcinoma, the reacquisition of stem cell properties by normal differentiated epithelial cells is poorly understood (Blanpain and Fuchs, 2014). Our results reveal that this may be regulated at the level of tissue organization through distinct combinatorial cell-cell and cell-ECM signals.

OPN, IL-8, and COL6A3 exposure upregulated EMT-related genes and COL6A3 supported breast cancer cell drug resistance. OPN is an N-linked glycoprotein and functions as an extracellular structural protein in many tissues. OPN expression is relatively low in normal mammary gland but it is induced during lactation and involution (Insua-Rodriguez and Oskarsson, 2016). OPN was reported to be part of the hematopoietic and neural stem cell niche (Haylock and Nilsson, 2006). IL-8 is a pro-inflammatory and pro-angiogenic factor, and is strongly associated with cancer progression. Genetic variation and increased expression of IL-8 correlates with increased risk of breast cancer as well as poor prognosis (Snoussi et al., 2006; Milovanovic et al., 2013). IL-8, among other cytokines, has been linked to regulation of the breast CSC state, and IL-8 can stimulate CSC self-renewal (Korkaya et al., 2011; Palacios-Arreola et al., 2014). COL6 is a widely distributed ECM macromolecule that plays a crucial role in tissue development, it was reported to be part of the normal breast and breast cancer microenvironments (Ferguson et al., 1992; Karousou et al., 2014). Upregulation of COL6 was shown to generate a microenvironment that promotes tumor progression (Chen D. et al., 2013). COL6 is a heterotrimer composed of three genetically distinct polypeptide chains: α1, α2, and α3 i.e., COL6A3. COL6A3 is the largest of these three chains and the cleavage of the C5 domain, also called endotrophin, has a crucial role in breast cancer development, and it is a ligand for ANTXR1-receptor, which is a putative biomarker for breast CSC (Chen P. et al., 2013; Karousou et al., 2014). COL6A3 represents a frequently mutated gene in triple negative breast cancers (Cancer Genome Atlas Network, 2012; Curtis et al., 2012; Shah et al., 2012). Based on our cell-based functional experiments, we suggest that OPN, IL-8 and COL6A3 are part of a CSC niche.

Cooption of epithelial plasticity mechanisms has emerged as a central challenge for current cancer treatments. In spite of advances in cancer therapies, most cancer patients still do not experience lasting clinical benefit. Tumor cells invariably elude treatment; reemerging as advanced, disseminated malignancy that is associated with increased mortality. This study highlights how malignant carcinoma cells adapt to different microenvironments by activating drug resistance via clinically actionable RTKs. Hence a deeper understanding the interplay between malignant cells and a dynamic microenvironment, and the underlying signaling pathways will inform new combination therapy approaches to prevent resistance.

MATERIALS AND METHODS

Contact for Reagent and Resource Sharing

Further information and request for resources and reagents should be directed to and will be fulfilled by the lead contact, Jim Lorens (Jim.Lorens@uib.no) or Mark LaBarge (mlabarge@coh.org).

Cell Culture

Human mammary epithelial cells were cultured in M87A media supplemented with cholera toxin at 0.5 ng/ml (Sigma-Aldrich) and oxytocin at 0.1 nM (Bachem, Switzerland) (Garbe et al., 2009). Cells were isolated from reduction mammoplasty specimen 184, a 21 year old Caucasian female, and maintained as previously described (Garbe et al., 2009; Labarge et al., 2013). Pre-stasis, passage 4,184 cells were sorted by flow cytometry, and a cKIT positive progenitor subpopulation was used for experiments. Immortal cell lineages were derived by using the chemical carcinogen benzo(a)pyrene (BaP) to overcome stasis. The BaP treated post-stasis 184Aa lineage clonally overcame the immortalization barrier to generate the non-malignant immortal 184A1 line (Stampfer and Bartley, 1985). The clonal tumorigenic cell line 184AA3 emerged from 184Aa following insertional mutagenesis in the p53 locus (Stampfer et al., 2003). Cells were cultured on 2D plastic dish (unless otherwise mentioned). In 3D cultures, a single cell suspension was embedded in growth factor reduced matrigel (Corning) or 1.5 mg/ml COL1 gel (rat tail, non pepsinized, 5 mg/ml, Ibidi), Culture medium was changed every other day, and after 12 days cells and gel were fixed and stained.

In Vivo Human Tissue Studies

The archival formalin fixed paraffin embedded (FFPE) tissues used in this study originates from the Department of Pathology, Haukeland University Hospital, Bergen, Norway, and the Regional Institute of Oncology, Iasi, Romania. Tissue histology and tumor classification were verified by trained pathologists at the respective institutions. Tissues from Haukeland University hospital has ethical approval REK (Regional Ethics Committee #2014/1984), and tissues from Regional Institute of oncology has approval from Ministerul sanatatii, IRO, Cod Fiscal 29067408.

Flow Cytometry

One hundred and eighty four passage 4 HMECs were cultured close to confluence and trypsinized. After that fluorescein conjugated Anti-CD117/cKIT-antibody (Biolegend, clone 104D2, 1:50) was added to cells in media for 25 min−1 h on ice, cells were washed with cold PBS and sorted with FACS Vantage DIVA or FACS Aria SORP (Becton Dickinson).

MicroEnvironment MicroArray (MEMA)

MEMA method is comprehensively presented here (Lin et al., 2012; Lin and LaBarge, 2017). Briefly, polyacrylamide (PA) gels were made on standard glass microscope slide etched with 0.1M NaOH. Slides were covered with 3-Aminopropyltriethoxysilane (APES, Sigma-Aldrich), and after 5 min slides were soaked in distilled H_2O. Then incubated 30 min at 0.5% Glutaraldehyde (Sigma-Aldrich) solution in PBS. After this, slides were dried and polyacrylamide gel 350 μl/slide was pipette on the slide and covered with cover glass. PA gel solution contained 5% acrylamide (Sigma-Aldrich) and 0.15% Bis-Acrylamide (VWR), final gel modulus was 4,470 ± 1,190 Pa. The MEMA master plate was prepared by diluting the ECM-combinations with printing buffer composed of 100 mM Tris-Acetate/20%

glycerol/0.05% TritonX-100 pH5.2. Protein information and used concentrations used are shown in (**Table 3**). SpotBotIII microarrayer (ArrayIt, CA, USA) was used to perform printing, with 5–20 replicate spots of each microenvironment were printed. After printing MEMAs were placed into 4-well plates (Nunc) and first washed with PBS with 50 U/ml of penicillin and 50 U/ml streptomycin (Gibco), followed by a second wash with cell culture medium. Cells were diluted to the desired concentration into 5 ml of media, plated over the MEMA slide, and incubated at +37°C with 5% CO_2. After 4 h one replicate MEMA was fixed with methanol:acetone (1:1) at −20°C for 20 min, to indicate cell attachment on MEMAs. For replicate MEMA, non-attached cells were washed away with culture media and fresh media was added to wells. After 24 h the media was changed again and after 48 h MEMAs slides were fixed, as described above. Fixed MEMA were blocked with PBS, 5% normal goat serum (Invitrogene), 0.1% Triton X-100 (Sigma-Aldrich), and incubated with anti-AXL (1:200, 10c9) and anti-cKIT (1:200, CD117, Biolegend) overnight at 4°C, then visualized with fluorescent secondary antibodies (VWR), and DRAQ5 DNA dye (Cell signaling). MEMA slides were imaged with InnoScan 1100 (Innopsys) or LSM710 confocal microscope (Carl Zeiss).

Immunohistochemistry

For *in vitro* immunofluorescence staining, cells were fixed in methanol:acetone (1:1) at −20°C for 20 min, blocked with PBS, 5% normal goat serum, 0.1% Triton X-100, and incubated with anti-Keratin14 (1:1,000, Covance, polyclonal) and anti-Keratin19 (1:200 AB7754, Abcam) overnight at 4°C, then visualized with fluorescent secondary antibodies (Invitrogen) incubated with sections for 2 h at room temperature.

Human formalin-fixed paraffin embedded (FFPE) tissue sections of normal mammary gland, invasive breast cancer and triple negative breast cancer (TNBC) were deparaffinized in xylene, and rehydrated according to standard protocols. Antigen retrieval was performed by boiling the sections in 0.01 M citrate buffer, pH6, for 25 min, followed by cooling to RT at the bench and a 10 min wash in dH_2O prior to staining. For detection of ECM components, COL4 (1:100, MAB3326), COL1 (1:100, ab34710), LAM5 (1:50, MAB19562), Pan laminin (1:100, L9393), EPCAM (1:100, 34202), and K14 (1:1,000, Covance, polyclonal) antibodies were diluted in permwash buffer (BD Bioscience) and incubated at +4°C overnight. Fluorescence labeled secondary antibodies and Hoechst nuclei label were diluted also in permwash buffer and incubated 2 h at RT. For detection of COL6A3 (NBP-71566, Novus Biologicals) in FFPE tissue sections of normal mammary gland and triple negative breast cancer (TNBC) specimens, DAKO EnVision™ System-HRP (DAB) for Rabbit primary antibodies (K4011, DAKO) was applied according to the manufacturer's instructions. Antibodies were diluted in antibody-diluent with background reducing components (S3022, DAKO). Stained sections were counterstained with haematoxylin, prior to mounting using Faramount Aqueous Mounting Medium (S3225, DAKO). Images were obtained on a Leica DMLB microscope equipped with AnalySIS software (Leica).

Dual RNA *in Situ* Hybridization

Simultaneous *in situ* detection of the *OPN*, *IL-8* and *AXL* mRNA on human normal mammary FFPE tissue sections and TNBC specimens were performed using the RNA scope technology. *OPN* and *IL-8* were detected by C1 probes and *AXL* by C2-probes in all experiments. Probes and reagents were provided by Advanced Cell Diagnostics (ACD, Hayward, CA). Briefly, freshly cut 5-μm thick human archival mammary gland tissue sections were deparaffinized in xylene, followed by dehydration in an ethanol series. Tissue sections were then incubated in citrate buffer (0.01 M, pH 6) maintained at a boiling temperature (100–103°C) using a hot plate for 15 min, rinsed in deionized water, and immediately treated with10 μg/mL protease (Sigma-Aldrich, St. Louis, MO) at 40°C for 30 min in a HybEZ hybridization oven (Advanced Cell Diagnostics, Hayward, CA). Hybridization with target probes, preamplifier, amplifier, label probe and chromogenic detection were performed according to the ACD recommendations. Sections were counterstained with hematoxylin, and mounted with EcoMount prior to imaging. Assays using archival FFPE specimens were performed in parallel with positive and negative controls, to ensure interpretable and reproducible results.

Drug Resistance Assay

For paclitaxel resistance experiments, 8-well chamber slides were coated with COL1 (calf skin, Sigma-Aldrich) 100 μg/ml with or without COL6A3 (MyBioSource) 10 μg/ml diluted in 50 mM Hepes. 184AA3 cells were plated 24 h prior to drug treatment to coated chambers, followed by 24 h culturing with paclitaxel (0.1 μM, Sigma-Aldrich). Proliferation rate was analyzed by using Click-iT® Plus Edu imaging kit (Molecular probes). For paclitaxel IC50 analysis, 96-well plates were coated with COL1 (calf skin, Sigma-Aldrich) 100 μg/ml with or without OPN 4 μg/ml. 184AA3 cells were plated 4 h prior to drug treatment to coated wells and culture media was supplemented with or without IL-8 (50 ng/ml) and OPN (50 ng/ml). Followed by 48 h culturing with 5 different concentration of paclitaxel (0.001–1 μg/ml, Sigma-Aldrich). Cell viability was analyzed by using CellTiter-Glo 2.0 Assay (Promega). Paclitaxel was dissolved to DMSO, and control cultures were treated with equally diluted DMSO-solution.

Real-Time PCR

Cells were cultured 24 h over the matrigel or COL1-gel (0.5 μg/ml, rat tail, non pepsinized, Ibidi) supplemented with or without 2 ug/ml COL6A3 (MyBioSource). Cell culture medium was supplemented with or without 10 ng/ml OPN (R&D systems), 10 ng/ml IL-8 (Abcam). Total RNA was purified with Trizol (Invitrogen). cDNA was synthesized with RT^2 First strand kit (Qiagen). Transcripts levels were measured by RT^2Profiler™ PCR arrays, human stem cell and human epithelial to mesenchymal transition (EMT) using RT^2 SYBR Green PCR master mix (Qiagen) and LightCycler480 (Roche). Fold expressions were calculated using the formula $2^{(-\Delta\Delta Ct)}$, where $\Delta\Delta C_t$ is $\Delta C_{t(\text{sample})} - \Delta C_{t(\text{control sample})}$, ΔC_t is $C_{t(\text{gene of interest})} - C_{t(\text{average from control gene setup})}$ and C_t is the cycle at which the detection threshold is crossed.

TABLE 3 | Key resources table.

Reagent of resource	Source	Identifier	Notes
ANTIBODIES			
Anti-CD117 (cKIT)	Biolegend	313201	
Anti-AXL (10c9)	BerGenBio, Bergen, Norway	N/A	
Cytokeratin 14 antibody	Thermo scientific	PA5-16722	
Anti-Cytokeratin 19	Abcam	AB7754	
Anti-Collagen type IV	Merck millipore	MAB3326	
Anti-Collagen I	Abcam	AB34710	
Anti-Laminin-5	Merck millipore	MAB19562	
Anti-Laminin	Sigma-aldrich	L9393	
Anti-human CD326 (EPCAM)	Biolegend	34202	
Anti-AXL (mAb: 1H12)	BerGenBio, Bergen, Norway	N/A	
Anti-COL6A3	Novus biologicals	NBP-71566	
BIOLOGICAL SAMPLES			
The human FFPE-healthy mammary gland and breast cancer tissues	The Department of Pathology, Haukeland University Hospital, Bergen, Norway, and the University Hospital of Iasi, Iasi, Romania	N/A	
Chemicals, peptides, and recombinant proteins			**Concentration in MEMA, Reference; relevance in cancer**
Bone morphogenetic protein 2/7 heterodimer (BMP-2/7)	R&D systems	3229-BM/CF	1 μg/ml, (Ye et al., 2009)
Bone morphogenetic protein 4 (BMP-4)	R&D systems	113-BP/CF	1 μg/ml, (Ye et al., 2009)
Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6)	R&D systems	3934-CM-050	1 μg/ml, (Beauchemin and Arabzadeh, 2013)
Carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM8)	Abnova	H00001088-P01	1 μg/ml, (Beauchemin and Arabzadeh, 2013)
CD44	R&D systems	3660-cd	1 μg/ml, (Karousou et al., 2014)
Collagen I (COL1)	Sigma-Aldrich	C8919	100 μg/ml, (Insua-Rodriguez and Oskarsson, 2016)
Collagen IV (COL4)	Sigma-Aldrich	C5533	100 μg/ml, (Insua-Rodriguez and Oskarsson, 2016)
CollagenXXIIIA1 (COL23A1)	R&D systems	4165-CL	1 μg/ml, (Spivey et al., 2012)
CollagenVIα3 (COL6A3)	MyBiosource	MBS958856	1 μg/ml, (Karousou et al., 2014)
E-Cadherin (ECAD)	Sigma-Aldrich	E2278	1 μg/ml, (Yu and Elble, 2016)
Epidermal growth factor (EGF)	Sigma-Aldrich	E9644	1 μg/ml, (Voudouri et al., 2015)
Fibroblast growth factor basic (FGF-2)	R&D systems	233-FB-025	1 μg/ml, (Zheng et al., 2014)
Fibronectin (FN1)	Sigma-Aldrich	f2518	100 μg/ml, (Insua-Rodriguez and Oskarsson, 2016)
Growth arrest specific 6 (GAS-6)	R&D systems	885-GS-050	1 μg/ml, (Mc Cormack et al., 2008)
Hepatocyte growth factor (HGF)	R&D systems	294-HG-005	1 μg/ml, (Ho-Yen et al., 2015)
Hyaluronan HMW (HA)	R&D systems	GLR002	100 μg/ml, (Karousou et al., 2014)
Insulin like growth factor-1 (IGF1)	R&D systems	291-Gi-250	1 μg/ml, (Voudouri et al., 2015)
Interferon- γ (IFN-γ)	Gibco	PHC4031	1 μg/ml, (Esquivel-Velazquez et al., 2015)
Interleucin-1β (IL-1β)	Biolegend	579404	1 μg/ml, (Esquivel-Velazquez et al., 2015)
Interleucin-6 (IL-6)	Biolegend	570804	1 μg/ml, (Esquivel-Velazquez et al., 2015)
Interleucin-8 (IL-8, CXCL8)	Biolegend	574204	1 μg/ml, (Palacios-Arreola et al., 2014)
Laminin-111 (LAM1)	Sigma-Aldrich	I2020	80 / 100 μg/ml, (Insua-Rodriguez and Oskarsson, 2016)
Laminin-332 (LAM5)	Abcam	ab42326	20 μg/ml, (Insua-Rodriguez and Oskarsson, 2016)
Leptin	Sigma-Aldrich	L4146	1 μg/ml, (Garcia-Robles et al., 2013)
Lumican	Sigma-Aldrich	2846	1 μg/ml, (Nikitovic et al., 2014)

(Continued)

TABLE 3 | Continued

Reagent of resource	Source	Identifier	Notes
Melanoma growth stimulating activity alpha (GRO-α/CXCL1)	Sigma-Aldrich	G0657	1 μg/ml, (Palacios-Arreola et al., 2014)
Nidogen1	R&D systems	2570-nd	1 μg/ml, (Insua-Rodriguez and Oskarsson, 2016)
Osteopontin (OPN)	Novus Biologicals	NBC1-21056	1 μg/ml, (Insua-Rodriguez and Oskarsson, 2016)
Osteoprotegerin (OPG)	R&D systems	185-OS-025	1 μg/ml, (Weichhaus et al., 2015)
Stem cell factor (SCF)	R&D systems	255-SC-010	1 μg/ml, (Mimeault et al., 2007)
Stromal derived factor-1 (SDF-1β/CXCL12)	Abnova	P4470	1 μg/ml, (Palacios-Arreola et al., 2014)
Tenascin C (TNC)	Chemicon	CC065	1 μg/ml, (Insua-Rodriguez and Oskarsson, 2016)
Tumor growth factor β (TGFβ)	Biolegend	580704	1 μg/ml, (Esquivel-Velazquez et al., 2015)
Paclitaxel	Sigma-Aldrich	T7191	
rat tail Collagen type I	Ibidi	50201	
Collagen type 1, calf skin	Sigma-Aldrich	C8919	
Matrigel, growth factor reduced	Corning	356231	
CRITICAL COMMERCIAL ASSAYS			
$RT^2Profiler^{TM}$ PCR arrays, human stem cell	Qiagen	PAHS-405ZF	
$RT^2Profiler^{TM}$ PCR arrays, human epithelial to mesenchymal transition (EMT)	Qiagen	PAHS-090ZF	
Click-iT® Plus Edu imaging kit	Molecular probes	C10337	
RT^2 SYBR Green PCR Master Mix	Qiagen	330503	
RT^2-First Strand Kit	Qiagen	330401	
Quick-RNA MicroPrep	Zymo Research	R1050	
CellTiter-Glo 2.0 Assay	Promega	G9242	
EXPERIMENTAL MODELS: CELL LINES			
HMEC progression series	Dr. Martha Stampfer, Lawrence Berkeley national Laboratory, CA, USA	184	
OLIGONUCLEOTIDES			
RNAScope probe for *AXL*	Advanced cell diagnostics	Probe-Hs-AXL-C2	
RNAScope probe for *IL-8*	Advanced cell diagnostics	Probe-Hs-IL8-C1	
RNAScope probe for *OPN*	Advanced cell diagnostics	Probe-Hs-SPP1-C1	
SOFTWARE AND ALGORITHMS			
Cell profiler	www.cellprofiler.org		
R-language, R-studio	www.R-project.org/		
Cytobank	cellmass.cytobank.org		
IC50 toolkit	www.ic50.tk		

Gene Expression Analysis

Total RNAs were isolated using Quick-RNA MicroPrep (Zymo Research). Sample preparation and Poly(A) enriched mRNA-sequencing were performed in City of Hope Comprehensive cancer center, Integrative genomics and bioinformatics core.

Data Analysis

R was used for all statistical analysis (R foundation for statistical computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/). To compare two population distributions *t*-tests were performed. Significance was established when: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. RNA sequencing data is normalized and results are presented as RPKM (Reads Per Kilobase Million).

MEMA images were analyzed for single cell data with the CellProfiler (Carpenter et al., 2006) pipeline that is included in **Supplementary File 1**. Briefly fluorescence channel images were analyzed as separated gray scale images. To normalize intensity of images, threshold method: Background was used. This method finds the mode of the histogram part of the image, which is assumed to be the background of the image, and choose a threshold at twice that value. Threshold value was subtracted from the remaining pixel intensities. Marker-based watershed segmentation was used to identify single cells. Fluoresence intensity, cell size and morphology and cell neighbors were measured for each cell. Data analyses were performed using R-software. AXL and cKIT intensities were presented as mean of pixel intensity values. (AXL^{+}/$cKIT^{+}$)-subset was calculated by using threshold from COL4-microenvironment

spots (mean+SD), cells expressing intensities above threshold were counted in ($AXL^+/cKIT^+$)-subset. Generalized linear model (GLM) was applied to decouple effects of multiple microenvironment components from each other, and then express ($AXL^+/cKIT^+$)-subset expression as a function of each microenvironment component. Tuckey's post Hoc test was performed after GLM to identify differences inside the microenvironmental factors. tSNE-method (Amir et al., 2013) in Cytobank portal (https://www.cytobank.org) was used to cluster and visualize MEMA data. For the clustering analysis, the mean value of each individual microenvironment was calculated from these data types: percentage of ($AXL^+/cKIT^+$)-subpopulation, fluorescein intensities in ($AXL^+/cKIT^+$)-subpopulation and in total population, cell number/spot, cell eccentricity and cell solidity.

IC50 was calculated by plotting and fitting data points to curve and regard the mid-point ligand concentration (IC50), curve fitting formula $y = a + [b\text{-}a]/[1+(x/c)^d]$ is presented in ic50.tk.

ADDITIONAL RESOURCES

Additional information on 184 HMEC progression series: http://hmec.lbl.gov/mindex.html.

AUTHOR CONTRIBUTIONS

TJ designed and performed the experiments and data analysis, also wrote the manuscript. AE performed COL6A3, *AXL*, *OPN*, and *IL-8* tissue immune staining's and RNAscope experiments and critically reviewed and helped with manuscript writing. AR provided technical support. FP provided support with data analysis and critically reviewed manuscript. JG provided support with HMEC culture system. MM performed RNA sequencing experiment. CT, DF, and LA provided breast tissue sections. MS provided support with HMEC culture system and critically reviewed manuscript. JL and ML supported, critically reviewed and helped with manuscript writing and experimental design.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00041/full#supplementary-material

Supplementary File 1 | Cell profile-pipeline used in this work.

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Microenvironmental Signals and Biochemical Information Processing: Cooperative Determinants of Intratumoral Plasticity and Heterogeneity

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Intra-tumor cellular heterogeneity is a major challenge in cancer therapy. Tumors are composed of multiple phenotypic subpopulations that vary in their ability to initiate metastatic tumors and in their sensitivity to chemotherapy. In many cases, cells can transition between these subpopulations, not by genetic mutation, but instead through reversible changes in signal transduction or gene expression programs. This plasticity begins at the level of the microenvironment where local autocrine and paracrine signals, exosomes, tumor–stroma interactions, and extracellular matrix (ECM) composition create a signaling landscape that varies over space and time. The integration of this complex array of signals engages signaling pathways that control gene expression. The resulting modulation of gene expression programs causes individual cells to sample a wide array of phenotypic states that support tumor growth, dissemination, and therapeutic resistance. In this review, we discuss how information flows dynamically within the microenvironmental landscape to inform cell state decisions and to create intra-tumoral heterogeneity. We address the role of plasticity in the acquisition of transient and prolonged drug resistant states and discuss how targeted pharmacological modification of the signaling landscape may be able to constrain phenotypic plasticity, leading to improved treatment responses.

Keywords: receptor, kinase, network state transition, neoplastic, epithelial-to-mesenchymal transition, stem cell, single-cell

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INTRODUCTION: TWO PERSPECTIVES ON TUMOR HETEROGENEITY

It has long been understood that tumors are composed of multiple cellular subpopulations that vary in their ability to initiate new tumors (Fidler, 1978) and in their sensitivity to chemotherapy (Heppner et al., 1978). When subpopulations of cells within tumors differ markedly in drug resistance, treatment becomes much more difficult, as a single cytotoxic therapy cannot eliminate all of the malignant cells. Heterogeneity in tumor initiation potential also complicates treatment, because even if a therapy kills the vast majority of tumor cells, it will ultimately fail if the few remaining cells are able to expand or disseminate to initiate new tumor sites. Understanding the

nature of heterogeneity and the factors that drive it would enable better prediction of effective therapeutic strategies.

Over the past decade, insight into cancer cell heterogeneity has emerged from two distinct fields. First, research into the tumor microenvironment (TME) has revealed that the behavior of tumor cells is dramatically modulated in response to their immediate surroundings; for extensive review see Bissell and Hines (2011) and Quail and Joyce (2013). Mapping of the cellular milieu of tumors in detail has revealed that molecular signals presented by neighboring stromal cells and extracellular matrix (ECM) engage receptors on the surface of tumor cells, triggering intracellular signaling pathways. The resulting induction of gene regulatory circuitry plays a determinative role in the phenotype expressed by a cancer cell. While these signaling pathways are often functionally modified by genetic mutations, DNA sequence alone is insufficient to capture the full range of potential for any cancer cell; mutant cells still respond to extracellular cues, albeit with altered sensitivity. Thus, tumor cell heterogeneity cannot simply be ascribed to genetic diversity within a tumor, but also to the broad variation in signaling cues derived from tumor cells themselves and the many stromal cells that make up the tumor ecosystem (Marusyk et al., 2014; Tabassum and Polyak, 2015).

In parallel, an emerging field has investigated the physical basis of cellular heterogeneity originating in the biochemistry of signaling. From the earliest studies of signal transduction using the *E. coli lac* operon as a model, it has been clear that genetically identical cells respond divergently to environmental stimuli (Novick and Weiner, 1957). At first glance, this variation could be ascribed simply to "noise" in the molecular processes of receptor binding and the relay of intracellular messengers (Korobkova et al., 2004). However, advances in live-cell fluorescence microscopy have made possible well-controlled cell culture experiments that have revealed a deep and intricate underlying structure to the diversity of signaling responses (Levine et al., 2013). Key among these results is the observation that an individual cell's potential to respond to a signaling cue varies from cell to cell and is non-genetic in nature, but is nonetheless heritable for one or more cellular generations (Spencer et al., 2009). Whereas these studies cannot reproduce the physiological complexity of a tumor, they have a clear implication: because the biochemistry of signaling drives variable responses in genetically identical cells even under controlled conditions, the same diversification probably occurs *in vivo* and contributes to the heterogeneity of tumor cells.

The common feature shared by both of these perspectives is the concept that tumor cell heterogeneity can arise from the unique, cell-specific operation of signal transduction pathways within each individual tumor cell. This concept contrasts with the current notion that ongoing genetic mutations are the primary source of heterogeneity in tumors. In reality, both genetic and non-genetic factors contribute substantially to the phenotypic diversity within tumors, but as of yet, there are few approaches that can definitively resolve their relative contributions. The role of intra-tumoral genetic heterogeneity has been reviewed extensively, and for the purposes of this review we defer to other discussions of this topic (Vogelstein et al., 2013; Alizadeh et al., 2015), acknowledging the importance of mutation as a parallel source of phenotypic diversity in tumors. We focus our attention here on how both complex microenvironments and physico-chemical properties of signal transduction cascades contribute to cellular heterogeneity, even in the absence of genetic differences, an important topic that has received more limited attention (Brock et al., 2015).

As an organizing theme, we present a thought experiment in which two genetically identical tumor cells, originating from the same cell division, experience different microenvironments, and integrate the respective extracellular signals in their gene expression programs, finally resulting in different drug responses (**Figure 1**). We discuss each stage in this hypothetical divergence, beginning with a discussion of the sources of heterogeneous signals in the microenvironment. We discuss what is understood about variability in the signaling process leading up to regulation of gene expression, followed by the gene expression programs that give rise to persistent phenotypic states and variation in drug resistance. We end with a discussion of how variability in drug sensitivity may be measured and targeted to improve therapeutic responses.

A LANDSCAPE OF HETEROGENEOUS SIGNALS

All cells reside in a microenvironment defined by both cellular and non-cellular components. The classic example is the stem cell niche which is a spatially and temporally ordered environment composed of ECM and stromal cells that provide signals to maintain a given cell state (Adams and Watt, 1989; Medema and Vermeulen, 2011; Sato et al., 2011). When a progeny of a stem cell division moves away from the niche, distances of even a few cell widths will expose it to new cues, setting it on a different phenotypic trajectory (e.g., differentiation). The general concept of a niche can be extended to any cell, in the sense that its phenotype is guided by cues from its local microenvironment.

In normal tissues and organs, the microenvironment is organized and maintained over time, supporting homeostasis. However, in cancer the microenvironment becomes corrupted, leading to the formation of many disorganized and heterogeneous niches (Bellail et al., 2004; Lu et al., 2012; Frenkel et al., 2015; Natrajan et al., 2016). As such, the local signals received by an individual tumor cell from fibroblasts, immune lineages, ECM, and/or vascular endothelium vary in composition and strength within the tumor stroma, over small scales. Proximity of tumor cells to stable vs. growing vasculature exposes them to different concentrations of nutrients, hormones, and other cues (Carmeliet and Jain, 2000; Ghajar et al., 2013; Marusyk et al., 2016). The composition of signals secreted by individual stromal cells is also variable due to fibroblast and myeloid subtypes that either co-exist in differing ratios, or are localized in specific regions within the TME (Kiskowski et al., 2011; Carmona-Fontaine et al., 2017). In the following section, we briefly discuss the cell types present in the tumor stroma, examples of their spatial distribution and subtypes, and the signaling cues they provide to shape tumor cell phenotype within the TME. Throughout our descriptions we consider how

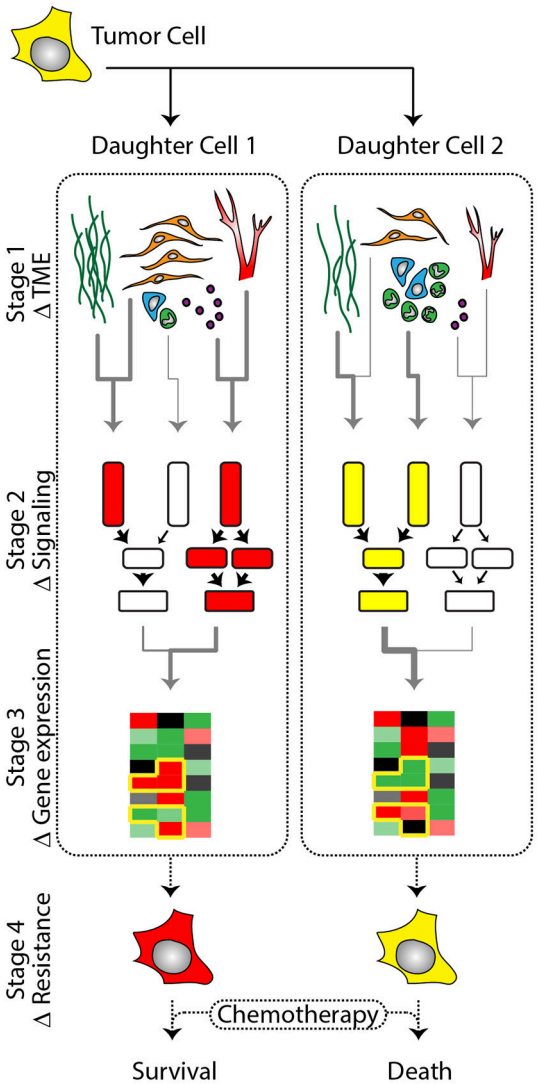

FIGURE 1 | A single tumor cell gives rise to genetically identical daughter cells that vary in phenotype based on exposure to heterogeneous signaling cues and intrinsic variation in signal integration. (Stage 1) Daughter cells are exposed to unique signaling cues in the dynamic tumor microenvironment (TME). Abundance of ECM (dark green), cancer associated- fibroblasts (orange), tumor associated immune cells (blue and green), vasculature (red), and exosomes (purple) vary in abundance and secretory composition throughout the TME, exposing tumor cells to unique signaling microenvironments. (Stage 2) Signals arising from the microenvironment are integrated by membrane receptors and transduced via downstream kinases that modulate transcription factor activation. Inherent cell-to-cell variation in the sensitivity of cells to signaling cues coupled with regional variation in microenvironmental signaling composition contributes to the differential regulation of transcription factors between single cells. (Stage 3) The factors described in Stage 1 and 2 are compounded by transcriptional noise and epigenetic variation leading to cell-to-cell variability in gene expression profiles. (Stage 4) The culmination of microenvironmental signaling and gene expression (Stage 1–3) results in the generation of heterogeneous tumor cell phenotypes (red vs. yellow cells) despite genetically identical backgrounds. As a consequence of phenotypic heterogeneity, some tumor cells will display altered sensitivities when exposed to chemotherapeutic agents, contributing to fractional killing.

two daughter cells, resulting from a tumor cell division, could remain in relatively close proximity, yet exhibit heterogeneous phenotypes secondary to the unique sets of extracellular signals within their respective niches (**Figure 1**, Stage I).

Cancer-Associated Fibroblasts (CAF)

Fibroblasts are the most abundant cell type in solid tumors and heavily influence the phenotypic behavior of tumor cells in their local proximity. A compendium of studies have shown that fibroblasts adopt a "cancer-associated fibroblast" (CAF) state, exposing tumor cells in their secretory radius to a host of phenotype-modifying growth factors; for extensive review see (Bhowmick et al., 2004b; Kalluri, 2016). For example, hepatocyte growth factor (HGF), and transforming growth factor β (TGFβ) are known to enhance tumor cell proliferation and promote the acquisition of invasive phenotypes, such as epithelial-to-mesenchymal transitions (EMT) (Stoker et al., 1987; Miettinen et al., 1994; Bhowmick et al., 2004a). Additionally, CAFs produce chemotactic factors and cytokines. Gradients of stromal-derived factor-1 (SDF-1) increase the migratory capacity of tumor cells and provide directionality, whereas cytokines, such as interleukin-6 (IL-6), modulate the proliferation and therapy response characteristics of tumors (Adams et al., 1991; Orimo et al., 2005).

CAFs are not a single entity; they are composed of multiple functionally distinct subtypes and exhibit regional variation in density within the TME (Sugimoto et al., 2006; Kiskowski et al., 2011; Rudnick et al., 2011; Brechbuhl et al., 2017). Early studies hypothesized that CAFs arose from resident fibroblasts that were reprogrammed by tumor-secreted factors. Indeed, it is likely that many CAFs are derived from resident cells. However, recent evidence supports more diverse origins that may contribute to the observed heterogeneity in this population of cells. It has been demonstrated that a proportion of CAFs arise from mesenchymal stems cells (MSC) recruited to the TME from the bone marrow (Worthley et al., 2009). Other mesenchymal cell types, including smooth muscle cells and endothelial cells, have also been implicated as sources of CAF generation (Madar et al., 2013). And, remarkably, tumor cells themselves appear to represent a large CAF reservoir through trans-differentiation from epithelial to mesenchymal states (Zeisberg et al., 2007). These diverse origins raise the possibility that "CAFs" represent not just functional subtypes, but distinct populations of cells that are yet to be defined beyond current morphological and marker expression standards (e.g., smooth muscle actin, vimentin).

As a consequence of their heterogeneity, the signals derived from fibroblast subtypes, and their relative strength in the TME, can alter the phenotypic characteristics of adjacent tumor cells in different ways. For example, in the breast, sub-populations of CD146 or prostaglandin E2 (PG2E) expressing fibroblasts have been identified. These studies show that an increased ratio of CD146(–) fibroblasts suppresses estrogen receptor (ER) expression and renders cells insensitive to tamoxifen (Brechbuhl et al., 2017). However, altering the ratio of CD146(+/−) fibroblasts over time can restore ER expression, and sensitivity

to chemotherapy, indicating a non-genetic mechanism by which the stroma can control ER expression in proximal tumor cells. In a similar manner, PGE2(+) fibroblasts enhance tumor growth through secretion of IL-6; thus, controlling the ratio of PGE2(+/−) fibroblast subpopulations can either enhance or suppress tumor growth in a breast cancer model (Rudnick et al., 2011). Finally, CAFs also exhibit focal effects on tumor phenotypes, regulating local growth and invasion characteristics (Gao et al., 2010; Puram et al., 2017). CAFs from the interface zone (i.e., the junction between the tumor and normal tissue) induce a highly invasive tumor phenotype compared to the effect of "normal" fibroblasts or CAFs isolated from the tumor parenchyma (Gao et al., 2010). While the regional and subtype-specific signals produced by these cells are not known, these findings provide compelling evidence for the ability of CAFs to modulate the phenotypic repertoire of tumor cells in a region-specific manner.

Vasculature, Nutrient Density, and Hypoxia

Vascular networks are of critical importance in tumorigenesis. They deliver oxygen and nutrients vital to sustain rapid growth of tumor cells and provide a conduit for the delivery of immune cells to, and dissemination of tumor cells from, the primary tumor. However, vessels are not passive participants in tumorigenesis; they actively signal to tumor cells to form functional niches (Carmeliet and Jain, 2000). For example, depending upon proximity to a vascular wall, or a growing tip, tumor cell phenotype can be vastly different. Tumor cells residing along a vascular wall niche often take on a cancer stem cell-like (CSC) state and exhibit relative quiescence, or even enter dormant states, based on the local signaling milieu (Calabrese et al., 2007; Ghajar et al., 2013; Malladi et al., 2016). Conversely, cells in niches established by growing vessel tips are exposed to regionally high levels of growth factors, such as TGFβ, and proliferate rapidly (Ghajar et al., 2013). Similarly, vascular integrity is also important. Compromise of the endothelial wall leads to a local influx of platelets and serum proteins into the microenvironment similar to a wound environment. These events are not benign. Platelets release TGFβ and other soluble factors, and serum proteins, such as albumin, contain functional domains that can bind membrane receptors and initiate signal transduction (Laursen et al., 1990; Ivens et al., 2007; Labelle et al., 2011). Nutrient abundance and oxygen tension are also impaired in these regions. The degree of metabolic state change in tumor cells, necrosis, and immune cell infiltration are enhanced by lack of functional vasculature (Helmlinger et al., 1997; Gatenby and Gillies, 2004; Carmona-Fontaine et al., 2017). Thus, signals arising from the vasculature (i.e., perivascular vs. growing tip), or its state of function, are major determinants tumor cell phenotype on a niche-specific basis.

Immune Cells

Immune lineages play important roles in the positive and negative regulation of tumor growth (Wels et al., 2008; Gajewski et al., 2013; Coffelt et al., 2016). Histopathologic characterization of tumors reveal that these cells are functionally and spatially heterogeneous throughout the TME, with the relative abundance of specific immune cell types, or functional sub-states, carrying significant prognostic value (Gooden et al., 2011; Heindl et al., 2015; Natrajan et al., 2016; Tashireva et al., 2017). Due to the complexity of tumoral immunity we will only touch briefly on a few aspects of this important TME component. However, the importance of the immune system in the balance of tumor growth vs. clearance cannot be underscored enough, as is evidenced by the recent surge in immuno-oncology-based therapeutics.

Like CAFs immune cells can take on tumor-associated states. For example, macrophages and neutrophils are reprogrammed to tumor-associated TAM and TAN states, respectively (Fridlender et al., 2009; Egners et al., 2016). Also, similar to CAFs, TAM, and TAN populations can be divided into functional sub-types, referred to as polarization states, which confer either pro- or anti-tumor behaviors to these cells. In the case of TAMs, signals from the TME can polarize their function either toward anti-tumor M1, or tumor promoting M2 states (Chanmee et al., 2014). Neutrophil polarization follows the same respective pattern toward N1 or N2 polarization (Fridlender et al., 2009). Importantly, these states coexist in the TME, with the relative ratios driven by regional signaling inputs.

One TME that favors the polarization of TAMs and TANs into their tumor promoting states is the hypoxic niche (Egners et al., 2016). In this niche, a host of cytokines and growth factors secreted by necrotic and ischemic tumor cells act as potent chemo-attractants (Murdoch and Lewis, 2005). Once recruited to these regions, direct sensing of local oxygen gradients and tumor cell-derived metabolites polarizes TAMs toward the M2 state (Carmona-Fontaine et al., 2017). TGFβ produced by tumor cells perpetuates this transition and also drives the polarization of TANs toward the N2 state (Fridlender et al., 2009). As a result, M2 and N2 cells become coordinate regulators of the local niche structure through production of pro-angiogenic factors, such as VEGF, and secretion of ECM remodeling enzymes. Concurrently, M2 cells release other growth factors, including EGF and TGFβ, that promote tumor proliferation and the migration of tumor cells away from the hypoxic niche. These events generate a microenvironment that promotes tumor expansion and supports metastatic dissemination (Wyckoff et al., 2004; Condeelis and Pollard, 2006; Bonde et al., 2012; Carmona-Fontaine et al., 2017). Therefore, it is not surprising that large necrotic regions and marked infiltration by these cells are clinically correlated with poor prognosis (Vaupel et al., 2001).

T-cells present yet another source of cellular and functional heterogeneity in the TME. Signals released from tumor cells and other immune cells act as potent chemo-attractants for T-cells. However, physical and chemical barriers in the TME impair T-cell localization or function on a region-to-region basis. This is particularly true in the hypoxic niche where signals released from necrotic tumor cells and infiltrating TAMs recruit cytotoxic T-lymphocytes (CTL) to this region (Haddad and Saldanha-Araujo, 2014). However, local derangements in vascular structure often impair infiltration of these tumor-killing cells (Nagy et al., 2009). CTLs that are able to reach this location are inhibited by high levels of TGFβ released from TAMs and tumor cells, and low

oxygen tension (Kim et al., 2008, p. 301; Anderson et al., 2017). This leads to impaired cell killing function, shifting the balance in favor of tumor growth within the hypoxic niche. In other regions of the tumor, T-cells are interspersed throughout the parenchyma where the relative ratio of their subtypes determines the net effect on tumor growth. This is particularly true with respect to ratios of CTLs to T regulatory (T_{reg}) cells. Like TAMs, T_{reg} cells produce significant amounts of TGFβ, creating a chemical barrier that inhibits CTL proliferation and cell-killing (Anderson et al., 2017). As a result of these actions, increased T_{reg} levels are linked with tumor growth and overall poor prognosis in the clinical population (Gooden et al., 2011).

These vignettes highlight only a limited number of aspects of immune function within tumor tissues, which continue to be investigated intensively. Nonetheless, they begin to establish a mechanism by which different immune components, and variation in their functional states and localization within the TME, carry the potential to modulate tumor cell behavior on a region-to region-basis.

Extracellular Matrix

The ECM provides the structural as well as the signaling foundation regulating normal tissue function. The concept that ECM signals via specific receptors to the nucleus, first proposed by Bissell et al. (1982) has now been demonstrated broadly (Lu et al., 2012). Disruption of ECM composition contributes to the generation of dysfunctional niches, leading to altered cell polarity and ultimately tissue morphology, which are the foundation of dysplastic and neoplastic transitions. We will briefly touch upon a few salient features of the ECM; however, for extensive review we defer to others (Bissell and Hines, 2011; Lu et al., 2012).

Extracellular matrix structure and composition are regulated by multiple cell types in the stroma and affect numerous aspects of tumor cell behavior. Stromal cells, such as fibroblasts, modify the ECM through via production of enzymes which degrade collagens and laminins. Degradation of the ECM releases matrix bound growth factors (e.g., TGFβ, VEGF) and ECM degradation products that stimulate the infiltration of immune cells, promote angiogenesis, and act directly on tumor cells (Bhowmick et al., 2004b). Concurrently, stromal cells produce new fibronectin and laminin forms altering the microenvironment. As such, changes in ECM structure and composition vary with the regional composition of stromal cells within the TME. For example, CAFs, which are enriched at the tumor interface zone, copiously secrete pro-invasive forms of laminin, leading to enrichment of laminin-332 and high levels of TGFβ in this region of the tumor (Kim et al., 2011). Similarly, the interface zone is also enriched with collagen I that is arranged in linear patterns which act as tracks for tumor cells to migrate along (Provenzano et al., 2006; Egeblad et al., 2010). Changes in ECM stiffness and composition are sensed and integrated by tumor cells through integrin-mediated signal transduction pathways that modulate cell polarity and invasive properties (Lühr et al., 2012; Acerbi et al., 2015). Importantly, sensing of ECM stiffness also modulates cellular response characteristics to growth factors, regulating the ability of tumor cells to respond to signaling cues, such as TGFβ, and adopt pro-invasive EMT states (Leight et al., 2012). Thus, extracellular cues arising from matrix composition and stiffness, coupled with its role in direct signaling and signaling modulation (e.g., growth factor storage), make the ECM multifaceted in its ability to control tumor cell fate.

Emerging Signaling Mechanisms

Extracellular vesicles have recently gained attention in the cancer field for their roles in intercellular communication; for extensive review see Raposo and Stoorvogel (2013). They are composed of several classes based on size. Microvesicles range in size from 100 to 1,000 nm and are formed by outward budding from the cell membrane (Cocucci et al., 2009). Exosomes range in size from ∼30 to 100 nm, are formed via the endocytic system, and are thought to be released by fusion of the multivesicular body with the cell membrane (Ostrowski et al., 2010). Membranous vesicles such as apoptotic bodies also fall under the umbrella of extracellular vesicles and have important biological function; however, we will not discuss them here. Unlike soluble growth factors, extracellular vesicles convey multiple molecular signals in a single packet. They can transport an array of cargo, such as DNA, mRNA, miRNA, and proteins, including functional receptors and multiple growth factors (Thakur et al., 2014; Hoshino et al., 2015; Becker et al., 2016). Importantly, their cargo is highly dependent upon cell type and state. Thus, extracellular vesicle cargos transfer quantitative phenotypic information about host cell state that modify the phenotype of adjacent cells, and importantly, cells in distant microenvironments throughout the body (Peinado et al., 2012; Lázaro-Ibáñez et al., 2017).

The recognized contributions of extracellular vesicles in cancer are growing rapidly. Currently, much of our knowledge comes from studies exploring the role of tumor-derived exosomes on the stroma. Nonetheless these studies provide compelling evidence for the capacity of extracellular vesicles to induce plasticity in their target cells. In the local TME, tumor-derived exosomes and microvesicles activate fibroblasts, myeloid lineages, modulate blood vessel growth and leakiness, and alter ECM structure and composition (Becker et al., 2016). At distant sites such as the lung and liver, tumor-derived exosomes are taken up by resident cells, which leads to phenotypic reprograming and the establishment of premetastatic niches (Peinado et al., 2012; Costa-Silva et al., 2015; Hoshino et al., 2015). Reprogramming of cell state often results in focally altered patterns of ECM deposition, such as increases of fibronectin expression, and regional patterns of immune cell invasion to these areas (Costa-Silva et al., 2015; Hoshino et al., 2015). As a result, circulating tumor cells more readily establish residence at these sites and are able to develop into large metastatic colonies over time.

The role of stromal-derived extracellular vesicles in regulating tumor cell phenotype is less developed; however, the existing data are striking. Current evidence suggests that extracellular vesicles are secreted by all cell types in the TME. CAF-derived exosomes have been shown to promote the sustained growth of tumor cells through transfer of metabolic intermediates, promote breast cancer cell invasion through activation of the cell migration pathways, and modulate therapeutic resistance (Boelens et al., 2014; Zhao et al., 2016; Donnarumma et al., 2017). Exosomes from other stromal sources, such as TAMs,

have similarly been shown to modulate the invasive potential of tumor cells (Yang et al., 2011), while endothelial cell exosomes induce vasculogenesis and modulate therapeutic response (van Balkom et al., 2013; Bovy et al., 2015). Finally, microvesicles released from the stroma have been shown to have similar effects compared to exosomes, modulating tumor dormancy vs. growth states and sensitivity to chemotherapeutic agents (Boelens et al., 2014; Sansone et al., 2017a,b). Thus, similar to the effect of soluble signals arising from the stroma, extracellular vesicles appear capable of inducing a wide variety of tumor cell phenotypes.

Microenvironmental Contributions to Therapeutic Resistance

Several drug tolerant phenotypes have been described in the literature, such as EMT and CSC-like states (Shibue and Weinberg, 2017). Local signals from the TME influence generation of these cell states in a region-specific manner. For example, TGFβ, IL-6, exosomes, and many other CAF-secreted cytokines and growth factors have been shown to drive mesenchymal transitions (Yamada et al., 2013; Boelens et al., 2014; Yu et al., 2014). This results in drug-resistant EMT phenotypes that are enriched in sites like the invasive tumor front where CAFs are densely localized (Nakayama et al., 1998; Puram et al., 2017). Similarly, the hypoxic niche creates a signaling milieu conducive to drug tolerant EMT states (Yang et al., 2008). Finally, regions like the perivascular niche are often enriched with CSCs, which are thought to favor a slow growing phenotype and chemo-resistance (Calabrese et al., 2007; Abdullah and Chow, 2013).

Targeted therapies have attempted to improve the efficacy of treatment by inhibiting specific pathways utilized by, or overexpressed, in cancer. For example, the receptor tyrosine kinase (RTK), epidermal growth factor receptor (EGFR), is frequently mutated in lung cancer, activating the Ras/ERK pathway and driving cell proliferation (Paez et al., 2004). Similarly, another RTK, vascular endothelial growth factor receptor (VEGFR), regulates vessel growth and tumor angiogenesis (Leung et al., 1989). In both cases targeted therapies exist to block the microenvironmental cues that stimulate these pathways (e.g., EGF and VEGF) and their proliferative and pro-angiogenic effects, respectively. However, resistance to these RTK targeted therapies, and even agents that act downstream of these receptors, is frequently observed. In part, this occurs secondary to the complexity of signals arising from stromal components (Junttila and de Sauvage, 2013). CAFs, TAMs, and other cell types in the stroma secrete multiple growth factors, such as EGF, HGF, and PDGF, which activate RTKs through a common Ras/ERK signaling pathway. As such, blockade of EGFR or VEGFR can be overcome by redundant activation of Ras/ERK signaling through other RTKs given the right microenvironmental signaling niche (Straussman et al., 2012; Wilson et al., 2012).

Changes in ECM composition and cell polarity sensed by integrins also drive chemotherapeutic resistance. β4-integrin mediated polarity has been shown to mediate therapy resistance through NF-κB signaling (Weaver et al., 2002). In a similar manner, several studies have shown the fibronectin and other ECM components are capable of inducing resistance through modulation of signaling pathways, such as PI3K/Akt (Pontiggia et al., 2012; Cho et al., 2016). As we have discussed, the ECM is dynamic and variable in composition throughout the TME, implying that resistance-inducing capabilities of the ECM may be variable on a cell-to-cell or regional level.

These brief highlights emphasize the regional variation in stromal composition and signals (cell extrinsic factors) that compose the TME. Returning to our hypothetical daughter cells, we can envision how the two cells, while remaining in relatively close proximity, could sample a variety of extracellular cues as a result of the dynamic and region-specific variability of the TME (**Figure 1**, Stage 1). In the following section, we will move on to explore how signals are integrated by individual tumor cells, and discuss how intrinsic variation could synergize with variation in signaling from the microenvironment to enhance phenotypic heterogeneity and modulate therapy response.

DYNAMICS AND DIVERSIFICATION IN SIGNAL TRANSDUCTION

Signal transduction pathways connect extracellular cues to the regulation of gene expression. As our two hypothetical cells receive distinct cues from the components of their TME niche, different intracellular pathways will be stimulated in each one (**Figure 1**, Stage 2), ultimately leading to different gene expression programs (**Figure 1**, Stage 3). In principle, this process of stimulated gene expression is profoundly determinative for cell phenotype; it is the orchestrated differential expression of genes that creates the broad diversity of cell types in the adult body. However, at the single-cell level there is inherent variability in signaling and gene expression response to the same signaling cue, blurring the lines between signal input and predictable gene expression output.

Inherent Variability in Signaling

Live-cell microscopy with genetically encoded biosensors has revealed that many biochemical events fluctuate continuously within individual cells, with diverse time scales and frequency patterns (Locke and Elowitz, 2009; Levine et al., 2013). Early work in bacteria and yeast explored the physical basis for this variability, and concluded that one potential source is the stochastic nature of biochemical reactions occurring on a very small scale (Elowitz et al., 2002; Volfson et al., 2006). Another source is the heritable propagation of cellular states or properties induced by transient exposure to stimuli unique to each cell. If a subpopulation of cells exposed transiently to a stimulus induces expression of a long-lived gene product, that elevated expression level can persist long after the stimulus is removed, and even through cell division events (Kaufmann et al., 2007). The elevated expression of that gene can then affect the reception and processing of subsequent signals. For example, exposure to a cytokine such as interferon-γ can elevate levels of TNF-receptor, making the cell responsive to TNFα for days after the interferon-γ is removed (Tsujimoto et al., 1986). Studies of cell-to-cell

variation have typically categorized these sources as "intrinsic" or "extrinsic," respectively (Swain et al., 2002).

More recently, studies have investigated the reliability of signaling pathways by quantifying the degree to which information about the concentration of an extracellular stimulus is preserved in the activation of downstream effectors (Cheong et al., 2011). Surprisingly, most signaling pathways have a measured channel capacity of only 1–2 bits, meaning they can respond differentially to, at most, 2–4 different concentrations of the extracellular stimulus (Uda et al., 2013; Selimkhanov et al., 2014). More work remains to be done to refine these measurements, but the emerging view is that the intracellular response to a given signal is far from an absolute and precise measurement of the extracellular cue; instead, it is contingent on the pre-existing state of the cell, the biochemical limits of the pathway (for example, saturation of a particular step in the pathway), and thermodynamically stochastic events.

Variation Over Time: Dynamic Heterogeneity in Signal Transduction

If we were able to directly monitor multiple signaling pathways within our two hypothetical cells, we would likely observe continual fluctuations and pulses of activity as they respond to both static and evolving cues in their microenvironment. Cancer-relevant signaling pathways, such as p53, Ras/ERK, and NF-kB, respond to constant stimuli in the form of discrete pulses that ultimately influence gene expression and ultimately cell phenotype; however, the characteristics of these responses vary on the cell-to-cell level.

Lahav and colleagues demonstrated that the transcription factor p53 is activated in discrete hour-long pulses following a DNA-damaging event (Lahav et al., 2004). Further investigation revealed significant heterogeneity in single-cell p53 responses to fixed concentrations of cisplatin. Under these conditions, cells that accumulated p53 at a rapid rate (1–2 days) underwent apoptosis, whereas cells that accumulated the same peak levels of p53 over several days survived (Paek et al., 2016). Investigation of the Ras signaling pathway has revealed a similar regulatory behavior, in which activation of the proliferative kinase ERK downstream of Ras occurs in discrete bursts, ranging from 20 min to several hours in length, with the duration and frequency of bursts modulated by growth factor concentration, autocrine signals, and cellular density (Aoki et al., 2013). Tracking of single cell responses showed substantial cell-to-cell variation in ERK signaling dynamics, prompting genetically identical sister cells to make different decisions to enter S-phase (Albeck et al., 2013). *In vivo* monitoring of ERK activity reveals similar patterns of pulsatile signaling, indicating that signaling operates in a similar manner under physiological conditions (Hirata et al., 2015). These surprising patterns of activity reveal a new level of complexity in the response to signaling cues, supporting the concept that time-dependent cell-to-cell variation in signaling dynamics contributes to the generation of heterogeneous phenotypes.

Divergence in Gene Expression

The functional output of many signaling pathways involved in cancer is the regulated expression of a defined subset of genes. Accordingly, recent studies have focused on correlating the dynamic activity in a signal transduction pathway to the resulting downstream changes in gene expression level (Tay et al., 2010; Lee et al., 2014; Porter et al., 2016; Wilson et al., 2017). At the level of transcription itself, tracking of mRNA abundance reveals that transcription of many genes occurs in the form of "bursts," in which multiple copies of mRNA are produced, and which are interspersed by dormant periods where no transcripts are made (Suter et al., 2011). The upsurge in mRNA in response to an upstream signal can come from either an increase in the length or frequency of bursts, or an increase in the rate of transcript production during the bursts; interestingly, both scenarios can be observed for the same gene in response to different stimuli (Molina et al., 2013). While a simple model might suggest that the pulses in upstream signals (activity of p53, ERK, NF-kb) correspond to bursts in mRNA production, this does not appear to be strictly the case; for instance, no transcriptional bursting was observed in ERK target genes even when bursts of upstream ERK activity are enforced using optogenetic stimulation (Wilson et al., 2017). Within the context of an ordered tissue, probabilistic gene expression can enhance the dynamic range of regulated gene expression, because the response of multiple neighboring cells can be averaged (Garcia et al., 2013). Theoretical studies also support the idea that cell-to-cell variability can enhance the reliability of signaling at the tissue level (Suderman et al., 2017).

Another potential driver of divergent gene expression between individual cells is the ability of time-dependent processes of mRNA and protein translation to discriminate transient input signals from chronic ones. This effect may be particularly important in Ras/ERK-stimulated gene expression, where ERK is known to control the expression of many of its target genes, such as Fra-1—a transcription factor controlling metastatic behavior—through a "feedforward" regulatory circuit that modulates multiple steps in the expression process (Murphy et al., 2004). For example, a sufficiently long burst of ERK activity may be capable of both stimulating mRNA production (by phosphorylating Elk-1 and other transcription factors) and stabilizing the Fra-1 protein product through phosphorylation once it has eventually been translated. Conversely, a shorter pulse of ERK activity long enough to stimulate the mRNA production step but terminating before translation has been completed would fail to produce phosphorylated (and stable) Fra-1 protein (Murphy et al., 2002). Given that Ras/ERK signaling is often highly dynamic (as discussed above), such temporal filtering may be important in determining the particular gene expression program resulting from a cue that stimulates the Ras pathway. It has also been proposed that expression of dual specificity protein phosphatases (DUSPs), ERK target genes that feed back to dephosphorylate nuclear ERK, may act to bias gene expression toward transient rather than constant activity (Wilson et al., 2017). Moreover, recent studies suggest that there may be yet more diversification in the expression process. Surveys of the mRNAs produced by ERK or p53 activation reveal a diversity of

parameter values, such as mRNA half-life, that result in different temporal responsiveness among targets of the same gene (Porter et al., 2016; Uhlitz et al., 2017). Together, these temporally-modulated sources of diversification may effectively allow the same pathway to induce very different gene expression profiles, depending on the duration of pathway activation. Accordingly, predicting single-cell gene responses to stimuli or inhibitors will require mapping each gene's input-output relationship at the single-cell level. However, such data are now in reach, using genome engineering to insert fluorescent reporters at endogenous gene loci and to track expression levels over time in response to defined signaling events (Gillies et al., 2017; Wilson et al., 2017).

Altogether, single-cell studies of signal transduction-mediated gene regulation have revealed many layers of output diversification in response to stimuli. Practically, for our two hypothetical cells, these sources of variation could allow them to exhibit different intracellular responses even in the absence of substantial variation in their microenvironment. This provides yet another possible mechanism for the generation of intratumoral heterogeneity, and more importantly, a mechanism by which cells arising from clonal populations can diverge in their sensitivities to chemotherapeutic agents, as we discuss below.

FROM GENE EXPRESSION TO DRUG RESISTANCE

After the signaling processes described thus far, our two hypothetical cells are likely to be quite different in their gene expression profile, even though their genomes remain identical in sequence. These expression differences may result in divergence in their threshold for drug tolerance, such that upon exposure to a similar concentration of a cytotoxic drug, one will cell survive while the other succumbs to the treatment (**Figure 1**, Stage 4). Similarly, the cells may have different capacities for surviving stressful situations that arise physiologically, such as hypoxia within the TME. In this section, we consider how the gene expression changes that have accumulated may result in these divergent survival responses.

Drug Resistance as a Function of Gene Expression

One major factor determining drug resistance is the expression of members the ABC transporter family, which include the multi-drug resistance (MDR) genes. These transporters are capable of exporting various compounds from the cytoplasm, including chemotherapeutic drugs, and their expression thus increases cellular tolerance of cytotoxic therapies. Expression of the ABC transporters is known to be regulated by Wnt signaling, multiple microRNAs, the transcription factors Nrf2 and Runx3, and the histone methyltransferase EZH2 (Chen et al., 2016), making it possible for microenvironmental signals to modulate transporter levels. There are at least three ABC transporter genes involved in cancer drug resistance—P-glyocoprotein/MDR1 (ABCB1), MRP2 (ABCC2), and BCRP (ABCG2)—which have distinct, but overlapping, spectra of substrates. Thus, the threshold of drug tolerance mediated by MDR expression can be expected to vary as a function of signals received from the microenvironment, but is difficult to predict for any individual cell due to its multi-factorial nature.

Also important in determining cellular drug sensitivity are regulators of apoptosis, including the Bcl-2 family of proteins, because many chemotherapeutic agents induce cell death through apoptosis. There are at least 17 genes in the Bcl-2 family in humans, with both pro- and anti-apoptotic roles, and the overall threshold for the induction of apoptosis is set by the aggregate levels of these opposing proteins (Certo et al., 2006). The expression of many Bcl-2 family members is under control of signaling pathways that lie downstream of TME signals (Holohan et al., 2013). Similarly, expression levels of the many components of the DNA damage repair machinery can determine the cellular tolerance for DNA-damaging therapies (Bouwman and Jonkers, 2012). Expression profiles can also affect drug sensitivity indirectly; for example, expression levels of cyclins and CDK inhibitors modulate the rate of cell cycle progression, which can in turn determine the sensitivity to chemotherapeutics, such as microtubule stabilizers, that target cells at specific stages of the cell cycle. Thus, the overall ability to tolerate drug exposure is determined by the composite expression levels of dozens of proteins, some of which have specificity for certain drugs or drug classes, and others which control cell death responses more generally. Drug resistance therefore behaves as a complex trait of individual cells.

Connecting Gene Expression Profiles to Cell States

With ∼20,000 genes in the human genome, there are an extremely large number of possible expression profiles for each cell, even if it is assumed that each gene has only two expression states ("on" or "off") and that many genes are coordinated in their expression status. Of course, many of these states are in reality either unstable or unreachable due to conflicting regulation, such as the simultaneous expression of two transcription factors that each inhibit the other's transcription (Brock et al., 2009). Conversely, certain states are self-reinforcing due to positive feedback regulation, leading to the concept that there are "attractors"—stable regions within the overall space of gene expression profiles where cells tend to cluster (Huang et al., 2005). Considering our two cells that began with the same expression profile, a key question is how far these two cells may diverge in their overall expression status—potentially crossing from one attractor state to another—and whether this divergence will affect their metastatic and chemoresistance properties.

Functional studies support the relevance of attractor states for tumor cells, and suggest that tumor cells may transition between discrete expression profiles correlating with drug resistance (Ponti et al., 2005; Chiba et al., 2006). Well known examples of such states and transitions include the EMT and CSC states identified within some cancer types, prompting the model of a dynamic equilibrium of cell states underlying tumor heterogeneity (Gupta et al., 2011). However, precisely defining

the expression profiles corresponding to these states remains challenging and will likely require single-cell resolution. For example, if high expression levels of genes A and B together create a drug resistant state, single-cell methods will be needed to detect such cells because population-based methods cannot distinguish whether A and B are co-expressed within the same cells or separately in two different subpopulations of cells. While genome-wide expression profiling for large populations of cells has been possible for more than 15 years, practical methods for single-cell profiling have only recently become widely available and remain limited by the inherent technical challenges in accurately sampling the ∼1 pg of mRNA present in each cell. Moreover, single-cell profiling provides only a static snapshot of the expression profile at the time of the assay and provides little information on dynamic transitions, making it difficult to link to functional properties such as drug resistance. Nonetheless, as they mature, single-cell profiling technologies promise to provide molecular clarity in mapping the cell states accessible to tumor cells. Of particular interest are methodologies capable of tracking dynamic cellular behavior over time and correlating this behavior with the genome-wide expression profile within the same cell (Lane et al., 2017).

A key question is therefore whether the expression profile of tumor cells can be used to accurately predict drug responsiveness. Such tests, based on population-level measurements of mRNA abundance, are clinically available and have prognostic value (van 't Veer et al., 2002; Paik et al., 2004; Drukker et al., 2014; Shah et al., 2017). However, single-cell resolution of gene expression profiles are now feasible and could in principle more accurately predict clinical of interest (Anjanappa et al., 2017), since resistant and metastatic cells may be present as minor subpopulations that are obscured by the bulk of the tumor and any contaminating non-tumor cells. A remaining challenge is to ascertain whether there are discrete cell states identifiable by expression signatures that are broadly predictive of tumor cell characteristics. In this regard, it will be critical to determine whether attractor states corresponding to drug resistance behavior do in fact exist and can be detected by their expression profile. An alternative possibility is that the many layers of variation in gene expression, as discussed above, create a continuous landscape of expression states rather than discrete cell types; this could substantially complicate the analysis of tumor subpopulations.

Maintenance of Drug-Resistant States Over Time

States of drug tolerance may persist for times ranging from hours to weeks. Some drug-resistant states have been attributed to epigenetic mechanisms (Sharma et al., 2010). Typically, the term epigenetics is used to refer to chromatin modifications, including DNA methylation and histone acetylation or phosphorylation that can modulate gene expression patterns and which persist across multiple cell generations (Easwaran et al., 2014). These covalent modifications can play a role in resistance to chemotherapeutics are often highly stable, allowing a particular gene expression profile to persist for weeks or longer. However, they may be reversed by inhibitors of chromatin-modifying enzymes, accelerating the loss of the resistant phenotype (Sharma et al., 2010).

Considered more broadly, the concept of epigenetics includes any heritable cellular trait controlled by factors other than nucleic acid sequence. For example, protein expression levels can vary between genetically identical cells, and these differences can be preserved through cell division, making related cells more likely to contain similar protein expression levels (Sigal et al., 2006). Life-or-death differences in cell fate can result, as fluctuations in the levels of Bcl-2 family and other proteins can determine sensitivity to apoptosis inducers such as TRAIL or chemotherapeutics (Spencer et al., 2009). Because of the relatively short time needed for protein turnover to reshuffle expression levels, such states tend to persist for shorter periods of time, typically from hours to a few days (Flusberg et al., 2013; Flusberg and Sorger, 2015). In such cases, the cells surviving a cytotoxic treatment repopulate the original distribution, enabling a similar fraction of cells to be killed by a second round of the same treatment. Quantifying the time needed for the redistribution of resistance properties can thus be useful for determining the optimal frequency of cytotoxic treatments.

LOOKING FORWARD

We have traced here the flow of information from heterogeneous extracellular signals originating in the microenvironment, through variance-prone signaling networks, to regulate cell fate at the level of gene expression, emphasizing that this process tends to diversify rather than constrict cellular responses to a narrow range (**Figure 1**, Stages 1–4). In the context of a normal tissue, such diversity may be important for maintaining proper tissue function, for example by maintaining subpopulations of cells prepared to respond to a broad range of stimuli or stressors, or by increasing the dynamic range of the mean response (Suderman et al., 2017). In the context of a tumor, where signals are spatially and temporally heterogeneous, the same properties likely contribute to tumor cell resilience by creating diverse subpopulations with selective survival advantages (relative to normal cells) as they disseminate to foreign environments and evade therapy. The key question that now arises is how these advances in understanding the molecular diversity of cancer cells can be translated into more effective therapies (Brock et al., 2009). We consider here how single-cell technologies may impact the diagnosis and the development of new compounds or treatment regimens for cancer.

Correlating Prognosis and Treatment Efficacy With Single-Cell Measurements

Ideally, transient therapy-resistant subpopulations (or subpopulations with the potential to become resistant) could be detected, and appropriate treatment strategies chosen depending on the distribution of single-cell profiles within each patient's tumor. Yet, while the technology for measuring such heterogeneity is now available, in the form of methods for single-cell sequencing of genetic and transcriptional profiles,

significant obstacles remain before this information can be fully deployed in predicting patient responses to therapy.

First, it remains to be determined whether accurate detection of non-genetic heterogeneity and characterization of subpopulations within a tumor is practically feasible within a clinical setting. Single-cell sequencing of genomic DNA from nuclei in frozen tumor sections has established technical feasibility of single-cell isolation and sequencing and has demonstrated that sampling of 50–100 cells was sufficient to capture all of the predominant clones with a high level of confidence (Gao et al., 2016). However, the greater variability in transcriptional profiles may require the sequencing of a substantially larger number of cells to detect rare subpopulations. This number will depend on both the complexity of the population as well as the size and spatial heterogeneity of the tumor. Identifying clear predictive trends in such data also faces a significant statistical barrier, because in increasing the complexity of tumor classification, it becomes difficult to include sufficient numbers of patients to power statistical tests. Finally, it will be necessary to integrate models of variability in the genome and transcriptome to understand clonal evolution of tumors over time. Modeling of the clonal diversity within tumors has revealed a complex interplay by which certain clones play a supportive role for other cells within the tumor through secreted factors, and elimination of these clones can broadly reduce tumor cell viability (Marusyk et al., 2014). However, such changes can also enhance the viability of other clones (Waclaw et al., 2015), making the overall outcome difficult to predict with our current understanding.

Regardless of the specific technologies used, much still depends on better models linking transcriptional profiles to cellular phenotypes. This remains a daunting challenge; even at the bulk tumor level, predicting treatment response from genetic or transcriptomic profiling remains difficult for the majority of cancers (Niepel et al., 2017), despite a small number of high-profile examples in which a driver mutation predicts drug responsiveness (e.g., HER2 amplification in breast cancer or B-Raf mutation in melanoma). Interestingly, measuring signaling responses provides a more effective means than genetic markers for predicting drug sensitivity in cell culture models (Niepel et al., 2013), suggesting that single-cell resolution could improve accuracy by revealing the constituent subclones and their sensitivities. Further advances will require moving from reductionist cell culture systems to more realistic models that incorporate the effects of the microenvironment.

Therapeutic Strategies to Counteract Cellular Heterogeneity

The concepts presented here imply that therapeutic strategies should target not simply the central tendencies and static genetic complement of a tumor, but also the many subpopulations of transient cell states co-existing within the tumor and their potential to change their signaling and transcriptional profile in response to drugs (often termed an adaptive response). Early attempts to address this complexity searched for drugs that selectively target cancer cells with high potential to reinitiate tumors (Gupta et al., 2009). Other studies, recognizing that drugs shift the signaling behavior of tumor cells to induce resistant cellular phenotypes, have identified pathways involved in this adaptation and demonstrated the effectiveness of simultaneous inhibition of these pathways (Tandon et al., 2011; Rexer et al., 2014). These advances notwithstanding, there remains further potential to use information on cellular heterogeneity to improve therapeutic responses.

A clear demonstration of the role of variability in drug sensitivity is the long-standing observation that multiple rounds of chemotherapy are typically more effective than single treatments. If genetic variability alone were the cause of drug resistance, the surviving cells would all be genetically resistant and no benefit would be achieved from additional rounds. By understanding the mechanisms and kinetics of transitions between sensitive and resistant states, the timing of drug treatments can be better tailored to maximize the number of cells responding (Flusberg and Sorger, 2013). Alternatively, therapeutic approaches that reduce the intracellular heterogeneity of gene expression prior to treatment with a cytotoxic drug could improve the efficacy of tumor cell killing. A number of compounds that alter chromatin modification, such as bromodomain inhibitors, may be useful in this regard by preventing cells from entering resistant transcriptional states (Sharma et al., 2010). Conversely, antibodies blocking the effects of extracellular components may be used to limit the impact of the microenvironment in generating intratumoral heterogeneity. While the first generation of such molecules, such as inhibitors of VEGF or matrix metalloproteases have limited efficacy, the potential remains for multi-pronged interventions to normalize the TME. One recent study has provided an exciting example of how crosstalk between tumor cells and CAFs orchestrates the divergence of basal and ER-positive subtypes of breast cancer and can be interrupted by inhibitors to revert tumor cells to a more easily treated subtype (Roswall et al., 2018). In addition, immune system-based approaches promise to provide a new arsenal of tools that could be less dependent on the native microenvironmental heterogeneity.

Further Advances in Understanding the Fundamental Mechanisms of Variation

A limitation of many of the studies discussed here is that they each primarily investigate a single pathway, and provide little insight into how signaling networks function under physiological conditions when multiple pathways converge. Significant technical limitations remain for understanding how multiple microenvironmental signals are integrated by individual tumor cells, in a dynamic way. At the imaging level, the maximum number of pathways that can be simultaneously interrogated in a live single-cell remains ∼4 (Regot et al., 2014). At the analysis level, methods for signal engineering have yet to be adapted for use on the relatively sparse and noisy data from live-cell experiments. Single-cell mRNA sequencing has provided a wealth of data on where cells cluster within the many-dimensional space of possible gene expression patterns. Application of this technology has already vastly expanded our

knowledge of the phenotypic and stroma cell states present in the TME. However, these data are snapshots and do not reveal how frequently transitions are made between cell states. Nonetheless, further studies using high-content imaging and single cell genomic approaches will allow interrogation of individual cells within a population to understand how signaling dynamics are integrated and determine gene expression programs. Combining these modalities with physiologically relevant 3-dimensional, and multicellular, culture models will allow us to measure tumor–stroma signal cross-talk with new precision. Importantly, further development of computational methods and models will be essential to interpret these complex experiments. Combining these approaches will more accurately determine the relative contributions of extrinsic and intrinsic factors to cell fate determination. In doing so we will gain valuable insight into how these factors contribute the plasticity of tumors and ultimately how to control them for therapeutic benefit.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Hyaluronan, Cancer-Associated Fibroblasts and the Tumor Microenvironment in Malignant Progression

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This review summarizes the roles of CAFs in forming a "cancerized" fibrotic stroma favorable to tumor initiation and dissemination, in particular highlighting the functions of the extracellular matrix component hyaluronan (HA) in these processes. The structural complexity of the tumor and its host microenvironment is now well appreciated to be an important contributing factor to malignant progression and resistance-to-therapy. There are multiple components of this complexity, which include an extensive remodeling of the extracellular matrix (ECM) and associated biomechanical changes in tumor stroma. Tumor stroma is often fibrotic and rich in fibrillar type I collagen and hyaluronan (HA). Cancer-associated fibroblasts (CAFs) are a major source of this fibrotic ECM. CAFs organize collagen fibrils and these biomechanical alterations provide highways for invading carcinoma cells either under the guidance of CAFs or following their epithelial to mesenchymal transition (EMT). The increased HA metabolism of a tumor microenvironment instructs carcinoma initiation and dissemination by performing multiple functions. The key effects of HA reviewed here are its role in activating CAFs in pre-malignant and malignant stroma, and facilitating invasion by promoting motility of both CAFs and tumor cells, thus facilitating their invasion. Circulating CAFs (cCAFs) also form heterotypic clusters with circulating tumor cells (CTC), which are considered to be pre-cursors of metastatic colonies. cCAFs are likely required for extravasation of tumors cells and to form a metastatic niche suitable for new tumor colony growth. Therapeutic interventions designed to target both HA and CAFs in order to limit tumor spread and increase response to current therapies are discussed.

Keywords: hyaluronan, cancer-associated fibroblasts, migration, tumor microenvironment, tumor initiation, circulating cancer-associated fibroblasts, metastasis

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INTRODUCTION

Historically, cancers have been studied as diseases whose initiation and progression are caused by the mutation of key oncogenic "driver" genes, loss of suppressor genes and increasing mutational load resulting in genomic instability, immortalization, unrestrained growth and acquisition of colonizing potential (Hanahan and Weinberg, 2011; Garraway and Lander, 2013; Tomasetti et al., 2013; Vogelstein et al., 2013). More recent studies predict this concept of cancer initiation and

progression is incomplete. Most genetic changes that are hallmarks of epithelial cancer are already present in pre-malignant lesions that rarely progress to frank cancer. For example, ultra-deep sequencing of 74 cancer genes in small biopsies of normal aged and sun-exposed human skin reveal a high mutation burden in most key drivers of cutaneous squamous cell carcinoma (Martincorena et al., 2015). These were estimated to be present in over a quarter of the keratinocytes in an epidermis that maintained its normal tissue architecture and physiological functions. A similar paradigm has been observed in other tissues. Endometriosis is a benign inflammatory lesion that is cancer-like in its local invasion and resistance to apoptosis but rarely transforms. Exome sequencing shows that over a quarter of these benign lesions harbor oncogenic driver gene mutations confined to the epithelial compartment that do not result in tumors (Anglesio et al., 2017). These clinical findings are remarkably consistent with experimental studies showing that the tumor phenotype is plastic. Tumor cells can be reverted into a normal growth state while retaining a highly mutated genome by blocking signaling pathways commonly activated by tumor microenvironment (Illmensee and Mintz, 1976; Hall et al., 1995; Wang et al., 2002; Kenny and Bissell, 2003; Postovit et al., 2008; Bizzarri et al., 2011; Northey et al., 2017).

Clues as to the factors required for a mutant genome to either manifest as a transformed phenotype or be restrained into apparent normalcy were initially provided by pioneering studies. The classic studies of B. Mintz brought initial attention to the plasticity of the mutant tumor phenotype and the key role of microenvironments in maintaining transformation (Illmensee and Mintz, 1976). Teratocarcinoma cells, injected into blastocysts, unexpectedly participated in normal tissue development rather than forming tumors. In another key report, chick embryos injected with an oncogenic virus only developed tumors at wound sites even though the viral genome was expressed in unwounded tissues (Dolberg et al., 1985). These original results predicted that while oncogenic insults (e.g., mutations, oncogenic viruses) are a first step toward initiation of cancer, the status of host microenvironment is critical and rate-limiting for disease initiation and progression. These predictions have fueled a synergistic interest in characterizing the properties of "cancerized" host tissue that collaborate with mutant epithelial cells to produce tumors, and drive progression and metastasis, as well as targeting these properties with novel therapeutics designed to manage this aspect of the disease (Radisky et al., 2007; Karn et al., 2015; Werb and Lu, 2015; Luo et al., 2016; Turley et al., 2016; Bridelance et al., 2017; Ghosh et al., 2017; Hutchenreuther and Leask, 2017; Zhan et al., 2017).

Host stroma is a complex mixture of phenotypically heterogeneous endothelial cells, pericytes, immune cells and fibroblasts. Normally, each of these cell types are required for tissue homeostasis, and contribute to the maintenance of tissue architecture and physiologically appropriate tissue functions. The collective paracrine signaling networks that sustain these functions have highly effective tumor-suppressor activity. Gene expression analyses have shown that the stroma surrounding tumors is altered from normal stroma, has lost its tumor suppressing activity and participates in rather than limits tumor initiation, growth and spread (Campisi, 1998; Dumont and Arteaga, 2002; Barsky and Karlin, 2006; Coppé et al., 2010; Bissell and Hines, 2011; Hinds and Pietruska, 2017). Expression differences in normal vs. cancer stroma have been mined to identify signatures that add independent prognostic information to classical epithelial biomarkers (Berdiel-Acer et al., 2014; Bedognetti et al., 2015; Nannini et al., 2015; Winslow et al., 2015; Colangelo et al., 2017; Petitprez et al., 2017). These unbiased analyses together with experimental evidence predict the critical importance of neovascularization, inflammation, immune tolerance and fibroblast activation in creating a "cancerized" microenvironment. In this review, we focus upon the roles of carcinoma-associated fibroblasts (CAFs), also known as tumor-associated fibroblasts (TAFs), in creating a remodeling extracellular matrix that drives tumor initiation and mediates tumor cell spread. We concentrate on the tissue polysaccharide, hyaluronan (HA), as a key contributing ECM component in stromal fibrosis and tumor progression. We conclude by reviewing current experimental interventions targeting both stroma ECM and/or CAF functions that may ultimately limit tumor spread and improve current therapies.

STROMAL EXTRACELLULAR MATRIX IN CARCINOMA INITIATION AND PROGRESSION

It is now well-accepted that carcinomas behave like wounds, which force the host tumor microenvironment into a constant state of fibrotic repair (Dvorak, 1986). As with wound repair, carcinoma-associated stromal tissues undergo dynamic changes in cellular composition and extensive remodeling of extracellular matrix (ECM) as they progress. A particular feature of stromal ECM in cancers particularly pancreatic, prostate, lung and esophageal is its highly fibrotic structure that significantly impacts on progression, metastasis and response-to-therapy (Keely, 2011; Tung et al., 2015; Werb and Lu, 2015; Jiang et al., 2017). Although less well-studied, evidence suggests that chronic inflammation and pro-fibrotic changes in host stroma precede and instruct primary tumor initiation or formation of metastatic colonies by creating a microenvironment or niche favorable for transformation and growth. As examples, in healthy individuals with BRCA1 mutations that are at risk for breast cancer, stromal fibroblasts exhibit a CAF-like activation state (Etzold et al., 2016). Similarly healthy individuals with Li Fraumeni syndrome who bear germ line mutations in TP53 and are at an elevated risk of cancer exhibit "cancerization" of their stromal tissues (Pantziarka, 2015). In a mouse model of colon tumor initiation, both a chronically inflamed and fibrotic stroma are an essential pre-requisite for tumor initiation (Sasaki et al., 2014; Tanabe et al., 2016). There has therefore been an intense effort to understand the dynamic changes in stromal ECM composition to identify the changes that impact on cancer progression, metastasis and resistance to therapies.

BIOMECHANICAL PROPERTIES OF TUMOR-ASSOCIATED STROMA

A major ECM component of all fibrotic stroma is type I collagen, which provides structural and biochemical cues to cells within the stroma (Keely, 2011; Tung et al., 2015; Werb and Lu, 2015; Jiang et al., 2017) (**Figure 1**). A notable property of "cancerized" stroma is the accumulation of type I collagen fibrils in the stroma that are extensively crosslinked by lysyl oxidase (LOX) and tissue transglutaminase (TG2) (Perryman and Erler, 2014; Lee et al., 2016). Collagen crosslinking confers proteolytic resistance to the fibrils and increases stroma stiffness, which promotes tumor cell migration, invasion and proliferation. Tumor-associated collagen signatures categorized by increased collagen density and orientation of mature collagen fibers parallel to or perpendicular to the tumor boundary offer prognostic information (Mellone et al., 2016). Orientation of the fibrils is the result of a process of prolonged mechano-signaling mediated by integrin/cytoskeletal linkages, activation of downstream adhesion pathway signaling components particularly focal adhesion kinase (FAK), phosphorylation of myosin light chain kinase and activation of Rho-Kinase (Schedin and Keely, 2011; Boyle and Samuel, 2016). Oriented collagen fibrils are an ominous biomarker of tumor cell invasion, metastasis and poor outcome (Schedin and Keely, 2011; Tung et al., 2015). In experimental models, non-transformed epithelial cell adhesion to stiff collagen matrices results in elevated activation of oncogenic pathways and increased expression of growth-promoting genes, emphasizing that the mechanical property of stiffness contributes to carcinoma progression (Paszek et al., 2005; Provenzano and Keely, 2011; Ray et al., 2017). Carcinoma cells cultured on stiff collagen gels grow as colonies with discrete boundaries, whereas the same cells cultured in oriented collagen gels of equal stiffness invade along these collagen fibers (Provenzano and Keely, 2011). These *in vitro* observations have been confirmed *in vivo* using multiphoton laser scanning microscopy and second harmonic generation imaging of live *ex-vivo* tumors (Provenzano and Keely, 2011). While fibrillar collagen is a major component of fibrotic stroma, many additional prognostic ECM factors impact the biological and biomechanical properties of tumor-associated stroma. One of these is HA, whose elevated accumulation in the tumor microenvironment contributes to cancer initiation, progression and therapy resistance (Karousou et al., 2014; Chanmee et al., 2016; Sato et al., 2016; Turley et al., 2016; Binder et al., 2017; Bourguignon et al., 2017; Safdar et al., 2017). These properties as they relate to tumor initiation and dissemination are discussed in the following sections below.

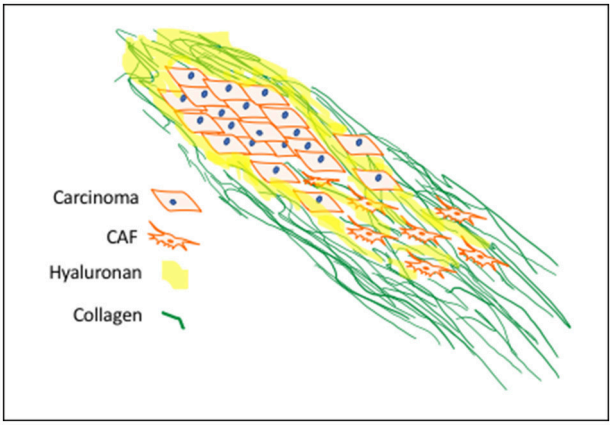

FIGURE 1 | Progression-Associated Fibrosis in Cancerized Stroma: Deregulated synthesis and deposition of ECM components, including HA and type I collagen, leads to tumor-associated fibrosis. HA, a major polysaccharide of provisional wound matrices, contributes to cancer initiation, progression and resistance-to-therapy. CAF activation sustains increased collagen synthesis, structurally oriented by fibroblast contractile forces. These provide structural and biochemical cues to enhance mechano-signaling for carcinoma motility and invasion.

CANCER-ASSOCIATED FIBROBLASTS

Cancer-associated fibroblasts (CAF) are the primary cell type in "cancerized" stroma and are a major source of ECM as well as cytokines/growth factors that impact upon both tumor susceptibility/initiation and progression (Kalluri, 2016; Liu et al., 2017; Santi et al., 2017; Yamauchi et al., 2018). CAFs are a heterogeneous mixture of multiple resident fibroblast subtypes and infiltrated circulating mesenchymal cells. Understanding the origin and nature of the fibroblasts that drive oncogenic initiation and progression has been hampered by a paucity of CAF-specific markers and thus their origin remains controversial. Mesenchymal stem cells (MSCs) and resident fibroblast progenitors of CAFs are recruited by chemokines/cytokines and growth factors to specific sites and ECM components at these sites activate these cells into CAFs (Mishra et al., 2008; Shinagawa et al., 2010; Mi et al., 2011). For example, knockdown of the HA receptor CD44 in MSCs blocks both their ability to be recruited to the tumor site, and their tumor promoting functions (Spaeth et al., 2013) Recent studies have identified CAF properties that are distinct from activation of normal fibroblasts responding-to-wounding. For example, CAFs activation status appears to be irreversible while wound repair fibroblasts activation is both reversible and dependent on wound-induced signaling. The secretome, ECM remodeling and tumor promoting properties of CAFs and injury-activated fibroblasts also differ (Kalluri, 2016).

CAFs are most commonly identified by their expression of fibroblast activation protein (FAP) and alpha smooth muscle actin (∂-SMA), however, additional markers including platelet derived growth factor receptor b (PDGFRB), fibroblast specific protein (FSP) and vimentin (VIM), all of whose expression in tumor stroma have, like ∂-SMA, been linked to poor outcome of many cancers, can also be expressed in CAFs (Jacob et al., 2012; Folgueira et al., 2013; Paulsson and Micke, 2014; Han et al., 2015; Peiris-Pagès et al., 2015; Corvigno et al., 2016; Gascard and Tlsty, 2016; Kuzet and Gaggioli, 2016; Hammer et al., 2017; Tao et al., 2017; von Ahrens et al., 2017). The roles of CAFs as promoters of tumor initiation, progression, epithelial to mesenchymal transition, stemness, tumor invasion, angiogenesis,

metastasis and drug resistance are well established (Kalluri and Zeisberg, 2006; Shekhar et al., 2007; Straussman et al., 2012) Experimentally, CAFs exhibit activity in all Hallmarks of Cancer categories (Salo et al., 2014; Tommelein et al., 2015; Attieh and Vignjevic, 2016; Mezawa and Orimo, 2016). Many studies of CAF participation in tumorigenesis have viewed their role as a reactive process that is a consequence of signals originating in the epithelial tumor, which results in a permissive environment for tumor cells to grow. A number of studies have demonstrated a more instructive role for CAFs in the initiation and dissemination of tumors. These studies have stimulated interest in the development of therapies that target CAFs and other stromal components of the tumor stroma. These CAF properties are reviewed here.

CAFS AND TUMOR INITIATION

In general, fibroblasts in normal stroma have tumor-suppressing properties (Bhowmick et al., 2004; Augsten, 2014; Klein, 2014; Rhee et al., 2015; Kubo et al., 2016; Lin and Lin, 2017; Mangge et al., 2017). However, when normal fibroblasts are activated (e.g., into myofibroblasts) or become senescent they lose these tumor-suppressing functions and under appropriate conditions convert into tumor-promoting and/or initiating CAFs. Experimentally, such cells can facilitate conversion of pre-malignant epithelial cells into tumors. An early example of this was provided by evidence that irradiated fibroblasts increase the incidence of tumors arising from pre-malignant mammary epithelial cells (Bhowmick et al., 2004; Ji et al., 2017). A number of more recent studies using experimental models provide direct evidence for the ability of CAFs to drive the initiation of cancer (Sasaki et al., 2014). Thus, loss or reduction of a notch effector (CSL) in stromal fibroblasts is sufficient for CAF activation and induction of keratinocyte tumors. Conversely, CCR5 blockade of fibroblast activation in colon tissue of a mouse model of colitis-associated carcinogenesis strongly reduces tumor initiation even though inflammation/colitis is still present. In experimental models, senescent fibroblasts have also been shown to enhance cancers including ovarian and keratinocyte transformation (Lawrenson et al., 2010).

Clinically, CAF-like fibroblast-induced stromal ECM changes have been reported to precede tumor formation and these early changes in ECM provide prognostic information that permit risk stratification. For example, high mammographic density is a strong risk factor in breast cancer (DeFilippis et al., 2012; Ghosh et al., 2017; Vinnicombe, 2017). Clinical features of this condition, which precede detectable tumor formation, include adipocyte loss and high ECM production. This condition has been linked to expression loss of the mesenchymal differentiation regulator CD36 in stromal fibroblasts, which phenocopies the clinical features of high mammographic density breast tissue. In clinical samples, CAFs exhibit loss of CD36 expression. (DeFilippis et al., 2012) and this in breast cancer tissue is strongly associated with poor outcome. Other examples include evidence that primary dermal fibroblasts exhibit a CAF-like state with a germ-line BRCA1 epi-mutation (Etzold et al., 2016). These fibroblasts stimulate rather than suppress epithelial proliferation and migration, express CAF markers including ACTA2, FAP, PDPN, and TNC, and are highly proliferative and migratory relative to normal counterparts from other patients. In early stage breast cancer, high stromal Heat Shock Factor 1 (HSF1) activation is associated with poor outcome and experimental data show that HSF1 expression is elevated/activated and results in potent enabling of malignancy (Scherz-Shouval et al., 2014). Genetic loci have been also identified that affect stromal properties and control mammary tumor susceptibility. These include genes that affect TGFß signaling (Zhang P. et al., 2015). Consistent with these findings, fibroblast-specific deletion of TGFßIIR in a transgenic mouse model results in repression of tumor suppressing functions of fibroblasts and a rapid development of aggressive prostate cancer (Li et al., 2012). HA is one ECM factor that is regulated by TGFß (Heldin et al., 2014) that is linked to tumor susceptibility, initiation and progression of many cancers and will be focused upon here.

STROMAL HYALURONAN IS LINKED TO TUMOR SUSCEPTIBILITY AND CAF ACTIVATION

HA is a simple extracellular matrix polysaccharide that a wealth of experimental approaches has demonstrated is an instructive factor in cancer initiation and progression (Heldin et al., 2014; Tolg et al., 2014; Zhang C. et al., 2015; Chanmee et al., 2016; Turley et al., 2016; Bohaumilitzky et al., 2017; Senbanjo and Chellaiah, 2017; Shih et al., 2017; Wight, 2017; Wong et al., 2017). For example, blocking HA synthesis (Itano et al., 2008; Hamada et al., 2017; Ikuta et al., 2017) or ablating the HA-binding function of one of its receptors RHAMM (gene name HMMR) (Hall et al., 1995), which has been strongly linked to tumorigenesis (Tolg et al., 2014; Turley et al., 2016), attenuates the transformed phenotype. Clinical analyses show that elevated HA accumulation in either the stroma or tumor parenchyma of many cancers is linked to tumor aggression and poor outcome (Sironen et al., 2011; McAtee et al., 2014; Chanmee et al., 2016; Sato et al., 2016; Turley et al., 2016; Bourguignon et al., 2017; Wu et al., 2017). Unexpectedly, HA has also recently been implicated as a stromal tumor-suppressing factor (Tian et al., 2013; Fisher, 2015; Triggs-Raine and Natowicz, 2015; Bohaumilitzky et al., 2017). These opposing effects are not well-understood but have been linked to differences in its metabolism and in particular the regulation of HA polymer size (Simpson and Lokeshwar, 2008; Tian et al., 2013; Khaldoyanidi et al., 2014; Tolg et al., 2014; Litwiniuk et al., 2016; Turley et al., 2016; Fouladi-Nashta et al., 2017).

HA is composed of repeating disaccharide units of N-acetylglucosamine and ß-glucuronic acid linked together by three highly homologous synthases (HAS1,2,3). These are most frequently located at the plasma membrane and the growing HA polymer is extruded directly into the extracellular space through pores in the plasma membrane formed by synthase oligomerization (Weigel, 2015) (e.g., **Figure 2**).

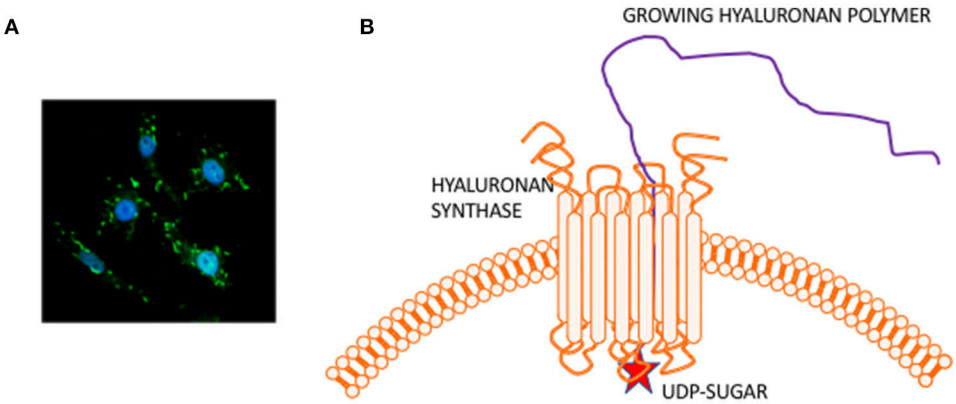

FIGURE 2 | Hyaluronan is a simple polysaccharide produced by cell membrane synthases. **(A)** Micrograph showing membrane localized hyaluronan synthase 2 in fibroblasts. **(B)** Hyaluronan synthase has multiple transmembrane domains that cluster to form pores in the cell membrane. UDP-sugars bind to the protein cytoplasmic face and growing polymer is extruded through the pore to the extracellular space.

Evolving evidence indicates that the biological effects of HA are primarily determined by size rather than conformational changes typically required for protein activation. In general, large HA polymers, which are mainly present in homeostatic tissues, are immunologically quiescent and contribute to enforcing cell survival and homeostasis. HA fragments (e.g., <100–200 kDa), which are generated by reactive oxygen/nitrogen species (ROS/RNS) and hyaluronidases produced during tissue stress, repair and chronic disease, are pro-inflammatory and pro-fibrotic (Simpson and Lokeshwar, 2008; Gaudet and Popovich, 2014; Cyphert et al., 2015; Sherman et al., 2015; Gaggar and Weathington, 2016; Maytin, 2016; Turley et al., 2016; Bohaumilitzky et al., 2017; Cowman, 2017; Frevert et al., 2017; Kavasi et al., 2017; Wight et al., 2017; Wu et al., 2017; Avenoso et al., 2018a,b) (**Figure 3**). The precise effect of specific sizes of HA fragments on immune and mesenchymal cells on such functions as gene expression appears to be cell-context and stimulus-specific, and is currently controversial (Cowman, 2017; Weigel, 2017; Weigel and Baggenstoss, 2017). HA fragment accumulation in quiescent homeostatic tissues is low. In contrast remodeling and diseased tissues such as cancers often contain an elevated level of HA (e.g., Teder et al., 2002; Koyama et al., 2007; Li et al., 2011; Tolg et al., 2017), clear evidence of fragmentation, and overexpression of HAS, hyaluronidases and HA receptors.

The tumor-resistance properties of high molecular weight HA were originally identified in the tumor resistant naked mole rat and resistance of fibroblasts to oncogenic transformation was shown to depend upon production of large HA polymers (Tian et al., 2013). Naked mole rat tissues contain larger HA polymers and less detectable fragmentation than tissues of the more tumor-susceptible mouse. HA-mediated tumor resistance of the naked mole rat is attributed to the ability of high molecular weight HA to hyper-sensitize cells to contact inhibition and induce p16 (ink4a) locus expression with consequent cell cycle arrest (Tian et al., 2015). Consistent with this explanation, HA overproduction has also been shown by other groups to regulate contact inhibition and adhesion in cultured non-malignant cells (Itano et al., 2008). Others have shown that excess production of HA by itself does not promote an aggressive tumor phenotype and can even be tumor-suppressing by blocking G1-S transition in the cell cycle (Bharadwaj et al., 2011). Similarly, exposure of tumor cells to hyaluronidases alone (e.g., HYAL1 or PH-20) can be growth-suppressing (Simpson and Lokeshwar, 2008) and increase response of tumor cells to therapy (Wong et al., 2017). Thus, high HA production combined with an increased capacity for polymer fragmentation appears to be responsible for oncogenic effects of this polysaccharide.

A number of studies using mouse models also predict that elevated HA production, primarily by fibroblasts, pre-disposes epithelial cells to tumor initiation. Examples include evidence that an HA-rich stroma precedes increased mammary tumor formation in transgenic mice expressing both MMTV-driven HAS2 and a c-neu proto-oncogene. HAS2/c-neu mice tumors notably produce higher levels of both high molecular weight and fragmented HA than the c-neu mice (Koyama et al., 2007). Using p38MAPK knock-in mice and tumor xenografts, others have shown that MAPK-driven HAS2 expression and consequent HA production by fibroblasts is required for their activation into CAFs and for loss of their tumor suppressing properties resulting in a pro-tumor niche and increased lung colonization (Brichkina et al., 2016). These studies suggest that the tumor suppressing effects of either HA or processing enzymes alone are converted into a pro-tumor stimulus when HA processing into fragments is enhanced and sustained by elevated expression of one or more HAS genes, and hyaluronidases, often HYAL1. Additional studies predict that the pro-tumor functions of HA also depend upon the display of specific receptors, notably the injury-related HA receptor, RHAMM (gene name HMMR), which activates oncogenic signaling pathways (Tolg et al., 2014; Misra et al., 2015; Nikitovic et al., 2015; Schwertfeger et al., 2015). In a pre-malignant stroma, these genes are expressed by CAF-like fibroblasts.

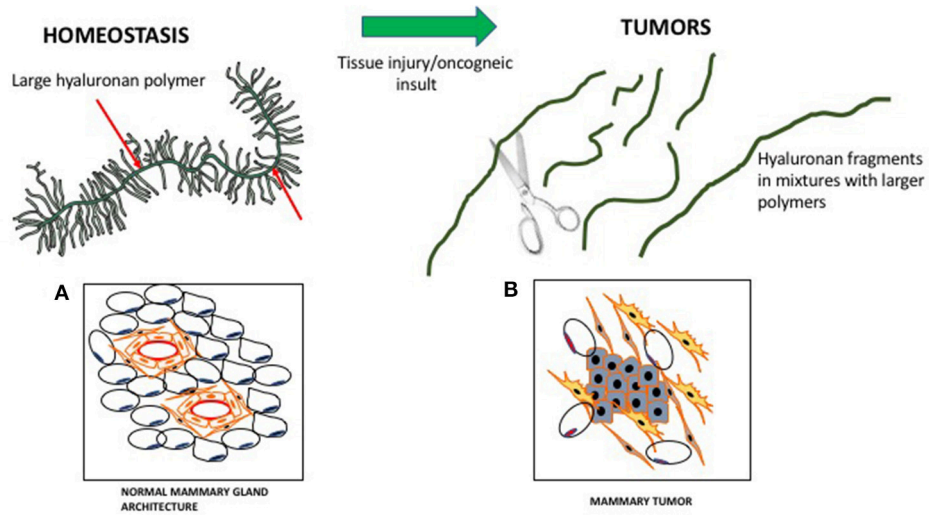

FIGURE 3 | Hyaluronan is a large polymer during tissue homeostasis and fragmented in wounds and tumors**.** In homeostasis, large hyaluronan polymers are decorated by proteins/proteoglycans, which contribute to normal tissue architecture. **(A)** Cartoon of a virgin mouse mammary gland. **(B)** Cartoon of a mouse mammary tumor (Blue, mammary tumor cells). During wounding, fragmented hyaluronan polymers produced by ROS/hyaluronidases activate fibroblasts and attract immune cells, which contribute to the loss of tissue architecture.

CAFS AND TUMOR DISSEMNATION

CAFs play a significant role in tumor dissemination by inducing an invasive phenotype in tumor cells, promoting motile phenotypes and remodeling the ECM. Invasion is achieved in part by CAF-driven EMT and consequent cell migration driven by factors such as TGF-B, HGF, and CXCL12/SDF-1(Kalluri, 2016). Paladin-expressing CAF create "tunnels" in the ECM which cancer cells migrate through (Brentnall, 2012). Under CAF guidance, tumor cells also migrate and invade as groups in the absence of apparent EMT. This collective migration and invasion is driven by heterotypic E-cadherin/N-cadherin interactions between tumor cells and CAFs (Labernadie et al., 2017) that results in a mechanically active adhesion. CAF-mediated ECM remodeling occurs as a result of secretion of collagen, proteases, and in particular, matrix metalloproteinases. ECM remodeling provides a microenvironment that further supports tumor cell migration and dissemination. Interestingly, CAFs from different breast cancer molecular subtypes including Luminal A, Her2-like, and triple negative/basal-like exhibit subtype-specific differences in stromal gene expression (Tchou et al., 2012), microRNA expression and secretory profiles (Shah et al., 2015). Furthermore, CAFs from more aggressive cancers induce more aggressive breast cancer cell phenotypes than CAFs from more indolent cancers (Shah et al., 2015).

Circulating tumor cell (CTC) clusters were originally described in the 1970's and are now considered to be precursors of metastatic colonies. In mouse breast cancer models, circulating tumor cell clusters exhibit higher metastatic capacity compared with individual or single CTCs (Aceto et al., 2014). Additionally, polyclonal breast cancer metastases have been suggested to arise from circulating tumor cell clusters composed of Keratin 14+ cells (Cheung et al., 2016). Quantification of these CTC clusters in breast cancer patients show that their presence correlates with reduced progression-free survival and poor outcome (Cheung et al., 2016; Jansson et al., 2016; Mu et al., 2016; Wang et al., 2017). Collective migration of tumor cell clusters into the circulation appears to offer a tumor cell survival advantage compared to entry of single tumor cells into the vasculature. CAFs are not only present in primary and metastatic tumor stroma but have recently been shown to occur in the circulation either as individual CAFs, part of CTC clusters or as CAF clusters. Circulating CAFs (cCAFs) likely contribute to CAFs found in pre-metastatic and metastatic niches. Mouse metastasis models suggest that circulating CAFs can exit either with groups of cancer cells or by themselves. In these models, the presence of CAFs from the primary TME promotes metastatic seeding and growth (Duda et al., 2010), likely by helping to create a suitable growth and survival microenvironmental niche for tumor cells and to aid in avoidance of immune surveillance. Additionally, since CAFs are present in pre-metastatic niches prior to the appearance of tumor cells, circulating CAFs likely also play a role in establishing or preparing a niche suitable for future tumor cell colonization. In a pilot study, cCAFs were detected in the blood from patient with Stage IV (metastatic) breast cancer but not from patients with Stage I disease with no evidence of relapse, while CTCs were detected in both patient samples (Ao et al., 2015). Furthermore, CTCs and cCAFs circulate in co-clusters in patient blood, and like CTCs, cCAFs can also cluster with each other (**Figure 4**). Jones and colleagues also found circulating CK-/CD45/VIM+ fibroblast-like cells in metastatic prostate cancer patient blood (Jones et al., 2013). The development of techniques for isolating circulating CAFs from mouse models of human breast cancer xenografts and mammary tumor susceptibility will greatly aid in characterizing both the origin and contribution of circulating CAFs to successful

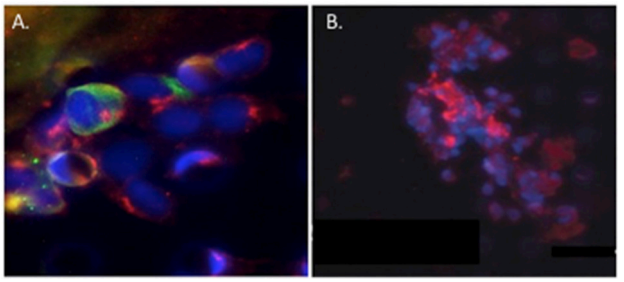

FIGURE 4 | Circulating cCAF/circulating tumor cell (CTC) clusters and cCAF clusters in breast cancer patient blood. **(A)** cCAF/CTC co-cluster and **(B)** cCAF cluster. Red: FAP, Green: CK. From Ao et al. (2015).

metastasis. Recent evidence suggests that at least a portion of CTCs are tumor cells transitioning between the epithelial and mesenchymal state (Yu et al., 2013) that possess stem cell-like properties and phenotypically plasticity (May et al., 2011). Functional characterization of these circulating cells/clusters will clarify the mechanisms of tumor cell dissemination and likely identify potential therapeutic targets for metastatic disease.

HYALURONAN AND TUMOR DISSEMINATION

A CAF property that appears to be critical to cancer cell invasion is their active motility and tropism toward tumor cells (e.g., Costea et al., 2013; Berdiel-Acer et al., 2014). These properties culminate in close physical heterotypic contact (Marusyk et al., 2016; Labernadie et al., 2017). Clinically, close proximity of CAFs to tumor cells is linked to poor outcome and resistance to therapy and supports migration and invasion of tumor cells by several mechanisms (Marusyk et al., 2016). HA is one CAF-produced ECM factor that appears to play a key role in these critical autocrine and paracrine migratory interactions of CAFs and tumor cells. Thus, highly motile CAF subtypes produce and rely upon HA for their motogenic properties (Costea et al., 2013) and ability to promote migration of tumor cells. We and others (e.g., Hamilton et al., 2007; Mele et al., 2017; Shigeeda et al., 2017) have also reported that highly aggressive breast cancer cells that have undergone EMT develop a CAF-like autocrine production of HA to sustain their high motility rates. Such tumor cells are able to invade independently from CAFs (Turley et al., 2016). Intriguingly, we have shown using fluorescent HA-probes that HA-binding to breast cancer cells and to activated fibroblasts is heterogeneous (Veiseh et al., 2014, 2015). FACS-sorted tumor cell subsets that bind high levels of HA are more motile, invasive and metastatic than subsets that bind low or no probe. A concept that emerges from these studies is that CAF subsets not only utilize HA to migrate close to tumor cells but that their autocrine production of HA also stimulates the migration of the HA binding tumor subpopulation (**Figure 5**). Expression of HA receptors CD44 and RHAMM is required for migration of these tumor cells, and we predict that these receptors also mediate HA-dependent, highly motile CAF subsets.

The role of HA and its receptors in circulating CAFs and tumor cells is currently understudied. However, several studies have reported that circulating tumor cells from cancer patients express the HA receptor CD44 (Grillet et al., 2017) and can be captured from circulation by adhering to HA, a process that is mediated by HA receptors (Xu et al., 2017). Interestingly, circulating cells with this dual phenotype are EpCAM- and are therefore distinct form the more commonly studied EpCAM+ circulating tumor cells (Mirza et al., 2017). EpCAM-/CD44+ cells may represent tumor cells that have undergone EMT and/or are circulating cancer stem cells (cCSCs). Circulating cells isolated from lung adenocarcinoma patients that had higher levels of markers such as RHAMM (HMMR) had shorter survival times (Man et al., 2014). CAFs also express CD44 and this CD44 plays important roles in CAF function. These collective results predict a critical importance of HA production and HA receptor display in cCAFs and CTCs to successful metastases.

TARGETING STROMA AND CAFS

Targeting key genetic or epigenetic alterations in tumors and/or the use of immune checkpoint inhibitors has significantly improved cancer therapy (Jiang et al., 2017). While these advances are encouraging, they are currently either effective in a minority of cancer patients, have significant pro-tumor side-effects or lack long-term durability. Thus, new approaches are necessary to expand the number of patients who will benefit clinically from chemotherapy and targeted therapy. Targeting the fibrotic stroma is emerging as a potentially key approach necessary to achieving therapeutic efficacy. This is particularly true for pancreatic cancer, which typically progresses with an extensive fibrotic stroma that can account for over 80% of the tumor volume (Yu and Tannock, 2012; Tan et al., 2015). Therapies that target the fibrotic stroma, including HA, are being developed and entering clinical trials (Provenzano and Hingorani, 2013; Jiang et al., 2017; Kumari et al., 2017).

High interstitial pressures in the fibrotic stroma of pancreatic cancers, which results from high production of collagen and HA, causes the collapse of the stromal vasculature in pancreatic cancers and impedes exposure of tumor cells to chemo- and immune therapies (Yu and Tannock, 2012). Multiple approaches to target fibrotic stroma are therefore being tested to overcome these delivery issues. One successful strategy is targeting HA. Systemic administration of a recombinant sperm hyaluronidase (PEGPH20), degrades hyaluronan in pancreatic cancer stroma (Provenzano and Hingorani, 2013). This destruction decreases interstitial fluid pressure, increases vasculature patency and improves the delivery of gemcitabine. Importantly, these hyaluronidase-mediated changes both decrease tumor volume and increase animal survival in experimental models of pancreatic cancer. PEGPH20 is now in phase III clinical trials for pancreatic cancer (Doherty et al., 2018). An alternative to the use of recombinant hyaluronidase has been of HA synthesis inhibitors (e.g., 4-methylumbelliferone), which also inhibits

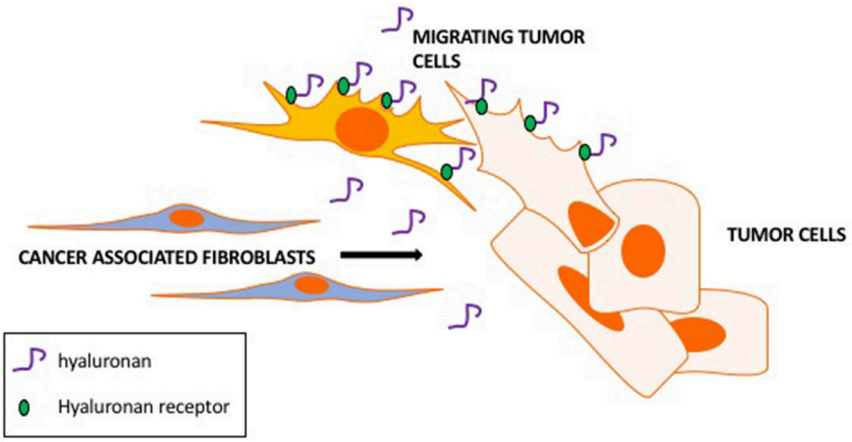

FIGURE 5 | Hyaluronan promotes CAFs motility toward tumor cells and tumor cell motility. CAF subsets produce hyaluronan as a motogenic stimulus for migrating toward tumor cells. Hyaluronan binds to tumor cell subsets via hyaluronan receptors (RHAMM and CD44) contributing to the migration and invasion of CAF-guided tumor cells.

tumor growth and could be used in alone or in combination with hyaluronidase to improve therapeutic response (Kudo et al., 2017).

CAF-targeted therapies are also being developed to blunt their fibrosis-activated signaling. For example, a selective FAK inhibitor (VS-4718) targets hyperactive FAK in stromal CAFs. This inhibitor reduces fibrosis, decreases the number of tumor-infiltrating immuno-suppressive cells and results in survival doubling in mouse models of pancreatic ductal adenocarcinoma (Jiang et al., 2016, 2017). Inhibiting FAK activation also increases responsiveness to chemotherapy and immune checkpoint inhibitors with resulting improved outcome. These pre-clinical successes have led to phase 1 clinical trials using this FAK inhibitor in combination with immune checkpoint inhibitors (Jiang et al., 2017). While FAK hyper-activation is a key feature of mechano-signaling in CAFs and provides a proof-of-concept for targeting the microenvironment, stromal immune cells also utilize FAK or the related PYK-2 for survival (Jiang et al., 2017). Off target effects of VS-4718 could contribute to immune-suppression and therefore compromise its effective utility in humans (Jiang et al., 2016).

Active investigations are also underway to target CAF survival in the fibrotic stroma. In contrast to carcinoma cells, CAFs are genetically normal cells that have been co-opted and modified by cancer cells into a state of constitutive activation. CAFs therefore have a less plastic genome than tumor cells limiting their ability to rapidly modify their genome but making them an attractive candidate for stable responses to targeted therapy. CAFs uniquely express FAP, which plays important roles in CAF function (Lai et al., 2012; Koczorowska et al., 2016). *In vivo* administration of a FAP enzyme inhibitor, Talabostat, in tumor-bearing mice results in tumor regression and upregulation of specific chemokines and cytokines that induce an anti-tumor immune response (Cunningham, 2007). Talabostat is well tolerated in healthy volunteers in both Phase I and II clinical trials but does not result in therapeutic benefit. A CAF-directed, anti-human FAP antibody, sibrotuzumab (Fischer et al., 2012), exhibits specificity and activity in preclinical mouse models (Fischer et al., 2012), and was well tolerated in early Phase I/II clinical trials (Hofheinz et al., 2003; Scott et al., 2003) but has failed to show therapeutic activity in patients with metastatic disease. FAP-targeted chimeric antigen receptor (CAR) T cells reduce ECM, vessel density, and growth of several types of human cancer xenografts and murine pancreatic cancers when introduced into mice by adoptive transfer (Wang et al., 2014; Lo et al., 2015). This technology has not yet entered clinical trials. FAP may be useful for targeting therapies to CAFs. Potentially the development of therapies that impede CAF survival/function in the circulation or their ability to migrate/enter the circulation (e.g., HA/RHAMM) may be a more promising approach.

In conclusion, despite recent advances in targeted therapies, metastases, recurrence and relapse remain as major clinical obstacles to successful cancer treatment. Carcinoma cell epigenetic and genetic heterogeneity are important factors that limit therapeutic efficacy. However, a wealth of studies has now demonstrated that tumor-associated fibrotic stroma is also a major contributing factor to therapeutic failure. The success of new approaches to targeting tumor cells will in the future likely have to include agents that compromise the pro-tumorigenic fibrotic ECM.

AUTHOR CONTRIBUTIONS

ET: Organized, referenced, edited contributions and wrote introduction, and sections on CAF-mediated tumor initiation, relationship of microenvironment and hyaluronan, prepared model **Figures 1**, **2**, **3**. JM: Edited contributions, wrote abstract and wrote/referenced sections on tumor dissembination, biomechanical properties of tumor stroma and therapeutic approaches to targeting CAFs and fibrotic stroma. DE-A: Edited

contributions wrote/referenced section on circulating tumor cells and CAFs, tumor dissemination and prepared **Figure 4**.

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CORRECTION
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Corrigendum: Hyaluronan, Cancer-Associated Fibroblasts and the Tumor Microenvironment in Malignant Progression

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Keywords: hyaluronan, cancer-associated fibroblasts, migration, tumor Microenvironment, tumor initiation, circulating cancer-associated fibroblasts, metastasis

A Corrigendum on

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In the original article, there was a mistake in **Figure 4** as published. We erroneously included a figure of unpublished data that should not have been included. A new figure demonstrating the same key point of heterotypic co-clusters of CTCs and cCAFs with corrected text describing the figure (see below), as well as a new figure legend is being provided. The corrected [**Figure 4**] appears below and a correction has been made to CAFS AND TUMOR DISSEMINATION, Paragraph Number 2:

Circulating tumor cell (CTC) clusters were originally described in the 1970's and are now considered to be pre-cursors of metastatic colonies. In mouse breast cancer models, circulating tumor cell clusters exhibit higher metastatic capacity compared with individual or single CTCs (Aceto et al., 2014). Additionally, polyclonal breast cancer metastases have been suggested to arise from circulating tumor cell clusters composed of Keratin 14+ cells (Cheung et al., 2016). Quantification of these CTC clusters in breast cancer patients show that their presence correlates with reduced progression-free survival and poor outcome (Cheung et al., 2016; Jansson et al., 2016; Mu et al., 2016; Wang et al., 2017). Collective migration of tumor cell clusters into the circulation appears to offer a tumor cell survival advantage compared to entry of single tumor cells into the vasculature. CAFs are not only present in primary and metastatic tumor stroma but have recently been shown to occur in the circulation either as individual CAFs, part of CTC clusters or as CAF clusters. Circulating CAFs (cCAFs) likely contribute to CAFs found in pre-metastatic and metastatic niches. Mouse metastasis models suggest that circulating CAFs can exit either with groups of cancer cells or by themselves. In these models, the presence of CAFs from the primary TME promotes metastatic seeding and growth (Duda et al., 2010), likely by helping to create a suitable growth and survival microenvironmental niche for tumor cells and to aid in avoidance of immune surveillance. Additionally, since CAFs are present in pre-metastatic niches prior to the appearance of tumor cells, circulating CAFs likely also play a role in establishing or preparing a niche suitable for future tumor cell colonization. In a pilot study, cCAFs were detected in the blood from patient with Stage IV (metastatic) breast cancer but not from patients with Stage I disease with no evidence of relapse, while CTCs were detected in both patient samples

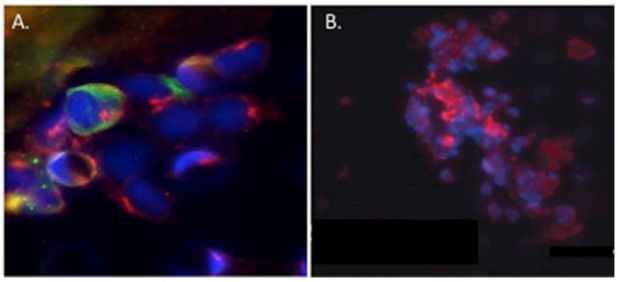

FIGURE 4 | Circulating cCAF/circulating tumor cell (CTC) clusters and cCAF clusters in breast cancer patient blood. **(A)** cCAF/CTC co-cluster and **(B)** cCAF cluster. Red: FAP, Green: CK. From Ao et al. (2015).

(Ao et al., 2015). Furthermore, CTCs and cCAFs circulate in co-clusters in patient blood, and like CTCs, cCAFs can also cluster with each other (**Figure 4**). Jones and colleagues also found circulating CK-/CD45/VIM+ fibroblast-like cells in metastatic prostate cancer patient blood (Jones et al., 2013). The development of techniques for isolating circulating CAFs from mouse models of human breast cancer xenografts and mammary tumor susceptibility will greatly aid in characterizing both the origin and contribution of circulating CAFs to successful metastasis. Recent evidence suggests that at least a portion of CTCs are tumor cells transitioning between the epithelial and mesenchymal state (Yu et al., 2013) that possess stem cell-like properties and phenotypically plasticity (May et al., 2011). Functional characterization of these circulating cells/clusters will clarify the mechanisms of tumor cell dissemination and likely identify potential therapeutic targets for metastatic disease.

In addition, there was an error in the Author Contributions Statement. It was stated that ET prepared the model for Figure 4 and DE-A for Figure 3 when it was in fact the other way around. ET prepared the model for Figure 3 and DE-A for **Figure 4**.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way.

The original article has been updated.

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HYPOTHESIS AND THEORY
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Aging in a Relativistic Biological Space-Time

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Here we present a theoretical and mathematical perspective on the process of aging. We extend the concepts of physical space and time to an abstract, mathematically-defined space, which we associate with a concept of "biological space-time" in which biological dynamics may be represented. We hypothesize that biological dynamics, represented as trajectories in biological space-time, may be used to model and study different rates of biological aging. As a consequence of this hypothesis, we show how dilation or contraction of time analogous to relativistic corrections of physical time resulting from accelerated or decelerated biological dynamics may be used to study precipitous or protracted aging. We show specific examples of how these principles may be used to model different rates of aging, with an emphasis on cancer in aging. We discuss how this theory may be tested or falsified, as well as novel concepts and implications of this theory that may improve our interpretation of biological aging.

Keywords: special relativity, aging, biological clocks, biological space-time, manifolds, time-contraction

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1. INTRODUCTION

The connection between one's chronological age and biological age is something that we all perceive. In a sense, it is the difference between the age you "feel" and the age you *are*. Some people look "young" for their age, while some become frail earlier than others (Ness et al., 2013). Molecular "clocks" and markers of "biological age" can change throughout one's lifetime. In some cases, the rate of change of a person's biological age is *greater* than the rate of change of their chronological age. For instance, some cancers have been shown to increase biological aging, which can be described as an increase in the speed of biological clocks (Horvath, 2013).

From a mathematical perspective, biological dynamics as they relate to aging are frequently modeled as periodic or oscillating "clocks" describing, for example, circadian rhythms (Klerman and Hilaire, 2007). When studied in isolation, biological clocks can be described and predicted with periodic functions, which may speed up, slow down, or even stop and start over the course of a persons' lifetime. The more complex case of multiple integrated biological clocks can be modeled with coupled oscillators and dynamical systems theory (Shiju and Sriram, 2017). However, a fundamental assumption of these approaches is that the rate of change of these biological clocks are measured with respect to a *linear passage of time* and that the rate is independent from the biological *space* in which the biological clocks operate.

Here we investigate a mathematical model of biological space-time which includes the effects of time dilation and contraction resulting from accelerated or decelerated biological clocks, which may provide a new theoretical foundation and perspective on rates of aging. The principle assumption of our model is that a dynamic biological process may be represented as the motion of a (massless) point along a trajectory on a manifold. We then investigate the consequence of time dilation and contraction in terms of accelerating or decelerating motion of the point along the trajectory, and relate these concepts to rates of biological aging, with a particular focus on aging in cancer. In particular, we put forth the hypothesis that a biological space-time may be used to model aging from an arbitrary biological viewpoint relative to a common frame of reference. A consequence of this hypothesis is that precipitous (faster than chronological time) or protracted (slower) aging may be modeled as relativistic corrections of dilation or contraction of time along the trajectory on the manifold in which the biological aging process occurs.

To the best of our knowledge, only a few groups have proposed similar concepts. Bailly and colleagues (Bailly et al., 2011; Longo and Montévil, 2014) have proposed a mathematical definition of "biological time" as a means to model biological rhythms and periodic biological processes. However, they do not define a biological "space" nor include the possibility of the dilation or contraction of time. Systems biology pioneer Denis Noble has proposed a theory of biological relativity, which asserts that there is no "privileged level of causation" in biology (Noble, 2012). Nobel's theory contends that biological processes occur on many scales in space and in time, and that these scales are coupled to each other and should not be separated; that no single scale is responsible for the dynamics of the whole. Noble's theory implicitly couples space and time, but does not include a definition of biological space or concept of dilation or contraction of time. Consequently, our work is among the first to introduce both the mathematical interpretation and specific concept of a relativistic biological space-time to be used to study rates of biological aging.

This manuscript is structured as follows: first we describe the mathematical objects which we use to define biological space-time. After defining the meaning of relativistic dynamics in this context, we show how relativistic corrections of dilation and contraction of time can be used to model precipitous or protracted aging. We then show examples of how these principles may be used to model aspects of the aging process with a particular emphasis on aging in cancer. We discuss the implications of this theory, including criteria that may be used to test or falsify the theory and suggest novel biological quantities that may improve our interpretation of biological aging.

2. BIOLOGICAL SPACE-TIME

Biology, and biological processes, are measured and observed in our conventional notion and understanding of physical space. Cells, tissues, and organisms move and change in a physical space that we can measure with length and time scales in conventional units. However, we may also consider the functional, or phenotype space in which biological processes can be represented as locations in the space. We refer to movement in a biological space as a sequence of locations in the space that form a trajectory. These general concepts have been used to characterize biological states such as hematopoietic differentiation, where 2- or 3-dimensional representations of biological space are constructed with dimension reduction techniques applied to high-dimensional single cell RNA-sequencing data (Mojtahedi et al., 2016; Nestorowa et al., 2016; Rizvi et al., 2017). The idea of the *relativity* of aging is to apply the special relativity machinery to provide a rigorously defined mathematical framework to represent biological dynamics as trajectories on manifolds moving at different acceleration rates relative to a common frame of reference. In other words, the difference in aging between two different people will be explained in terms of different dynamics in biological space-time.

2.1. A Manifold $\mathfrak{M}$ and Submanifolds $\mathfrak{M}_i$

In order to provide a conceptual picture of our mathematical framework, we imagine the space related to a biological process identified by the index i to be a smooth manifold $\mathcal{M}_i$. A manifold is a mathematical object that is locally, but not necessarily globally, Euclidean. The canonical example of a manifold is the Earth: locally flat, globally round. If a point on Earth represents an unique locus, a point on $\mathcal{M}_i$ represents a unique possible configuration or a state of a biological process. The evolution of the biological process in time, is then represented by a collection of points which form a curve, or trajectory, on the manifold $\mathcal{M}_i$. If the biological process does not change in time, the trajectory degenerates to a point, with the consequent lack of motion in the biological space. We then identify the i-th *biological space-time* manifold by mathematically combining the time component and the spatial components of the i-th biological process.

Given a subset U_i of a topological space (i.e., a set in which at each point it is possible to associate a neighborhood) $\mathcal{M}_i$, a d_i-dimensional chart is an injective (one-to-one) function $\varphi_i : U_i \subset \mathcal{M}_i \longrightarrow \mathbb{R}^{d_i}$. A point $\boldsymbol{q}_i$ on $\mathcal{M}_i$ is identified by a set of d_i spatial coordinates $(q_i^1, q_i^2, \dots q_i^{d_i})$. An atlas on $\mathcal{M}_i$ is the set $\mathcal{A}_i = \left\{\varphi_{i,\alpha} : U_{i,\alpha} \longrightarrow \mathbb{R}^{d_i}\right\}$ for some finite values of the index α, where the union $U_{i,1} \cup U_{i,2} \cup \dots$ is the whole space $\mathcal{M}_i$. A space $\mathcal{M}_i$ equipped with an atlas $\mathcal{A}_i$ is a d_i-dimensional differential manifold. For a more detailed discussion see (Tu, 2011).

A trajectory, or curve, γ_i on $\mathcal{M}_i$ is a smooth map:

$$\gamma_i : \mathcal{I}_i \subset \mathbb{R}^+ \longrightarrow \mathcal{M}_i$$
$$t \longmapsto \gamma_i(t) = \boldsymbol{q}_i(t) = (q_i^1(t), q_i^2(t), \dots, q_i^{d_i}(t)). \quad (1)$$

Here $t \in \mathcal{I}_i \subset \mathbb{R}^+$ is a parameter that is interpreted as the time variable associated to the manifold $\mathcal{M}_i$. The curve γ_i represents the time evolution of the i-th biological process starting from its beginning to its end (**Figure 1**). If the point $\boldsymbol{q}_i(t)$ does not change for all $t \in \mathcal{I}_i \subset \mathbb{R}^+$, the trajectory degenerates to a point. We stress the fact that the variable t is a parameter by which is possible to parametrize the curve $\gamma_i(t)$.

Given the manifold $\mathbb{R}^+$, we now define the i-th biological *space-time* manifold $\mathfrak{M}_i$ as the $(d_i + 1)$-dimensional Lorentzian

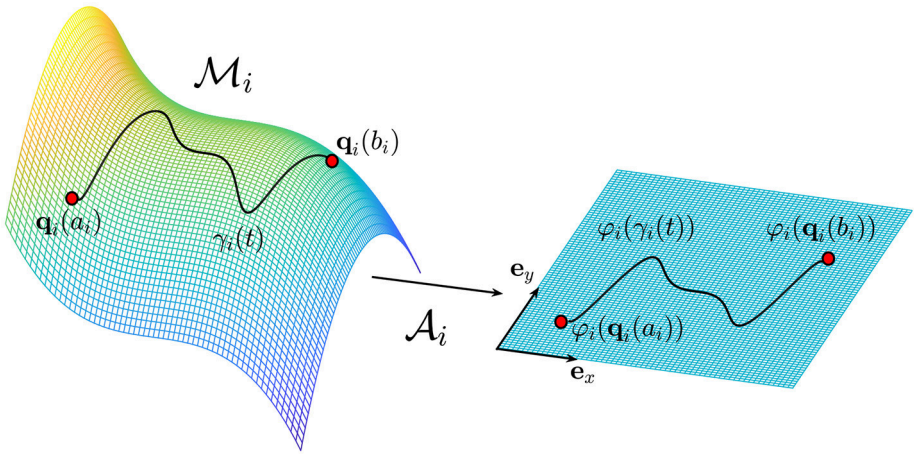

FIGURE 1 | The trajectory γ_i on the manifold $\mathcal{M}_i$ represents the evolution of the i-th biological process which starts at $\boldsymbol{q}_i(a_i)$ and ends at $\boldsymbol{q}_i(b_i)$, where a_i, $b_i \in \mathbb{R}^+$. The atlas $\mathcal{A}_i$ provides the connection with the Euclidean space $\mathbb{R}^2$ identified by the two unit vectors $\boldsymbol{e}_x$ and $\boldsymbol{e}_y$.

manifold given by the Cartesian product (the set of all ordered pairs) (see Tu, 2011):

$$\mathfrak{M}_i = \mathbb{R}^+ \times \mathcal{M}_i = \left\{(t, \boldsymbol{q}_i(t)) | t \in \mathbb{R}^+, \boldsymbol{q}_i(t) \in \mathcal{M}_i\right\}. \quad (2)$$

A point $\mathcal{Q}_i$ on the resulting manifold $\mathfrak{M}_i$ is then identified by a set of $d_i + 1$ coordinates $(t, q_i^1(t), q_i^2(t), \ldots, q_i^{d_i}(t))$. The invariant square of an infinitesimal line element between the points $\mathcal{Q}_i$ and $\mathcal{Q}_i + d\mathcal{Q}_i$, referred to as *space-time interval*, is then evaluated by:

$$ds_i^2 = \sum_{\mu,\nu=0}^{d_i} g_{i,\mu\nu} d\mathcal{Q}_i^\mu d\mathcal{Q}_i^\nu, \quad (3)$$

where $g_{i,\mu\nu}$ is the metric tensor of the i-th biological manifold whose entries are functions of the local coordinates, $(t, q_i^1(t), q_i^2(t), \ldots, q_i^{d_i}(t))$. Since the metric tensor is an intrinsic property of the manifold, its provides information on the geometry of the manifold and vice-versa. It is therefore a key quantity which characterizes the biological space-time and it can be evaluated once the structure of the manifold is known or hypothesized. What we emphasize is that the notion of distance is not necessarily the usual Euclidean distance and therefore, when dealing with biological processes, distances can be measured only once the entries of the metric tensor are known.

Since a large number of biological processes take place in the body of an individual, we assume the existence of several manifolds which may be indexed $\mathcal{M}_i$ and associated with individual or aggregate biological processes. We then consider all such biological processes by constructing the following manifold:

$$\mathcal{M} = \mathcal{M}_1 \times \mathcal{M}_2 \times \cdots \times \mathcal{M}_N, \qquad \dim(\mathcal{M}) = \sum_{i=1}^{N} d_i = m. \quad (4)$$

A point $\boldsymbol{q}$ on $\mathcal{M}$ is then identified by the set of coordinates $(\boldsymbol{q}_1, \boldsymbol{q}_2, \ldots, \boldsymbol{q}_N)$ where each $\boldsymbol{q}_i \in \mathcal{M}_i$, $i = 1, 2, \ldots, N$ and it represents a possible configuration, or state, in which the human body can be found. Following the definition given by Equation (1), a trajectory Γ on $\mathcal{M}$ is a smooth map:

$$\Gamma : \mathcal{I} \subset \mathbb{R}^+ \longrightarrow \mathcal{M}$$
$$t \longmapsto \gamma(t) = \boldsymbol{q}(t) = (\boldsymbol{q}_1(t), \boldsymbol{q}_2(t), \ldots, \boldsymbol{q}_N(t)), \quad (5)$$

where $t \in \mathcal{I} \subset \mathbb{R}^+$ is a parameter that is interpreted as the time variable associated to the manifold $\mathcal{M}$ and the curve Γ represents the entire life of a person from its birth, to its death. The connection between the curve Γ on $\mathcal{M}$ and the curve γ_i on $\mathcal{M}_i$ will be clarified later, when the projection map will be introduced.

In analogy with Equation (2), the biological space-time $\mathfrak{M}$ for all biological processes occurring in a body of an individual is then defined by the $m+1$-dimensional Lorentzian manifold given by:

$$\mathfrak{M} = \mathbb{R}^+ \times \mathcal{M} = \left\{(t, \boldsymbol{q}(t)) | t \in \mathbb{R}^+, \boldsymbol{q}(t) \in \mathcal{M}\right\}. \quad (6)$$

We consider the Cartesian product $\mathfrak{M} = \mathbb{R}^+ \times \mathcal{M}$ instead of $\mathfrak{M} = \mathfrak{M}_1 \times \mathfrak{M}_2 \times \cdots \times \mathfrak{M}_N$ because in the latter case it is unclear how all time variables, one per each submanifold $\mathfrak{M}_i$, will combine together and define the time on the resulting manifold $\mathfrak{M}$. A point $\mathcal{Q}$ on the manifold $\mathfrak{M}$ is then identified by a set of coordinates as follows:

$$\mathcal{Q} = (\overbrace{t}^{\in\mathbb{R}^+}, \underbrace{q_1^1, q_1^2, \ldots q_1^{d_1}}_{\in\mathcal{M}_1}, \underbrace{q_2^1, q_2^2, \ldots q_2^{d_2}}_{\in\mathcal{M}_2}, \ldots, \underbrace{q_N^1, q_N^2, \ldots q_N^{d_N}}_{\in\mathcal{M}_N}). \quad (7)$$

The idea for which time flows at different rates for different biological processes is now mathematically modeled by introducing a set of projection maps Π_i such that:

$$\Pi_i : \mathbb{R}^+ \times \mathcal{M} \longrightarrow \mathbb{R}^+ \times \mathcal{M}_i$$
$$\mathcal{Q} \longmapsto \mathcal{Q}_i = \Pi_i(\mathcal{Q}) = (\tau_i(t) = t_i, \pi_i(\boldsymbol{q}) = \boldsymbol{q}_i), \quad (8)$$

where the spatial part $\pi_i : \mathcal{M} \longrightarrow \mathcal{M}_i$ is the canonical projection map which maps the curve Γ on $\mathcal{M}$ onto the curve γ_i on $\mathcal{M}_i$ while the temporal part $\tau_i : \mathbb{R}^+ \longrightarrow \mathbb{R}^+$ provides the connection between the time t on the manifold $\mathfrak{M}$ and the time t_i on the i-th manifold $\mathfrak{M}_i$ (**Figure 2**).

We emphasize that the relationship between t and t_i is not necessary linear. In fact, in the next section we will show that the time measured in a particular manifold depends upon the acceleration of the particle along a trajectory and it will produce a non-linear relation between t and t_i.

2.1.1. Example: Decomposing the Torus

We now provide a simple example which can be easily visualized in a three-dimensional space. We consider a manifold, the torus embedded in the three dimensional space $\mathbb{R}^3$, which can be decomposed into two circles, the two submanifolds. In this particular example, this is the only possible decomposition (Tu, 2011).

The torus, embedded in $\mathbb{R}^3$, is defined by the following set of equations:

$$\begin{cases} x(\theta,\phi) = (R + r\cos\theta)\cos\phi \\ y(\theta,\phi) = (R + r\cos\theta)\sin\phi \\ z(\theta,\phi) = r\sin\phi \end{cases} \tag{9}$$

and is decomposed into the two submanifolds S^1_1 and S^1_2 given by:

$$S^1_1 = \left\{ re^{i\theta} : \theta \in [0, 2\pi) \right\}, \quad S^1_2 = \left\{ Re^{i\phi} : \phi \in [0, 2\pi) \right\}, \tag{10}$$

where, θ identifies the poloidal direction (along the orange vertical circle in **Figure 2**), ϕ identifies the toroidal direction (along the blue horizontal circle in **Figure 2**), R the major radius (distance from the center of the torus), and $r < R$ is the minor radius of the torus (radius of the tube). These two circles can represent two submanifolds related to two different biological processes, and their Cartesian product produces the torus. Therefore, the two biological space-times associated to this particular example, and hence to these two biological processes, will be given $\mathfrak{M}_1 = \mathbb{R}^+ \times S^1_1$ and $\mathfrak{M}_2 = \mathbb{R}^+ \times S^1_2$. A trajectory Γ on the torus, identified by the equations:

$$\begin{cases} x(t) = (R + r\cos(2\pi nt))\cos(2\pi mt) \\ y(t) = (R + r\cos(2\pi nt))\sin(2\pi mt) \\ z(t) = r\sin(2\pi mt) \end{cases} \tag{11}$$

where $t \in \mathbb{R}^+$, is then decomposed onto two trajectories:

$$\begin{aligned} s_1(t) &= \left\{ re^{2\pi nit} : t \in [0,1) \subset \mathbb{R}^+ \right\}, \\ s_2(t) &= \left\{ Re^{2\pi mit} : t \in [0,1) \subset \mathbb{R}^+ \right\}, \end{aligned} \tag{12}$$

where n and m are the winding numbers associated with the poloidal and toroidal directions, respectively. The two submanifolds (two circles) have two different trajectories defined by Equation (12). However, when their Cartesian product is considered, the resulting manifold (the torus) has a single unique trajectory on it defined by Equation (11). In this particular example, the magnitude of the velocity and the acceleration of the projected motion on the two submanifolds are given by:

$$\begin{cases} v_1 = 2n\pi r \\ v_2 = 2m\pi R \end{cases} \quad \text{and} \quad \begin{cases} a_1 = 4n^2\pi^2 r \\ a_2 = 4m^2\pi^2 R \end{cases} \tag{13}$$

which implies $a_2 > a_1$ for the same value of n and m. The position, the velocity and the acceleration on the torus are projected onto the two subspaces in which the dynamics are identified by different values for the position, velocity and

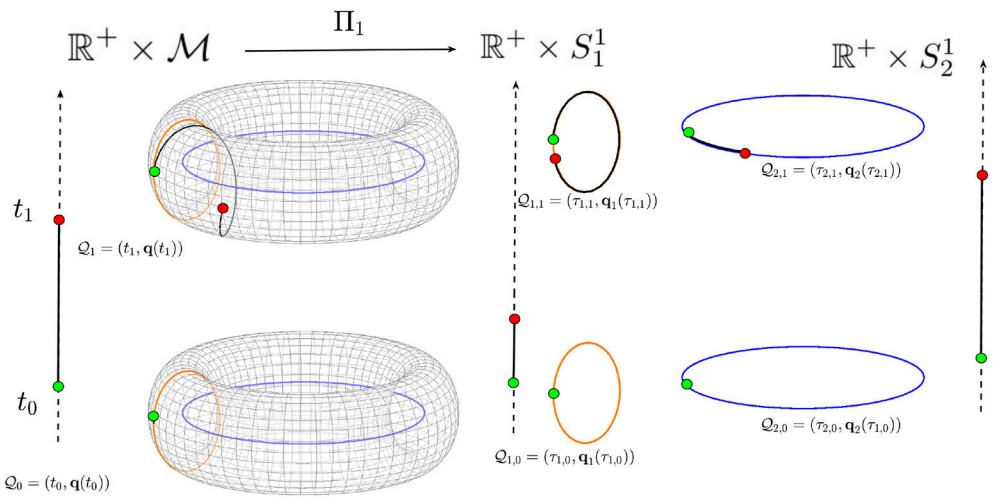

FIGURE 2 | A torus is shown as an example of a possible biological space-time manifold that may be decomposed into submanifolds. The time $\mathbb{R}^+$ is represented by the vertical arrows. The torus is decomposed into the two circles (submanifolds) $M_1 = S^1_1$ and $M_2 = S^1_2$. The point $\mathcal{Q}_0$ at time t_0 is mapped onto the points $\mathcal{Q}_{1,0}$ and $\mathcal{Q}_{2,0}$ while the point $\mathcal{Q}_1$ at time t_1 is mapped onto the points $\mathcal{Q}_{1,1}$ and $\mathcal{Q}_{2,1}$ in the two space-time submanifolds $\mathfrak{M}_1 = \mathbb{R}^+ \times S^1_1$ and $\mathfrak{M}_2 = \mathbb{R}^+ \times S^1_2$. The trajectory on the torus is then mapped onto two different trajectories on the two circles from the initial point (green circle) to the final point (red circle).

acceleration. If in Equation (11) we set $m = 0$, the trajectory reduces to a circle around the poloidal direction. In this case, Equation (13) become:

$$\begin{cases} v_1 = 2n\pi r \\ v_2 = 0 \end{cases} \quad \text{and} \quad \begin{cases} a_1 = 4n^2\pi^2 r \\ a_2 = 0 \end{cases} \tag{14}$$

This example shows that there can be motion on the torus and on one submanifold, but an absence of motion on the other submanifold.

The example of the torus illustrates how trajectories on submanifolds (e.g., representations of different biological dynamics) with different accelerations may be combined and interpreted as a single trajectory on a larger manifold, and vice versa. It must be noted that in this decomposition, the time t in the submanifolds is a parameter along the curve and not the biological time. In fact, as we will see in the next section, because the accelerations in the two submanifolds are different we expect time to flow at different rates in each submanifold.

3. DILATION AND CONTRACTION OF TIME

The fact that the Maxwell's equations are invariant under the Lorentz transformation implies that any inertial observer will measure light moving at the same constant speed c. Moreover, the Lorentz transformations define an invariant quantity, called *proper time*, which represents a space-time interval which assumes the same value for any inertial (non-accelerating) observer. In physics, the presence of fundamental equations such Maxwell's equations, helps us in understanding the type of coordinate transformations that leave the equations unchanged and can be used to define invariant quantities. Here, we deal with a biological space-time for which the existence of fundamental equations, coordinate transformations, and corresponding invariant quantities, are unknown.

Although many investigators have posed the question of whether or not governing laws exist in biology (Ruse, 1970; Brandon, 1997; Wagner, 2017), we contend this question has yet to be conclusively answered. Rather, we believe a more fundamental question is to investigate the nature of the space-time in which any biological phenomenon occurs. In the context of Special Relativity, it has been shown by Levy-Leblond (1976) that if the space is homogeneous and isotropic, the Lorentz transformations are characterized by a parameter with the dimensions of a velocity which is an intrinsic property of the space. Moreover, the meaning of this parameter is related to the maximum velocity at which information can travel or be transmitted (Brunner et al., 2004) and this velocity turns out to be the speed of light c.

3.1. Biological Invariants and Information

Although information in the context of biological processes can not be easily defined (Gatenby and Frieden, 2007), it can be related to the efficiency in the conversion of energy into order (Frieden and Gatenby, 2011). In our biological context, assuming our biological space-time to be homogeneous and isotropic, there will be a parameter analogous to c, denoted by $\tilde{c}$, which can be related to the rate of conversion of energy into order. We stress the fact that, although we are not sure about the meaning of information in this particular context, we assume that the meaning of $\tilde{c}$ is to provide an upper limit for the information to be transmitted. This upper limit is what we believe is an invariant quantity in the biological space-time.

As described in section 2.1, a point $\boldsymbol{q} \in \mathcal{M}$ defines the state of a biological process. In what follows we assume the manifold to be a flat plane and to be constructed such that two points $\boldsymbol{q}(t)$ and $\boldsymbol{q}(t + dt)$ represent the information of the system at time t and $t + dt$, respectively (see **Figure 3**). The existence of a value $\tilde{c}$ defines a circular region Ω, centered at $\boldsymbol{q}(t)$ with boundary $\partial\Omega$ given by the circumference with radius $r = \tilde{c}dt$, such that $||\boldsymbol{q}(t) - \boldsymbol{q}(t + dt)|| < \tilde{c}dt$. In other words, the value of $\tilde{c}$ defines a front of information which propagates in time along the manifold (see **Figure 3**). In the case of a generic manifold $\mathcal{M}$, we will have a generic region Ω bounded by a curve $\partial\Omega$ which will depend on the geometry of the manifold. In this case the distance can be evaluated once the metric tensor $g_{\mu\nu}$ is known. Moreover, the existence of such a parameter defines the non-relativistic and the relativistic limits in this context. The former occurs for velocities $v << \tilde{c}$ while the latter occurs for velocities $v \sim \tilde{c}$ (see **Figure 3**, top and bottom left). In analogy with the Special Relativity case, we refer to relativistic corrections, as those corrections which need to be made in order to correctly describe and characterize the dynamics of the moving particle with velocity $v \sim \tilde{c}$. We want to clarify the fact that at this stage we do not have a procedure to build a manifold for a given biological process, rather, we assume the existence of the manifold.

3.2. Special Relativity and Biological Processes

Special relativity is universally recognized as a theory which describes properties of the ordinary space-time in which physical phenomena occur. In the case of a biological process, unlike the physical phenomenon, the biological space-time is not known a priori and it needs to be mathematically constructed. In this section we assume the existence of such a space and we consider the motion of two frames of reference whose coordinates are related by a set of coordinate transformations. In particular we investigate the dilation and contraction of biological time resulting from accelerated motions. This approach is used to model and to explore different rates of aging.

As shown by Levy-Leblond (1976), Lorentz transformations are the only set of transformations of coordinates in a homogeneous and isotropic space which also preserve the group structure and the causality condition. Therefore, assuming our space to be homogeneous and isotropic, we assume their validity also in a biological space-time. In this case, instead of the speed of light c, the Lorentz transformations will depend on the biological invariant $\tilde{c}$.

We consider two frames of reference S and S' in the flat Minkowski (the space given by considering the time component and the ordinary three-dimensional space) space-time $M^4 = \mathbb{R} \times \mathbb{R}^3$. In general, the motion of a particle is along a curvilinear trajectory, and the two frames of reference can be in a relative

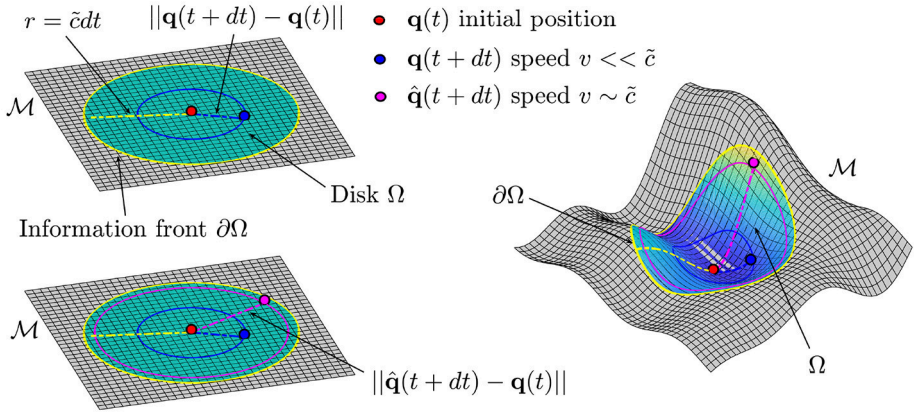

FIGURE 3 | Top left. Representation of the information front $\partial\Omega$ propagating at speed $v = \tilde{c}$ from the center of the disk Ω on the flat plane $\mathcal{M}$. The distance traveled by a point in the interval of time dt is given by $||\boldsymbol{q}(t+dt) - \boldsymbol{q}(t)||$ and it will be always less than $\tilde{c}dt$ (dashed yellow line), where $\tilde{c}$ is the maximum speed of information. **Bottom left**. The analogous of the relativistic corrections are needed if we want to fully characterize the dynamics of a particle which is moving at speed $v \sim \tilde{c}$ Right. In the case of a generic manifold $\mathcal{M}$ the information front $\partial\Omega$ will be the boundary of a region Ω.

motion like the one represented in **Figure 4** on the left. However, for simplicity, we assume a relative motion with a constant relative velocity v along the x-direction. We denote with (t, x, y, z) and (t', x', y', z') an event recorded by S and S', respectively. These two events are linked by the Lorentz transformations

$$x' = \gamma(x - vt), \quad y' = y, \quad z' = z, \quad t' = \gamma\left(t - \frac{vx}{\tilde{c}^2}\right),$$
$$\gamma = \frac{1}{\sqrt{1 - v^2/\tilde{c}^2}}, \tag{15}$$

where $\gamma > 1$ is the Lorentz factor and $\tilde{c}$ is the biological invariant previously introduced. It is straightforward to show that these transformations leave invariant the space-time interval $d\tau^2 = \tilde{c}^2 dt^2 - dx^2 - dy^2 - dz^2$ which we will call *biological proper time*. The invariance of the biological proper time will lead, also for biological systems, to the well known phenomenon of *time dilation*:

$$dt' = \gamma\, dt. \tag{16}$$

The time interval dt' measured from an observer in motion is dilated by the factor γ and we say that a moving clock runs slower. It has to be remarked that this effect is reciprocal when both frames of reference are inertial (there is a relative motion at constant speed between them). In that case, no preferred frame of reference exists and each observer can argue that the other one is moving. For this reason, we do consider accelerating frames of reference in which this ambiguity is solved. We now consider the particular case in which a point is moving along a straight line oriented along the x-axis with a constant acceleration a'_x (**Figure 4** on the right). We assume S' to be located at the position of the particle and therefore, its velocity is the same as the velocity of the particle and it increases in time. In this particular situation, it can be shown, (see Appendix A), that the time t' of the accelerated particle, and hence in the accelerated frame of reference, is given by:

$$t' = \frac{\tilde{c}}{a'_x} \operatorname{arcsinh}\left(\frac{a'_x t}{\tilde{c}}\right). \tag{17}$$

This equation tells us how much time t' elapses in the accelerated frame of reference S', in terms of the time t elapsed in the inertial frame of reference S. This function represents the time component $\tau_i(t)$ of the projection map Π_i introduced in Equation (8). In the case of an accelerating frame of reference, the phenomenon of time dilation is not reciprocal and the observer in the frame of reference S' who experiences the constant acceleration cannot argue that the other observer is moving and he is still. Therefore, the time t' of the accelerating frame of reference measured from the observer at rest in the frame of reference S appears to run slower according to Equation (17). In what follows we will apply this idea to few biological processes always assuming that the time dilation is described by Equation (17).

3.3. Biological Age

Many markers have been used to define biological age. In our modeling framework, biological time is defined as the rate at which biological processes take place, as measured against chronological time. Therefore, our model characterizes one's biological age by the degree of dilation or contraction of time resulting from the acceleration or deceleration of biological processes that are associated with biological age.

One marker of biological age is the methlyation state of the genome, composed of varying degrees of hyper- or hypomethlyated states (Bocklandt et al., 2011; Horvath, 2013). The "epigenome" is a component of gene regulation and has been proposed to contain information relevant to the overall state of the biological system (Jenkinson et al., 2017). Linear age-related epigenetic drift has been associated with cancer incidence (Curtius et al., 2016), and statistical methods have been proposed

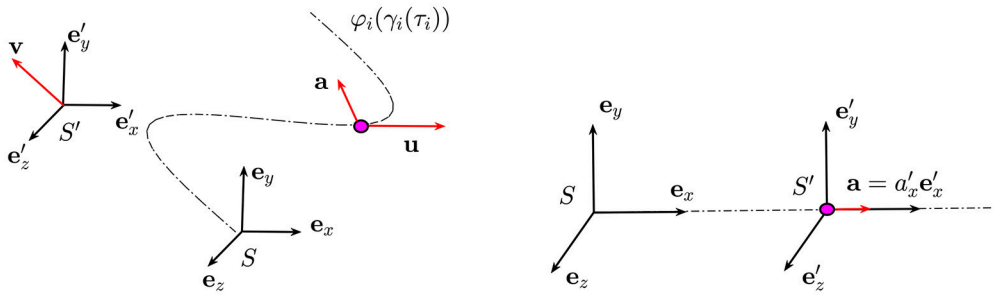

FIGURE 4 | On the left) The particle (purple dot) is moving along a trajectory with velocity $\boldsymbol{u}$ and acceleration $\boldsymbol{a}$. On the right) The frame of reference S' is moving at constant velocity $\boldsymbol{v}$ away from S: the components of the velocity and the acceleration of the particle in these two frames of references are related by Equations (A.1) and (A.2).

to identify deviation from time linearity in epigenetic aging, although without a theoretical rationale to describe—or predict—the nature of the non-linearity (Snir et al., 2016).

In order to explain the process of changing methylation states with age, and the use of methylation state as a surrogate marker of biological age with our model, we first consider the trajectory on a manifold which describes the methylation process of a living person and we assume that this trajectory is mapped onto a straight line in the Minkowski space. We now consider one frame of reference S at rest in the origin of the trajectory and one frame of reference S' that is moving at constant acceleration a'_x for which the time t' is given by Equation (17). We then assume that the frame of reference slows down to an acceleration $a''_x < a'_x$ for which the time is t'' and it is given by Equation (17). We define the rate of methylation dM/dt' and dM/dt'' when the frame of reference moves with acceleration equal to a'_x and a''_x, respectively and we have:

$$\frac{dM}{dt'} = \frac{dM}{dt}\frac{dt}{dt'}, \qquad \frac{dM}{dt''} = \frac{dM}{dt}\frac{dt}{dt''}. \tag{18}$$

If $a'_x > a''_x$ it can be shown that $dt'/dt < dt''/dt$, and therefore, we conclude that:

$$\frac{dM}{dt'} > \frac{dM}{dt''}. \tag{19}$$

The slowing down of the rate of demethylation is therefore interpreted as a decelerating frame of reference. We note that this model does not assume any specific functional form for the methylation trajectory. In fact, during the lifetime of a person, Equation (18) permits the acceleration or deceleration of age-related changes to the methylation state, which can be accentuated or modified in the context of cancer.

4. EXAMPLES OF SPACE-TIME DYNAMICS IN BIOLOGY

In this section we connect our model to different rates of aging between individuals, and discuss examples of precipitous or protracted aging within an individual.

4.1. Aging

We define aging as the functional and structural decline of an organism, resulting in an increasing risk of disease, impairment and mortality over time. At the molecular and cellular level several hallmarks of aging have been proposed to define common characteristics of aging in mammals (López-Otín et al., 2013). These hallmarks include: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, altered nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered cell-cell communication. These processes are interdependent and influenced by cell microenvironmental cues.

Ultimately, the rate of age-related decline varies depending on how genetic variation, environmental exposure and lifestyle factors impact these mechanisms. Consequently, age, when measured chronologically, is often not a reliable indicator of the body's rate of decline or physiological breakdown. Over the years, the idea of quantifying the "biological age" based on biomarkers for cellular and systemic changes that accompany the aging process have been explored (Levine, 2013), although without a rigorous mathematical treatment of biological age. Major efforts have been made to dissect the individual contribution of each of these hallmarks and factors on aging, but the major challenge remains how to determine how their interconnectedness as a whole impacts the aging dynamics. Here we connect concepts in our mathematical model to biological and chronological aging. We note that for the sake of clarity, we will refer to aging as precipitous (faster than chronological aging) or protracted (slower) in order to clearly associate the terms "accelerated" and "decelerated" to quantities in our modeling framework.

As we derived in the previous section, an unambiguous time dilation effect requires an accelerating frame of reference. We now imagine a range of accelerations representing a distribution within a population between the values a_{min} and a_{max} (green region in **Figure 5** on the left). The dashed and dotted curves corresponds to Equation (17) by using the values a_{min} and a_{max}, respectively. Any acceleration within this range will correspond to a curve in the green region. An interval of time Δt on the x-axes corresponds to a reduced interval of time $\Delta t'$ on the y-axis and therefore, the ticking of the second clock is slowed down. The existence of a distribution, or range of values of acceleration is

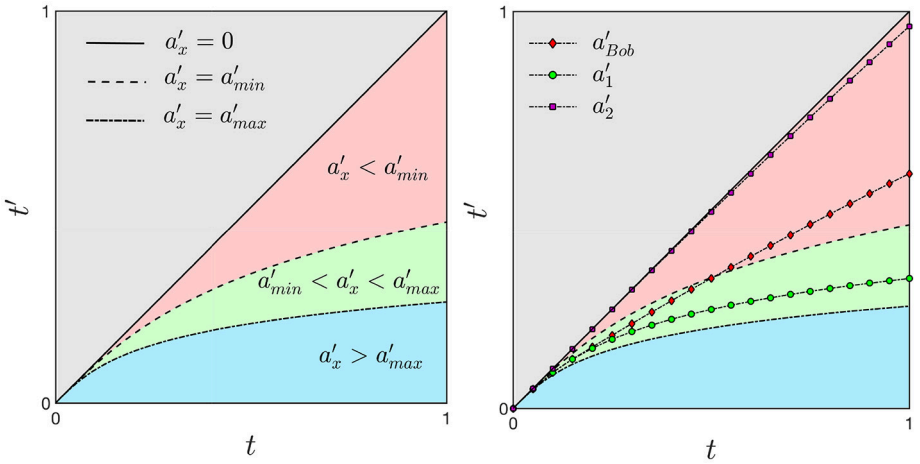

FIGURE 5 | On the left) For an accelerating frame of reference a time interval $\Delta t = 1$ corresponds to shorter $\Delta t'$ with an consequent dilation of time. The green region represents a distribution of accelerations associated with an average range of accelerations that is used as a common reference for accelerated and decelerated processes. Smaller values of the acceleration are related to time contraction (pink region, $a'_x < a'_{min}$) while large values are associated with time dilation (blue region, $a'_x > a'_{max}$). On the right) In the mental illustration of the lifetime of Bob, a deceleration will bend Bob's trajectory (black line with red diamonds markers) out of the green region with a corresponding contraction of time.

necessary to capture both features of time dilation, (green region in **Figure 5** on the left) and time contraction, when the value of the acceleration is less than a_{min} (pink region). Moreover, values of the acceleration much larger than a_{max} can be used to define the arrest of biological time such as in a hibernating state or cryogenic freezing (blue region). The case of zero acceleration is given by Equation (16) and is not considered in this model context.

4.1.1. Alice and Bob

We illustrate the contraction and dilation of time in biological space-time (i.e., on a submanifold) and how they can be related to the presence of a disease, we consider the following thought experiment involving Alice and Bob, two individuals with different rates of aging. For simplicity, we assume that their dynamics occur on a flat torus $\mathcal{T}^2$ defined as the Cartesian $\mathbb{R}^2$ plane under the identifications $(x, y) \sim (x + 2\pi, y) \sim (x, y + 2\pi)$. The opposite edges x, $x + 2\pi$ and y, $y + 2\pi$ of the domain are identified and therefore, they must be interpreted as the same point (see red and blue edges in **Figure 6**). In other words, the trajectory that comes out from the right edge of the plane, will come in from the left edge and the same applies from the upper and lower part of the plane. In this case, the manifold is trivially mapped into $\mathbb{R}^2$ with the identity map, and hence the dynamics on the manifold corresponds to the dynamics in the Minkowski space-time.

The point on the manifold corresponding to the birth of Alice and Bob is indicated in **Figure 6** by the green circle in which we assume to place a frame of reference S identified by the three unit vectors $(\boldsymbol{e}_x, \boldsymbol{e}_y, \boldsymbol{e}_z)$. The dynamics of Alice is represented by the motion of a purple point in which we imagine to place a frame of reference S'_A. We also assume that the point is moving along a straight line with constant acceleration given by $\boldsymbol{a}'_A = a'_A \boldsymbol{e}'_x$. The same conditions apply to Bob, but in this case the frame of reference is S'_B and the particle is moving with a constant acceleration $\boldsymbol{a}'_B = a'_B \boldsymbol{e}'_x$ (see **Figure 6**).

We now can apply the results obtained in the previous section to infer that the time in S'_A and S'_B are given by Equation (17):

$$t'_A = \frac{\tilde{c}}{a'_A} \operatorname{arcsinh}\left(\frac{a'_A t}{\tilde{c}}\right), \quad t'_B = \frac{\tilde{c}}{a'_B} \operatorname{arcsinh}\left(\frac{a'_B t}{\tilde{c}}\right). \tag{20}$$

Assuming the acceleration of Alice to lie within the range $[a_{min}, a_{max}]$, her proper time t'_A lies in the green region (see **Figure 5** on the right). On the other hand, Bob's frame of reference is moving with acceleration $a'_B < a'_A$ and its proper time $t'_B > t'_A$ deviates from the green region toward an older biological age in the pink region. With respect to Alice, Bob is moving with a slower acceleration and hence, his clock is running faster, resulting in a time contraction with respect to Alice's clock. If instead we assume $a'_B > a'_A$, Bob's proper time t'_B will be larger than t'_A and it deviates from the green toward the blue region. In this case Bob's clock will run slow with a consequent more evident effect of time dilation. Therefore, different rates of aging could be generally compared between individuals by determining their accelerations as compared to a frame of reference at rest.

We now consider only Bob and we assume his frame of reference S'_B to have an acceleration a'_1 within the range $[a'_{min}, a'_{max}]$. Suddenly, his acceleration is decreasing to the value a'_2 and we therefore want to know what will happen to its proper time t'. In particular we hypothesize the acceleration to change as follows:

$$a'_B = \begin{cases} a'_1 & t < t_1 \\ \alpha + \beta \cos(\pi (t_1 - t)/(t_2 - t_1)) & t_1 \leq t \leq t_2 \\ a'_2 & t > t_2 \end{cases}$$

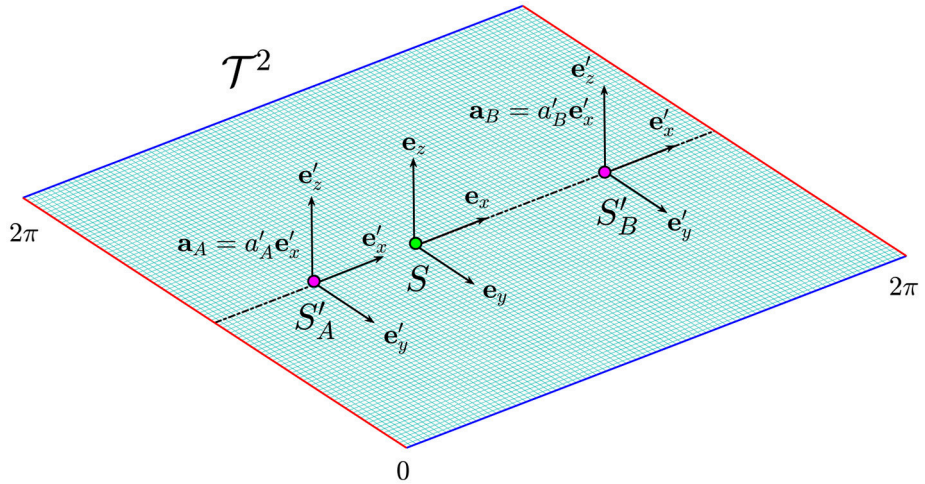

FIGURE 6 | The flat torus $\mathcal{T}^2$ in which the dynamics of Alice (S'_A) and Bob (S'_B) occur along the straight dotted line. The green and the purple dots represent their birth and their actual position on the manifold. The effect of time dilation is more evident for Alice whose frame of reference moves at higher acceleration.

where $\alpha = (a'_1 + a'_2)/2$ and $\beta = (a'_1 - a'_2)/2$. By solving Equations (A.5) and (17) we obtain the time t' for the decelerating frame of reference shown in **Figure 5** on the right: the line marked with green circles and the line with violet squared markers represent the trajectory corresponding to the accelerations a'_1 and a'_2, respectively. However, due to the deceleration, the line marked with red diamonds will not follow the line with the green circles but it will move outside the green region. The deceleration of Bob's frame of reference is reflected to a contraction of time while a larger time dilation is a consequence of a frame of reference with an increasing acceleration.

4.2. Cancer as Inducer of Precipitous Aging

Major pathologies, such as cancer, diabetes, cardiovascular disorders and neurodegenerative diseases have an impact on aging. Cancer and chemotherapy in particular are known to accelerate the aging process (Alfano et al., 2017). As described above, aging involves multiple complex changes at molecular and cellular level that lead to decline in physiologic reserve capacity across virtually all organ systems (Seals et al., 2016). In cancer, accumulation of cellular damage aggravates the hallmarks of aging and accelerates aging. In cancer patients, the trajectory of decline worsens not only because of the direct physiologic insult inflicted by cancer, but also because of the injuries induced by anti-cancer therapies, such as chemotherapy or radiation, to systems that maintain physiologic reserves (Koelwyn et al., 2012; Hurria et al., 2016). In this context, there is a dose-dependent effect, whereby the more intensive the treatment of cancer or more vulnerable and frail the physiologic state, the steeper the decline in physiologic reserves (Henderson et al., 2014; Kohanski et al., 2016). Below, we use these two variables, intensity of therapy and frailty, to illustrate our model of accelerated aging.

It is not surprising that the two cancer populations most affected by precipitous aging caused by cancer are survivors of childhood cancer who are typically exposed to intensive multi-agent therapy at a young age (Henderson et al., 2014; Ness et al., 2015) and adult patients who undergo hematopoietic cell transplantation (HCT) for refractory hematologic malignancies (Arora et al., 2016). These two scenarios can be explained by our model as follows. The early onset of advanced biological age in childhood cancers corresponds to a rapid contraction of time that deviates away from the green region, similar to Bob's modified trajectory, but with an earlier onset (see **Figure 5** on the right). The case of allogeneic HCT, in which case the transplanted cells come from another person, corresponds to a discontinuity in biological space-time. The recipient's trajectory on the hematopoietic manifold and/or the biological age is reset to that of the donor. If the biological ages of the donor and recipient of the HCT do not agree, the recipient will experience an abrupt and persistent change in the rate of biological aging corresponding to the discontinuity in both biological space and time.

Frailty, characterized by a cluster of measurements of physical states, is the best described measure of aging in a population, and identifies individuals who are highly vulnerable to adverse health outcomes and premature mortality. Although frailty is not a perfect corollary with biological age, it is a measure of abnormal aging at a population level. In long-term survivors of childhood cancer [median age 33 years (range 18–50 years)], the prevalence of frailty (Rockwood and Mitnitski, 2011) has been shown to be comparable to that reported among adults greater than 65 years of age (Ness et al., 2013). Comparable high rates have been reported in adult survivors of HCT (Arora et al., 2016). In fact, frail HCT recipients have a three-fold increased risk of subsequent mortality compared with the non-frail counterparts, (Arora et al., 2016) which is similar to the downstream consequences of frailty seen in the general population such as adverse health outcomes (Rockwood and Mitnitski, 2011), and early mortality (Fried et al., 2001; Hogan

et al., 2003). From the lens of our model, frailty can be associated with a biological age and therefore defined as a threshold in time (t'_f) so that the onset of frailty in the cancer population (t_c, t'_f) will appear sooner relative to the general population (t_g, t'_f) where $t_c < t_g$.

4.2.1. Chronic Inflammation

The normal process of aging is associated with chronic low grade inflammation and with cumulative oxidative stress, independently of disease. Inflammation and oxidative stress are critical responses in host defense and injury repair and are essential for normal body functions. However, with advanced age there is a loss of sensitivity in the injury-repair cycle leading to persistent chronic inflammation, and a natural decline in the endogenous anti-oxidant capacity leading to cumulative oxidative stress (Mittal et al., 2014). These two process are interdependent, and contribute to the hallmarks of aging, influencing telomere length, mitochondrial function, epigenetics and stem cell self-renewal.

Chronic inflammation and oxidative stress are also common underlying factors of age-associated diseases. Inflammation, particularly chronic low-grade inflammation, has been found to contribute to the initiation and progression of multiple age-related pathologies such as type II Diabetes, Alzheimer's disease, cardiovascular disease and cancer (Mantovani et al., 2008). In addition, chronic diseases, and in particular cancer, elicit and promote an inflammatory tumor microenvironment that increases cancer fitness. Therefore, chronic inflammation has been suggested to underlie and accelerate biological aging (Fougère et al., 2017), in particular when associated with disease. The concept of "inflamm-aging," inflammation-associated aging, can be used to provide a systemic perspective of biological aging in the human population (Franceschi et al., 2007).

On the level of tissue homeostasis, the best characterized example is hematopoietic aging associated with chronic inflammatory signaling in the bone marrow microenvironment. In fact, the "age" of a young hematopoietic stem cell can be "reprogrammed" when transplanted into an aged or inflammatory environment (Kovtonyuk et al., 2016), highlighting the impact of "inflamm-aging" and the plasticity of molecular clocks. Aging is associated with clonal hematopoiesis and accumulation of mutations in hematopoietic progenitors (Steensma et al., 2015), likely due to the underlying inflammation in the aged bone marrow niche. Indeed, an inflamed bone marrow can induce pre-leukemic conditions in mice similar to those occurring in elderly patients (Wang et al., 2014; Dong et al., 2016). Our model can be applied to measure how microenvironmental cues, such as inflammation, impact aging of hematopoietic cells.

4.3. Protracted Aging

In contrast to precipitous biological aging which corresponds to the contraction of biological time, protracted aging corresponds to the dilation of biological time. This can be illustrated by prolonged periods of near zero biological activity, for instance in freezing conditions or hibernation which is part of a continuum of biological and metabolic states (van Breukelen and Martin, 2015). How can this situation be explained in a relativistic framework? We imagine a *cryogenic*-manifold mapped onto a Minkowski space. If in this space the frame of reference is moving with a high acceleration in the direction of motion then the interval of time in its frame of reference tends to zero: a frozen cell corresponds to a frame of reference moving at very *large acceleration relative to its unfrozen state* on this particular biological manifold. **Figure 5** shows the limiting case of a near perfectly frozen (cryogenic) biological process (blue region), which would correspond to an extreme dilation of time, or equivalently, a very large relativistic correction.

5. DISCUSSION

Here we have investigated a mathematical model of biological aging. We define a biological space-time by mathematically combining manifolds and submanifolds, and apply the principles of relativity to compare different rates of biological aging. We illustrate the concepts of precipitous and protracted aging as relativistic corrections of biological time with a mental illustration comparing the lifespans of two individuals, Bob and Alice. This analogy provides a framework to compare the rates of aging between individuals by determining their rate of acceleration as compared to a common frame of reference.

A critical component of our theory is the construction of "biological space-time," hence a submanifold that represents a biological process. Following the work of (Levy-Leblond, 1976) we believe that the "biological space-time" should have the properties of being homogeneous and isotropic. If we also require that the transformations between frames of reference in this space preserve the group composition law and causality, the transformations are characterized by a parameter $\tilde{c}$, that has the dimensions of a velocity, which is an intrinsic property of the space (Levy-Leblond, 1976). In analogy with the case of the Lorentz transformation for which this parameter is the speed of light c and it represents the upper limit for the information to be transmitted (Brunner et al., 2004), we believe that $\tilde{c}$ represents the maximum speed at which information can be transmitted in the biological space-time. However, it might be possible that a space which satisfies the above constraints can not be built and hence, our theory would not apply.

On the other hand, given our ability to determine the value of $\tilde{c}$ and to represent dynamics along a trajectory on a manifold, our theory can be falsified or verified by considering the following cases. The first case occurs if the particle on the manifold moves with a constant velocity v. In this case, no accelerations are present and hence our theory based on accelerations does not apply. The second case takes place if the particle has an acceleration but its velocity $v << \tilde{c}$. The particle is in a non-relativistic regime and hence, relativistic corrections are not needed. On the other hand, if accelerations are present and if $v \sim \tilde{c}$, we are in the relativistic regime and hence, relativistic corrections must be taken into account if we want to properly describe the dynamics on the manifold. We note that we construct this theory with the intention of using it to better understand biological aging. We therefore postulate that a $\tilde{c}$ may exist—or be assumed—on the same scale of observed velocities from human aging data so that we may use the theory to better study the data.

Once the submanifolds are constructed, then it is always possible to combine them considering their Cartesian product defined by Equation (4). Given our ability to construct these submanifolds, the comparison between the dynamics along different trajectories can be done if and only if they do belong to the same submanifold. So for example, if we are interested in comparing different rates of aging of two individuals with respect to the methylation status, we will need to construct the methylation status manifold, evaluate the trajectories of their methylation status in time, and analyze the dynamics on each of them. In particular, the comparison between the accelerations along both trajectories may help in understanding the difference between the rate of aging of the two individuals. In this sense, the importance is the different dynamics along each trajectory and not the fact that there are two different trajectories.

Other groups have proposed a framework for biological time to explain biological rhythms and other oscillating or biological processes that repeat periodically (Bailly et al., 2011). Our biological space-time model is more general, and is aimed towards explaining non-linear biological phenomena that are not necessarily periodic or oscillating, although these can be considered special cases of more generalized trajectories. In complement to Noble's hypothesis that there exists no privileged level of causation in biology (Noble, 2012), we suggest that the multiple scales of biology may be interpreted as trajectories on distinct manifolds that may be combined and coupled to each other. Although generalizations of relativity and space-time have been proposed, here we adapt these mathematical structures in order to interpret trajectories on manifolds to represent biological processes (O'Neill, 1983). We want to note that, as described in section 2.1.1, there can be submanifolds in which the velocity and the acceleration are identically zero. However, this does not imply an absence of motion on the other submanifolds (the other circle S_2^1) or on the principal manifold (the torus). In this case, it will be impossible to draw any conclusion about the possible difference between chronological and biological time for the process that takes place in the manifold in which the velocity, and hence the acceleration, is zero.

A novel concept which naturally follows from our approach is the notion of *force* in a biological context. A biological force may be a generalization of a physical force in a biological context which changes the dynamics of a particle on a manifold. We may hypothesize then, that accelerated dynamics on the manifold may be the effect of biological forces acting on the particle. For example, cancer and chemotherapy may be interpreted as forces which combine and have a resultant which may change the dynamics of the point on a manifold with a consequent impact on the rate of aging.

The shape of the manifolds which characterize biological space-time also affect our notion of distance, which requires the knowledge of the metric tensor of the manifold. Although a manifold is defined to be locally Euclidean, two biological processes or objects that are sufficiently different from each other (in space-time) may not be measured by using the conventional definition of Euclidean distance. The degree to which we must redefine our notion of a *metric* in the biological space-time may also affect our ability to interpret long-time dynamical behaviors of biological systems and whether or not relativistic corrections are required in order to properly analyze these dynamics. We therefore hypothesize that the underlying geometry of the manifold, or equivalently, the elements and structure of the metric tensor, may provide valuable insights into the biological process.

6. SUMMARY

In summary, we have proposed a mathematical framework and criteria that may be used to define and construct a biological space-time. We use this as a tool to model and study different rates of biological aging based on the concept of relativistic corrections of time due to the acceleration or deceleration of biological dynamics relative to a common frame of reference. We discuss some examples of biological processes that illustrate these concepts, provide criteria that may be used to test or falsify the theory, and discuss implications and novel hypotheses that are generated by this model.

AUTHOR CONTRIBUTIONS

RR and DM: Conception, design, and approval of final manuscript. All authors: Manuscript writing and editing.

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APPENDIX A

By differentiating Equation (15) we obtain the transformations for the velocity

$$\begin{aligned} u'_x &= \frac{dx'}{dt'} = \frac{dx - vdt}{dt - vdx/\tilde{c}^2} = \frac{u_x - v}{1 - vu_x/\tilde{c}^2}, \\ u'_y &= \frac{dy'}{dt'} = \frac{u_y}{1 - vu_x/\tilde{c}^2}, \\ u'_z &= \frac{dz'}{dt'} = \frac{u_z}{1 - vu_x/\tilde{c}^2}. \end{aligned} \tag{A.1}$$

where $u_x = dx/dt$, $u_x = dx/dt$ and $u_x = dx/dt$ are the velocities along the corresponding direction. An additional differentiation leads to the transformations of the acceleration

$$\begin{aligned} a'_x &= \frac{du'_x}{dt'} = \frac{du'_x}{dt}\frac{dt}{dt'} = \frac{a_x\left(1 - vu_x/\tilde{c}^2\right) + (u_x - v)\left(va_x/\tilde{c}^2\right)}{\gamma(1 - vu_x/\tilde{c}^2)^3} \\ &= \frac{a_x(1 - v^2/c^2)}{\gamma(1 - vu_x/\tilde{c}^2)^3} = \frac{a_x}{\gamma^3(1 - vu_x/\tilde{c}^2)^3} \\ a'_y &= \frac{a_y}{\gamma^2(1 - vu_x/\tilde{c}^2)^2} - \frac{u_y va_x/\tilde{c}^2}{\gamma(1 - vu_x/\tilde{c}^2)^3}, \\ a'_z &= \frac{a_z}{\gamma^2(1 - vu_x/\tilde{c}^2)^2} - \frac{u_y va_x/\tilde{c}^2}{\gamma(1 - vu_x/\tilde{c}^2)^3}, \end{aligned} \tag{A.2}$$

where we have evaluated dt'/dt by considering the last of Equation (15) and we used the definition of the Lorentz factor γ. The sets of Equation (A.1) and (A.2) provide the connection between the velocity and the acceleration of an object measured by an observer in S and another observer in S'. We now consider the particular case in which a point is moving along a straight line oriented along the x-axis with a constant acceleration a'_x (**Figure 4** on the right). We assume S' to be located at the position of the particle and therefore, its velocity is the same as the velocity of the particle and it increases in time. Hence, by setting $v = u_x$ the x-component of the acceleration a'_x becomes

$$a'_x = \frac{a_x}{\gamma^3(1 - u_x^2/\tilde{c}^2)^3} = \frac{a_x(1 - u_x^2/\tilde{c}^2)^{3/2}}{(1 - u_x^2/\tilde{c}^2)^3} = \frac{a_x}{(1 - u_x^2/\tilde{c}^2)^{3/2}}, \tag{A.3}$$

and the acceleration in S is given by

$$a_x = a'_x(1 - u_x^2/\tilde{c}^2)^{3/2}. \tag{A.4}$$

By integrating the above equation with respect to time and by imposing $v(0) = 0$ we obtain the velocity

$$u_x = \frac{a'_x t}{\sqrt{1 + a'^2_x t^2/\tilde{c}^2}}. \tag{A.5}$$

By integrating once again with respect to time and by setting $x(0) = \tilde{c}^2/a'_x$ we obtain the position

$$x(t) = \frac{\tilde{c}^2}{a'_x}\sqrt{1 + \frac{a'^2_x t^2}{\tilde{c}^2}}. \tag{A.6}$$

The time t' of the accelerated particle, and hence in the accelerated frame of reference, is given by integrating Equation (16)

$$\begin{aligned} t' &= \int_0^t \sqrt{1 - u_x^2(s)/\tilde{c}^2}ds = \int_0^t \frac{ds}{\sqrt{1 + (a'_x s/\tilde{c})^2}} \\ &= \frac{\tilde{c}}{a'_x}\,\mathrm{arcsinh}\left(\frac{a'_x t}{\tilde{c}}\right). \end{aligned} \tag{A.7}$$

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REVIEW
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Sleeping Beauty and the Microenvironment Enchantment: Microenvironmental Regulation of the Proliferation-Quiescence Decision in Normal Tissues and in Cancer Development

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Cells from prokaryota to the more complex metazoans cease proliferating at some point in their lives and enter a reversible, proliferative-dormant state termed quiescence. The appearance of quiescence in the course of evolution was essential to the acquisition of multicellular specialization and compartmentalization and is also a central aspect of tissue function and homeostasis. But what makes a cell cease proliferating even in the presence of nutrients, growth factors, and mitogens? And what makes some cells "wake up" when they should not, as is the case in cancer? Here, we summarize and discuss evidence showing how microenvironmental cues such as those originating from metabolism, extracellular matrix (ECM) composition and arrangement, neighboring cells and tissue architecture control the cellular proliferation-quiescence decision, and how this complex regulation is corrupted in cancer.

Keywords: quiescence, proliferation, growth, microenvironment, extracellular matrix, tissue architecture, cancer, cell cycle

INTRODUCTION

Proliferation is one of the most conserved and fundamental traits of cells. However, all cells from prokaryota to mammals stop proliferating at some point during their lifetime in a controlled and reversible process called quiescence (O'Farrell, 2011). But why did quiescence triumphed during evolution and how is it controlled? For a number of unicellular organisms, the answer is very straightforward: environmental limitations. These limitations, mainly in the form of nutrient scarcity, acted as selective pressures that favored the success of those unicellular organisms that could quit proliferation and then "wake up" later on when the conditions were more suitable.

The gift of "sleeping" and thus remaining viable in adverse situations was vital to the perpetuation of genetic material and the success and ubiquity of unicellular organisms and also to the appearance of their multicellular descendants. While several key traits of unicellular quiescence (e.g., core signaling pathways, survivability, and reversibility) remain in higher organisms, in more complex living systems, such as mammals in which most cells are found to be quiescent, (macro)environmental pressures do not seem to contribute to quiescence. Cells become quiescent even in the presence of abundant resources (Spencer et al., 2011, 2013; Valcourt et al., 2012; Fiore et al., 2017).

Under the optimal conditions of nutrients, growth factors, and mitogens, cells "know" when to proliferate, but they need to be "told" when to stop. In other words, although proliferation might be considered a default setting (Parr, 2012), cells retain a built-in program of quiescence, which is set off extrinsically. This ability to become proliferative-dormant is essential to the acquisition of the function, defined geometry, and size of complex organs such as the heart, kidney, brain, and mammary glands. In these organs, a cell resides in a milieu composed of extracellular matrix (ECM) molecules, soluble factors, and other cells that produce various chemical and physical signals. This milieu is termed as the microenvironment, and it influences all aspects of a cell's life. It is the microenvironment that "tells" the cell to stop proliferating and activates the quiescence program.

Changes in the microenvironment too can "wake up" cells in a controllable fashion; as is the case in situations where cells need to proliferate to perform their functions (e.g., dermal fibroblasts upon wound healing and lymphocytes during the immune response) or to compensate for cell loss (e.g., intestinal epithelial cells and epidermal cells). Of course, the cell itself can also influence its microenvironment. A cell can instruct and modify its microenvironment by remodeling the ECM, and physically and chemically networking with its neighboring cells. Nevertheless, the reciprocal exchange between the cell and its microenvironment (Bissell et al., 1982), that finely regulates all aspects of cell behavior and fate including quiescence, is disrupted in cancer. The microenvironment "enchantment" is lost and cells "wake up" to resume proliferation, but this time in an unrestrained manner.

Here, we consider molecular aspects of cell cycle regulation and discuss how the cooperation of different microenvironmental signals are critical to the proliferation-quiescence decision, and how this orchestrated regulation goes awry in cancer. Because cellular quiescence programs are actively triggered by tissue- and conditional—specific factors, we argue that uncontrolled cell division, seen frequently in cancer, does not solely depend on oncogenic activation of the cell cycle, but also occurs due to loss of control over quiescence imposed by the microenvironment.

CELL CYCLE REGULATION AND DEFINITION OF CELLULAR QUIESCENCE

Proliferation is one of the most conserved and elemental attribute of a living system. Because of its importance for reproduction, tissue growth, and regeneration, and also its status as a hallmark of cancer, a lot is known about the molecular mechanisms that control cell proliferation. To divide, cells must go through a series of necessary steps termed the cell cycle. The cell cycle is divided into four phases characterized by a set of discrete events: growth and preparation for DNA replication (G1), DNA replication (S), preparation for mitosis (G2), and mitosis (M) and culminates with cell division (**Figure 1**). Although a large number of signals can trigger cell cycle entry and their molecular details may vary, key elements of the cell cycle are extremely well-conserved from yeast to mammals (Harashima et al., 2013). For instance, as a rule, the cell cycle is triggered by growth-factor signaling pathways that activate cyclin-dependent kinases (CDKs) which in turn are cyclically activated and mainly regulated by another class of proteins called cyclins (Bloom and Cross, 2007). The content of cyclins is mainly controlled at the gene expression level and post-translationally by degradation via proteasome (Bloom and Cross, 2007; Harashima et al., 2013). Another layer of regulation is provided by the two groups of cyclin-CDK inhibitors, the INK4 inhibitors (p15, p16, p18, p19), and the kip/Cip proteins (p21, p27, p57) (Polyak et al., 1994a,b; Pagano et al., 1995; Sherr and Roberts, 1999), and by the retinoblastoma protein (pRb). pRb represses the transcription factor E2F, which initiates the transcription of cell cycle activators. CDK-mediated hyperphosphorylation of pRb releases E2F promoting cell cycle progression (Fischer and Muller, 2017).

Unlike proliferation, little is known about the molecular biology of cellular quiescence. While proliferating cells from different tissues share many similarities, quiescent cells differ a lot in their expression programs (Bissell, 1981; Coller et al., 2006; Coller, 2011). There exist several types of quiescence, differing from organism to organism, cell to cell, and at different times and locations within an organ (O'Farrell, 2011). Few studies have focused on defining the molecular markers and/or signatures of different types of quiescence induction (Johnson et al., 1975; Williams and Penman, 1975; Schneider et al., 1988; Gos et al., 2005; Coller et al., 2006; Liu et al., 2007; Coller, 2011). In general, quiescent cells possess a transcriptional profile different from cycling cells, achieved by downregulating proliferation and cell-cycle progression genes, and upregulating genes that are not only related to cell cycle inhibition but also give the cells new properties (Coller et al., 2006; Coller, 2011; O'Farrell, 2011). Furthermore, depending on the type of inhibition of proliferation, the group of upregulated genes in quiescence can vary considerably (Coller et al., 2006).

Given the non-uniform nature of cellular quiescence (Coller et al., 2006; O'Farrell, 2011) and hence, the lack of a universal marker and/or genetic signature thereof, the assessment and definition of quiescence pose a difficult task and is subject to ongoing debate. To determine quiescence, some researchers have relied on the expression of CDK inhibitors like $p27^{kip1}$, which are usually elevated in quiescent cells (Polyak et al., 1994a,b; Coller, 2011). However, high levels of CDK inhibitors are also associated with entry into senescence and terminal differentiation (Ruas and Peters, 1998; Sherr and Roberts, 1999; Bringold and Serrano, 2000). Additionally, overexpression of CDK inhibitors does not reproduce the transcriptional signature of quiescent cells (Coller et al., 2006; Sang et al., 2008). It is also disputed whether cells in quiescence enter a non-cycling "compartment" termed G0 or halt in G1 (Smith and Martin, 1973; Shields and Smith, 1977; Spencer et al., 2013; Arora et al., 2017; So and Cheung, 2018). Although presenting the same amount of non-replicated DNA, quiescent cells spend longer periods of time between mitosis exit and S phase and express different sets of genes, when compared to active cycling cells in a canonical G1 (Coller et al., 2006; Sang et al., 2008; Coller, 2011; Spencer et al., 2013; Arora et al., 2017).

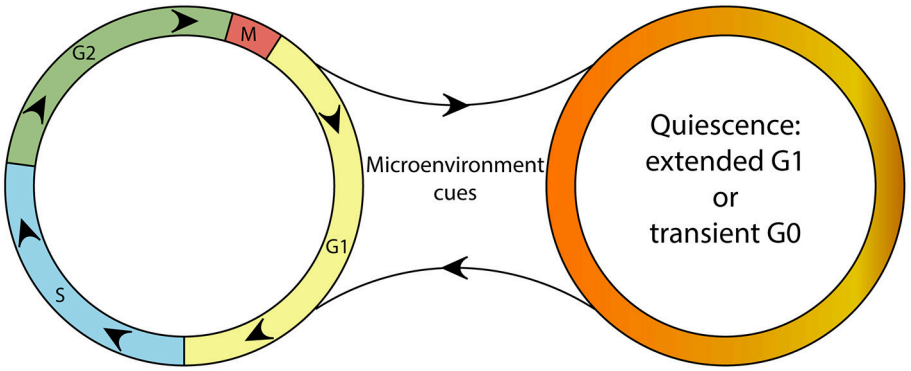

FIGURE 1 | A schematic depicting the continuity of the cell cycle and the quiescence compartment. Although a continuous cell cycle resulting in proliferation might be considered the "default program" of cells, microenvironmental cues can trigger the "optional setting" of quiescence. Quiescent cells are able reenter the cell cycle upon changes in their microenvironment.

We support the idea that the best way to identify quiescent cells relies on properties not detected in cycling cells and absence of proliferative traits such as cell cycle activating factors, DNA synthesis, and mitotic markers (O'Farrell, 2011). Also, there is some confusion when distinguishing quiescence from replicative senescence. Senescent cells are found in an essentially permanent growth arrest induced by extrinsic and genotoxic stresses (Campisi and d'Adda di Fagagna, 2007; Rodier and Campisi, 2011) and express markers not found in quiescent cells like senescence-associated β-galactosidase, nuclear foci containing DNA damage proteins (DNA-SCARS) and senescence-associated heterochromatin foci (SAHF) (Rodier and Campisi, 2011). Quiescent cells are those uncommitted to any proliferation-related activity but are also not in irreversible states such as senescence, terminal differentiation, or apoptosis. While the only other option for senescent and terminally differentiated cells is cell death, quiescent cells are capable of many cell fates such as reverting to proliferation, differentiating, senescing, or dying.

The ability of becoming quiescent is found in many different cells types and conditions, including non-malignant, malignant, undifferentiated, and differentiated cells from distinct tissues in normal and aberrant situations. For example, differentiated hepatocytes in a normal liver have the capacity to reenter the cell cycle upon physical (i.e., resection) and chemical injury (Kim et al., 1997; Michalopoulos and DeFrances, 1997; Presnell et al., 1997; Dong et al., 2007); Quiescence is also crucial for maintenance of adult stem cells (Wilson et al., 2008; Fu et al., 2017). Stem cell microenvironmental cues can either trigger quiescence or direct stem cells toward proliferation and functional differentiation (Cheung and Rando, 2013). In cancer dormancy, residual cancer cells disseminated from the primary tumors survive in a quiescent state in distant organs. These dormant cells appear to be responsible for metastases that occur years or even decades after tumor surgery and treatment (Ghajar et al., 2013; Sosa et al., 2014).

Cellular quiescence was defined in the past as a default state of inactivity (Cheung and Rando, 2013) acquired passively when conditions are not optimal for proliferation. But, a growing body of compelling evidence compiled herein shows that quiescence is a molecularly diverse, non-terminal and tissue-specific state that can be activated and sustained by the cell microenvironment.

CHANGES IN SYSTEMIC, TISSUE AND CELL METABOLISM REGULATE THE PROLIFERATION-QUIESCENCE DECISION

In metazoans, in addition to favorable nutritional conditions, ligands like growth factors, mitogens, and conditional signals are necessary to trigger a receptor response to promote and regulate growth and proliferation (Valcourt et al., 2012). Core cell growth pathways like PI3Kinase/AKT/TOR are conserved from yeast (a unicellular eukaryote) to Metazoa and connect biochemical cues and nutrient availability with the cell cycle re-entry of quiescent cells (Vivanco and Sawyers, 2002). In yeast, growth pathways are not coupled to extracellular signaling molecules. Rather, they proliferate if provided with sufficient nutrients and appropriate pH, temperature, and pressure (Soto et al., 2016).

Quiescence signaling in animal cells can be triggered even when conditions are suitable to sustain proliferation, such as with sufficient nutrients and growth factors (Weaver et al., 1997; Wang et al., 1998; Liu et al., 2004; Spencer et al., 2013). For example, during formation of 3D acinar structures in a reconstituted basement membrane, cells cease proliferating despite the culture media containing and being regularly replenished with all the additives required for cell growth and proliferation (Weaver et al., 1997; Wang et al., 1998; Liu et al., 2004). Most likely, quiescence in complex organisms is not a consequence of the absence of growth stimuli, but instead an active process of growth suppression (Parr, 2012).

Quiescent cells preferentially oxidize carbon compounds to produce ATP to drive basic cellular processes. Proliferating cells, however, shift their metabolism to more anabolic pathways so they can generate biomass and commit to cell division (Vander Heiden et al., 2009; Palm and Thompson, 2017). The first metabolic signature associated with cancer was that tumors

take up glucose and break it down into lactate more quickly than normal tissues, even in the presence of an abundance of oxygen that would otherwise lead to the complete oxidation of glucose in the mitochondria (Rabinowitz and Coller, 2016). This phenomenon is called the Warburg Effect, named after Otto Warburg, a biochemist who first observed the phenomenon by comparing lactate production in normal and tumor tissues (Kim and Dang, 2006; Koppenol et al., 2011). Proteins downstream of growth factor signaling regulate metabolism and display oncogenic potential. Pathways centered on PI3K-AKT and RAS proteins, for instance, are critically involved in the abnormal metabolism of cancer cells, including the Warburg effect, enhanced proliferation, and survival (Thompson, 2009; Pavlova and Thompson, 2016). On the other hand, tumor suppressors, like PTEN, a PI3K signaling inhibitor, and p53 can oppose unrestrained proliferation, and therefore, mutations in these genes also contribute to abnormal metabolism (Thompson, 2009).

Altered metabolic routes, including not only that of carbohydrates but also amino acid and lipid metabolism, are a hallmark of cancer and are essential to sustain the uncontrolled proliferation and survival of tumor cells (Hirschey et al., 2015; Coloff et al., 2016; Keckesova et al., 2017) (Hanahan and Weinberg, 2000, 2011). For example, proliferating cells metabolize significantly more glutamate via transaminases, whereas quiescent cells consume less glutamine and have reduced non-essential amino acid (NEAA) synthesis (Coloff et al., 2016). Highly proliferative tumors couple glutamine usage to NEAA production to sustain biosynthesis (Coloff et al., 2016). The Weinberg lab (Keckesova et al., 2017) performed transcriptome analysis of experimentally induced quiescent and differentiated muscle cells to identify that Lactamase B (LACTB), a mitochondrial protein, was upregulated in relation to undifferentiated and cycling cells. LACTB was able to strongly inhibit proliferation in multiple breast cancer cell lines by changing mitochondrial lipid metabolism and reducing the levels of mitochondrial phosphatidylserine decarboxylase (Keckesova et al., 2017).

The metabolic shift displayed by tumors was first considered exclusively as a consequence of cancer cell-autonomous processes such as mutations in proto-oncogenes and tumor suppressors that are primarily involved in growth signaling and cell cycle regulation. However, growing epidemiological and experimental evidence has shed light on the role of disrupted metabolism in oncogenesis (Onodera et al., 2014; Hirschey et al., 2015). Metabolic diseases such as type II diabetes and obesity correlate with an enhanced risk of developing many types of cancer (Giovannucci et al., 2010; Doerstling et al., 2017). The molecular mechanisms behind this correlation are still unclear and may be linked to aberrant growth factor-stimulated signaling, hyperglycemia, chronic inflammation associated with obesity and diabetes or indeed a combination of these processes (Giovannucci et al., 2010). Experimentally, Onodera et al. showed that increased glucose uptake and glycolysis can elicit malignant phenotypes in non-malignant breast cells, including activation of oncogenic pathways like EGFR, β1-integrin, PI3K, and MAPK, loss of tissue polarity and importantly, loss of quiescence. Reducing glucose uptake in malignant cells, conversely, resulted in gain of polarity and cell cycle arrest (Onodera et al., 2014). This may help explain why metformin, a drug used to treat type II diabetes, reduces the incidence of cancer in diabetes patients and is regarded as a potential treatment for some types of cancer (Ben Sahra et al., 2011; Loubière et al., 2013; Rosilio et al., 2014; Higurashi et al., 2016). The experimental data together with epidemiological evidence indicate that a hyperglycemic microenvironment may contribute to exit from quiescence of already genetically altered cells in the early stages of tumorigenesis and the consequent development of neoplastic lesions (**Figure 2**).

THE COMPOSITION AND PHYSICAL PROPERTIES OF THE EXTRACELLULAR MATRIX ARE KEY DETERMINANTS OF CELLULAR QUIESCENCE

The ECM is composed of a complex network of biochemically diverse molecules including glycoproteins, non-glycosylated proteins, proteoglycans, and polysaccharides that assemble into three-dimensional scaffolds with a myriad of biochemical and biomechanical properties. By acting as ligands of cell surface receptors like integrins, the ECM molecules not only make the structural frame that determines the morphology and physical properties of tissues and organs but are also crucial in tuning cell signaling pathways, including growth factor-stimulated pathways, that control all aspects of cell behavior (Hynes, 2009; Lu et al., 2011, 2012; Pickup et al., 2014). Cell to ECM adhesion mediated by heterodimers of integrins is required for cell proliferation. Responses to growth factors depend on the cell being anchored via integrins to a component of the ECM (Schwartz and Assoian, 2001; Hynes, 2002; Ivaska and Heino, 2011; Pickup et al., 2014). For example, insulin stimulation promotes binding of the integrin pair αvβ3-integrin with insulin receptor substrate-1 (IRS-1) and this linkage is necessary to induce DNA synthesis (Vuori and Ruoslahti, 1994). Blocking β1-integrin binding to ECM inhibited CDK activity leading to an accumulation of hypophosphorylated pRb (Day et al., 1997). Furthermore, there are numerous studies showing that intracellular signaling induced by integrin-binding to the ECM are analogous to, and intersect with, growth-factor pathways like RAS-MAPK and PI3K-AKT (Yu et al., 2001; Cabodi et al., 2004, 2010; Ivaska and Heino, 2011). Notably, the ECM also functions as a "reservoir" of a large number of growth factors and cytokines. The ECM can, in this way, store molecules that can be released by proteases and can induce proliferation in specific locations (Bonnans et al., 2014).

The ECM is divided into two main components: the interstitial matrix and the basement membrane. The interstitial matrix contains fibrillar collagens and different types of proteoglycans and glycoproteins made mostly by stromal cells (**Figure 2**). The basement membrane (BM) is a specialized thin layer of ECM produced by both epithelial cells and stromal cells composed almost exclusively of laminins, type IV collagen, nidogen, and proteoglycans (Lu et al., 2012). Besides separating

the epithelial and stromal compartments, the BM plays key roles in instructing the epithelial cells to differentiate, function, and survive. For example, laminin-rich ECM (lrECM), a surrogate for the basement membrane, induces tissue polarization and quiescence and also reprograms gene expression in mammary gland epithelial cells (Barcellos-Hoff et al., 1989; Streuli and Bissell, 1991; Boudreau et al., 1995, 1996; Streuli et al., 1995; Petersen et al., 1998; Kenny and Bissell, 2003; Akhtar et al., 2009; Spencer et al., 2011; Fiore et al., 2017). Additionally, the basement membrane suppresses apoptosis of mammary epithelial cells (Boudreau et al., 1995). These findings support the notion that quiescent cells not only cease proliferating but also undergo genetic reprogramming acquiring new properties (Coller et al., 2006; Coller, 2011; O'Farrell, 2011) and that quiescence programs might overlap with survival signaling resulting in resistance to cell death.

Normal epithelial cell lines grown in 3D laminin-111 rich gels have been shown to form quiescent acinar structures with polarized cells. In contrast, malignant cells grow as disordered proliferating structures (Rizki et al., 2008; Fiore et al., 2017; Wessels et al., 2017). Laminin-111 levels are reduced and show irregular distributions in several stages of breast cancer (Petersen et al., 1992). Increased expression and aberrant deposition of collagen I and IV and fibronectin induced proliferation, loss of polarity, and a malignant phenotype in several types of epithelial cells (Hoffman et al., 1996; Zhang et al., 2002; Wozniak et al., 2003; Provenzano et al., 2009; Malik et al., 2010; Schedin and Keely, 2011; Espinosa Neira and Salazar, 2012; Kim and Gumbiner, 2015). In non-malignant cells from the mammary gland, signals from laminin-111 increase the levels of nitric oxide and active p53 (Furuta et al., 2018) and decrease the level of nuclear actin via export by exportin-6 (XPO6) (Fiore et al., 2017), resulting in polarized and quiescent acini. Actin in the nucleus is essential for RNA synthesis and RNA levels (Percipalle, 2013; Virtanen and Vartiainen, 2017) which, in turn, are crucial to sustain cell proliferation (Spencer et al., 2011; Fiore et al., 2017). In malignant cells; XPO6 activity is not enhanced, nuclear actin and RNA levels are not decreased, and malignant cells are unresponsive to quiescence-inducing cues from laminin-111 (Fiore et al., 2017). Interestingly, inhibition of PI3K results in increased N-actin export (Fiore et al., 2017). Although quiescence involves genetic reprogramming leading to the acquisition of new properties (Coller et al., 2006; Coller, 2011), it is still uncertain whether the cooperation of laminin-111 signaling and nuclear actin influences the functional differentiation of the mammary gland. In addition, it is unclear which configuration of nuclear actin, either monomeric or filamentous, regulates cell proliferation and quiescence.

Cancer cells have the ability to remodel their own ECM by secreting pro-proliferation ECM components like fibronectin (Hynes and Naba, 2012; Naba et al., 2014, 2017). The nature of the ECM receptors bound to the cancer cell-secreted ECM may enhance proliferation-promoting pathways and/or attenuation of growth inhibitory signals. Additionally, active degradation of laminin-111 and the consequent breakdown of the supramolecular structure of the BM by matrix metalloproteinases (MMPs), enzymes that are commonly overexpressed in different stages of tumor development of several cancers (Kessenbrock et al., 2010; Bonnans et al., 2014), result in re-activation of the cell cycle (Beliveau et al., 2010) leading to subsequent development of tumors (Bissell et al., 2005; Calvo et al., 2013). Importantly, key genetic alterations (e.g., oncogene activation and loss of tumor suppressors) commonly found in malignant cells induce changes in the microenvironment and on how cells sense their surroundings. For instance, $BRAF^{V600E}$ mutation induces expression of both α3/α6 integrins (Nucera et al., 2010) and thrombospondin-1 (*THBPS1*) in thyroid papillary carcinoma, as well as in melanome, eliciting hyperproliferation and cell invasion (Nucera et al., 2010; Jayachandran et al., 2014).

Despite carrying several genetic alterations, disseminated tumor cells (DTCs) can survive in a reversible dormant state for decades in secondary sites (Sosa et al., 2014). Strong evidence from mouse and co-culture experiments has shown that the ECM of microvascular niches is crucial to influence the proliferation-quiescence decision in breast DTCs. Thrombospondin-1 in the BM of mature endothelial cells sustained quiescence, whereas sprouting neovasculature is rich in active TGF-β1 and periostin induced proliferation of breast cancer cells (Ghajar et al., 2013).

Epithelial cells in normal and neoplastic tissues reside in a cell-rich microenvironment containing resident fibroblasts, endothelial cells, pericytes, and immune cells. These cells have active roles in the tissue microenvironment biology, especially in ECM remodeling. Cancer-associated fibroblasts (CAF) and tumor-associated macrophages (TAM) are abundant in the tumor microenvironment playing major roles in remodeling the ECM and establishing paracrine signaling with neoplastic cells that support cancer development (Kalluri, 2016). CAFs derived from human prostate tumors were able to induce malignant transformation and proliferation of non-malignant, but genetically initiated, prostate epithelial cells (Olumi et al., 1999). Human mammary epithelial cells transfected with SV40 large-T antigen, the telomerase catalytic subunit, and an H-Ras oncoprotein when mixed with Matrigel (a commercial ECM) or primary human mammary fibroblasts displayed increased capacity to form tumors in immunocompromised mice (Elenbaas et al., 2001). In part, CAFs communicate with cancer cells via secretion of cytokines and exosomes, small vesicles that contain proteins, nucleic acids, and metabolites that can modulate the behavior of cancer cells (Hoshino et al., 2015; Kalluri, 2016; Zhao et al., 2016; Matei et al., 2017) (**Figure 2**). Macrophages are key players in chronic inflammation associated with oncogenesis and tumor progression (Balkwill et al., 2005). Noteworthy, chronic inflammation triggered by infectious or chemical agents and tissue-intrinsic mechanisms increase the risk of cancer, and many neoplasms are believed to initiate at sites of chronic inflammation (Balkwill et al., 2005; Karin and Greten, 2005; Kenny et al., 2007; Tyan et al., 2011). TAMs remodel the ECM via secretion of MMPs and promote cell cycle reactivation by producing growth factors and cytokines (Balkwill et al., 2005; Goswami et al., 2005; Deryugina and Quigley, 2015; Vinnakota et al., 2017) (**Figure 2**).

The ECM is not only a bystander in the tumor microenvironment (Pietras and Ostman, 2010) but rather actively contributes to cancer initiation and progression, and

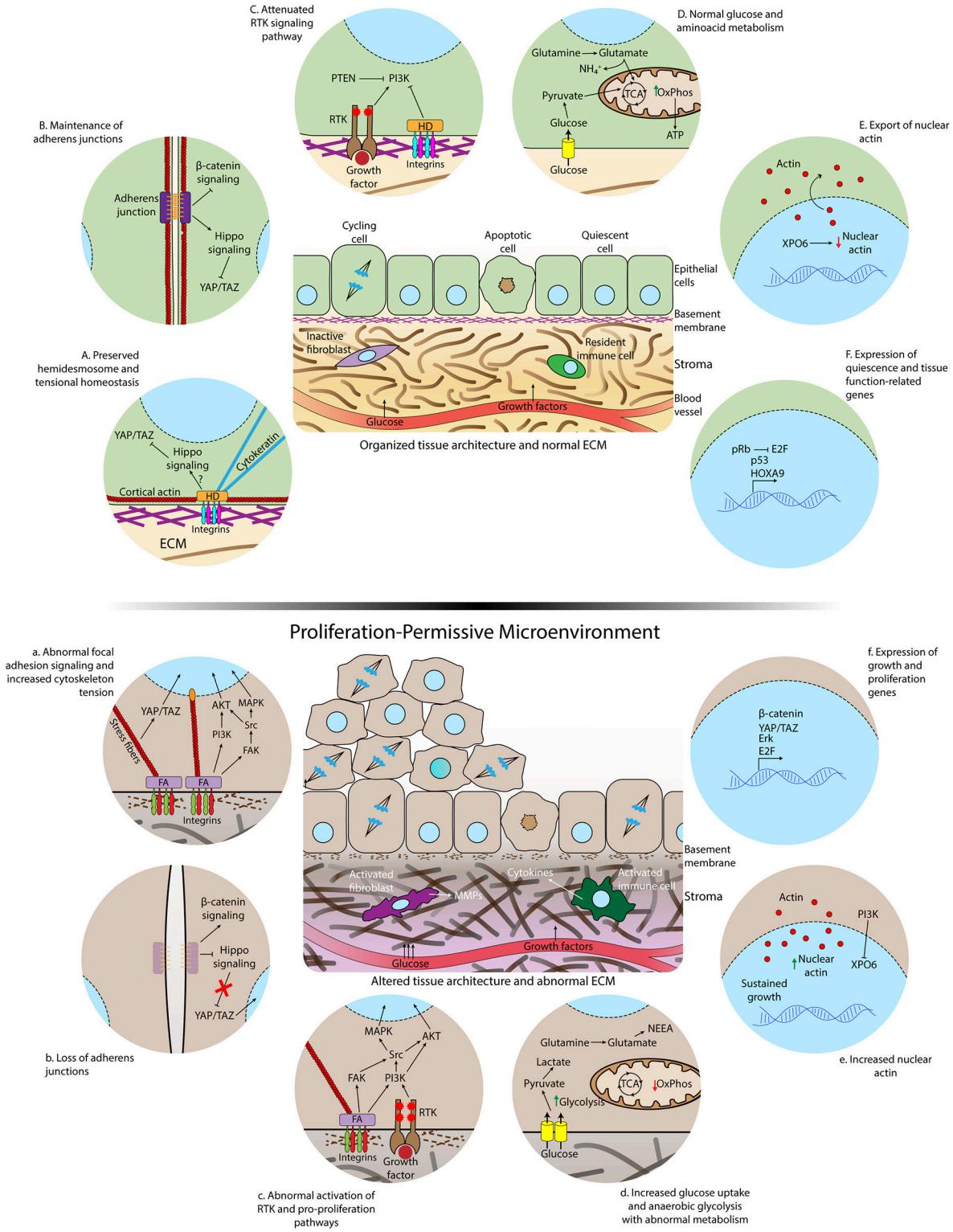

FIGURE 2 | Differences in microenvironmental and subcellular signaling between homeostatic conditions and loss of cell quiescence. **(Top)** Quiescence-inducing microenvironment showing normal extracellular matrix, intact basement membrane, organized tissue architecture, and resident immune cells and fibroblasts. The rates of proliferation and apoptosis are comparable to maintain homeostasis. **(Below)** Altered and proliferation-permissive microenvironment displaying abnormal

(Continued)

FIGURE 2 | extracellular matrix with altered composition and structure, disrupted basal lamina, activated fibroblasts in the stroma, inflammatory infiltrate by activated macrophages and other cytokines-secreting activated immune cells. The rate of proliferation is increased due to loss of quiescence regulation by the tissue microenvironment. Details of epithelial cells residing in a healthy **(A-F)** or aberrant microenvironment **(a–f)**. A normal ECM and correct tissue architecture induces the formation of hemidesmosomes connecting the ECM to cytokeratin filaments, cell-cell junctions, cortical actin cytoskeleton, and polarized epithelium **(A,B)**. Consequently, the Hippo pathway, that inhibits translocation of YAP/TAZ to the nucleus, is activated, receptor tyrosine kinase (RTK) activity is attenuated **(C)** and nuclear actin export is enhanced **(E)**. Glucose and glutamine are completely metabolized by the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OxPhos) **(D)**. Quiescence gene expression programs are triggered during quiescence acquisition **(F)**. Aberrant ECM signaling due to altered ECM composition and stiffness increases formation of focal adhesions (FA), loss of cortical distribution of the actin cytoskeleton, enhanced activation of FAK, formation of actomyosin stress fibers, translocation of YAP to the nucleus **(a)** and accumulation of nuclear actin **(e)**. Loss of adherens junctions allows the translocation of beta–catenin to the nucleus **(b)**. Overactivation of growth factor signaling occurs as a consequence of intersection between integrin and RTK-triggered signaling **(c)**. Glucose uptake and anaerobic glycolysis are further increased due to exacerbated activation growth factor pathways, glutamine is converted to NEAA and biosynthetic precursors in the TCA and oxidative phosphorylation is reduced in the aberrant microenvironment **(d)**. Activation of genes involved in cell-cycle entry is increased **(f)**.

this is an accepted fact. Indeed, it has been suggested that the ECM should be seen as a modulator of all hallmarks of cancer (Pickup et al., 2014) and we argue that an aberrant ECM should also be considered a hallmark of cancer. Similar to the biochemical traits of the ECM, biomechanical cues, specifically ECM stiffness and topology, are also sensed by cells, triggering signal transduction cascades affecting many aspects of cell behavior. Tumor stroma is typically more rigid than normal stroma due to increased collagen deposition and crosslinking between collagen fibers and other ECM molecules catalyzed by abnormal activities of the enzyme lysil oxidase (LOX) (Levental et al., 2009; Lu et al., 2012). Women with dense tissue in 75% or more of the breast have an elevated risk of developing breast cancer, in comparison with women with little or no dense tissue (Boyd et al., 2007). Moreover, patients with pancreatic ductal adenocarcinoma display intense desmoplasia, a process that involves a considerable increase in collagen types I and V, myofibroblastic pancreatic stellate cells, and immune cells, which are associated with cancer progression and poor survival (Pandol et al., 2009). In addition, leiomyomas, a common type of benign tumor, are characterized by neoplastic growth and excessive collagen I and III deposition (Wolanska et al., 1998).

How then does microenvironmental stiffness influence the proliferation-quiescence decision? Focal adhesions (FAs), intracellular protein complexes of protein kinases, and adaptors linked to filamentous actin (F-actin) are formed when cell surface receptors bind ECM molecules (Howlett et al., 1995; Giancotti and Ruoslahti, 1999; Zhao and Guan, 2011; Hynes and Naba, 2012). These complexes are modulated by substrate rigidity in response to intracellular tension built and stored in the actomyosin cytoskeleton (Wozniak et al., 2004; Halder et al., 2012). The critical proteins in FAs are the heterodimers of integrins (usually containing β1-integrin chains) and FAK (Focal Adhesion Kinase). FAK phosphorylates multiple substrates and helps integrate integrin and growth factor signaling pathways (Cabodi et al., 2010). In addition to growth factor-responsive receptor tyrosine kinases, PI3K is activated by other kinases, such as FAK and integrin-linked kinase (ILK), which also relay signals from the ECM (Wang et al., 1998; Grant et al., 2002; Reif et al., 2003; You et al., 2015). Aberrant β1-integrin signaling and increased expression and activity of FAK are frequently associated with tumorigenesis (Desgrosellier and Cheresh, 2010; Zhao and Guan, 2011) and inhibition of either EGFR or β1-integrin can induce the formation of quiescent acinar structures in malignant breast cells (Weaver et al., 1997; Wang et al., 1998; Nisticò et al., 2014). Furthermore, FAK phosphorylation activates pro-proliferative pathways like Src-RAS-MAPK (Zhang et al., 2002; Provenzano et al., 2009). Cell cycle progression requires integrin binding to the ECM that promotes activation of RAS and ERK signaling, re-initiating the cell cycle (Schwartz and Assoian, 2001; Pickup et al., 2014). In response to increased ECM rigidity, cells increase FAK activation that activates cell cycle progression via RAC stimulation (Bae et al., 2014; Pickup et al., 2014). In addition, the activation of FAK promotes nuclear localization of YAP, an effector of the Hippo pathway, through activation of SRC-PI3K signaling (Kim and Gumbiner, 2015). Conversely, inhibition of β1 integrin signaling with the AIIB2 blocking antibody inhibits FAK activation, reverting breast epithelial tumor cells to a normal-like phenotype reassembling the basement membrane and reestablishing cell to cell junctions resulting in decreased cyclin D1 and upregulation of p21 (Weaver et al., 1997; Wang et al., 1998).

In general, these data demonstrate that alterations in the composition of the extracellular matrix may be an important trigger for the activation of FAK-mediated pro-proliferative pathways, thus promoting a mechanism of escape from quiescent programs. Indeed, in leiomyoma cells, the interplay between collagen signaling and the growth factor-stimulated MAPK pathway regulates cell cycle progression (Koohestani et al., 2013). In mammary epithelial cells, a stiff ECM promotes malignant phenotypes by inducing miR-18a, which decreases levels of the transcription factor HOXA9 and the tumor suppressor PTEN, resulting, respectively, in cell proliferation (Gilbert et al., 2010) and enhanced PI3K activity (Mouw et al., 2014) (**Figure 2**).

While increased FA formation and FAK activity correlate with increased deposition and crosslinking of collagen and aberrant cell-growth pathways, hemidesmosomes seem to counteract these effects on physiological stiffness and are linked with a cell cycle arrest phenotype (Weaver et al., 2002; Chaudhuri et al., 2014; Nisticò et al., 2014). Hemidesmosomes are multiprotein structures mediating cell-ECM adhesion. They possess the integrin pair α6β4 at their core and connect the BM to cytokeratin filaments (Walko et al., 2015). Perturbation of hemidesmosomes is involved in the development and progression of certain cancers (Rabinovitz and Mercurio, 1996;

Nisticò et al., 2014). Remarkably, although ECM stiffness induces malignant phenotypic transformation, including escape from cellular quiescence (Paszek et al., 2005; Chaudhuri et al., 2014), this effect is lost when combined with an increase in available basement-membrane ligands (Chaudhuri et al., 2014). Thus, ECM rigidity and composition seem to act in conjunction with one another to regulate malignant phenotypes. This line of evidence indicates that altered physical properties of the ECM and the ECM composition itself should be jointly considered when evaluating the risk of development of hyperproliferative lesions and cancer prognosis (Branco da Cunha et al., 2014; Chaudhuri et al., 2014). Moreover, depending on the biological context, the composition and structural properties of the ECM may drive cell cycle progression as is the case in tissues possessing high densities of collagen fibers. Conversely laminin-111 together with an intact BM can induce a resting state and preclude cells from quitting quiescence and possibly establishing neoplastic lesions.

INFORMATION PROVIDED BY THE TISSUE ARCHITECTURE AND GEOMETRY ARE CRITICAL REGULATORS OF CELL PROLIFERATION AND QUIESCENCE

One of the most important physical properties of an organ is its architecture itself. The normal function of an organ is dependent on its shape and structural features. Indeed, loss of tissue architecture is one of the diagnostic traits of cancer (Nelson and Bissell, 2006). Interestingly, a healthy organ morphology can itself function as a tumor suppressor, suppressing malignancy even in cells possessing several mutations and aneuploidies (Mintz and Illmensee, 1975; Howlett et al., 1995; Weaver et al., 1997, 2002; Wang et al., 2002; Kirshner et al., 2003; Nelson and Bissell, 2006).

Cell quiescence and cytoarchitecture are also exquisitely coupled. The biology of epithelial tissues is a classic example of this relationship. The polarized distribution of cell–cell junctions, organelles, and molecules are the defining morphological traits of epithelial tissues (Inman and Bissell, 2010) and epithelial polarity can have a crucial regulatory effect on cell proliferation (Zeitler et al., 2004). The BM offers a platform of cell anchoring and a source of molecular cues for the correct orientation of apical–basal polarity in epithelia (Weaver et al., 1995; O'Brien et al., 2001; Bissell et al., 2002; Halaoui and McCaffrey, 2015). Reciprocally, cell polarity influences intracellular molecular pathways, providing a mechanism for cells to sense, and assimilate cues from their microenvironment to control metabolism and cell growth pathways and consequently the proliferation-quiescence decision (Jansen et al., 2009; Martin-Belmonte and Perez-Moreno, 2011; McCaffrey and Macara, 2011; Nance and Zallen, 2011; Halaoui and McCaffrey, 2015).

Perturbation of epithelial structure by injury can re-activate the cell cycle. However, epithelial cell–cell interactions induce quiescence when the final organ size is attained (Bryant and Simpson, 1984; Johnston and Gallant, 2002; Zeitler et al., 2004; Zhao et al., 2011), even in the presence of abundant nutrients and growth factors. Nevertheless, loss of cell junctions and polarity is a trait of tumors occurring at the onset and at pre-invasive stages of epithelial cancers (Bissell et al., 2002; Martin-Belmonte and Perez-Moreno, 2011; McCaffrey and Macara, 2011). Several proteins, especially the protein complexes Crumbs, Par, and Scribble, are essential for cell polarity and are deregulated in cancer (reviewed in doi: 10.1038/onc.2014.59 Halaoui and McCaffrey, 2015). In imaginal discs of Drosophila, deletion of *scribble* disrupts epithelial architecture and induces uncontrolled proliferation (Zeitler et al., 2004; Halaoui and McCaffrey, 2015). In mammalian epithelia, depletion of Scrib (a homolog of the Drosophila Scribble) leads to luminal filling due to high rates of proliferation (Zhan et al., 2008) and is sufficient to predispose mice to loss of quiescence control and prostate neoplasia (Pearson et al., 2011). Moreover, loss of the polarity protein Par3 induces mammary tumor growth and metastasis (McCaffrey et al., 2012). Malignant breast cells can be phenotypically reverted from disorganized epithelium to normal-like quiescent acini by inhibiting PI3K signaling. By contrast, PI3K-signaling effectors RAC1 and AKT, respectively, induce epithelial polarity perturbation and unrestrained proliferation via enhanced PI3K activity (Liu et al., 2004). Notably, forcing nuclear actin accumulation in 3D cultures of non-malignant mammary cells resulted in larger and proliferative epithelial structures displaying partially disrupted apical polarity but preserved basal polarity (Fiore et al., 2017). Structures with high levels of nuclear actin had a filled lumen resembling the effects of induced overexpression of ERBb2 or other oncogenes in non-malignant cells (Muthuswamy et al., 2001), which suppress quiescence without perturbing epithelial basal polarity (Spancake et al., 1999; Muthuswamy et al., 2001; Debnath et al., 2002; Liu et al., 2004; Leung and Brugge, 2012; Fiore et al., 2017). These data indicate that acquisition of both basal and apical polarity is required to induce quiescence in epithelial structures (Fiore et al., 2017).

The availability of space within tissues is an important regulator of cell death, quiescence, and proliferation. For instance, cells divide rapidly to fill open spaces and the resultant spatial constraints induce normal cell quiescence maintaining homeostasis (Streichan et al., 2014). Restricting the area available for growth is found to induce cell death, while a wider area increases cell proliferation (Chen et al., 1997). When cultured at high density, cells become quiescent. Tumor cells gradually lose the ability to recognize surrounding tissue architecture and exhibit motility independent of geometrical constraints (Kushiro et al., 2017) such as cell density. But, furthermore, cells residing in tissues with complex anisotropic morphologies have differential access to gradients of growth factors, mitogens, and growth inhibitors, resulting in diverse cell states and fates in different regions of the same tissue (Nelson et al., 2006; Gomez et al., 2010; Hannezo et al., 2017). For instance, Nelson and colleagues showed that tissue geometry dictates concentration gradients of autocrine TGFβ. TGFβ levels were found to be high at the trunk of the microfabricated tubules where cellular quiescence predominated, but were low at the branching/outgrowing tips, resulting in increased invasion and proliferation (Nelson et al., 2006).

It is only in the last two decades that the molecular details of how cells sense density have begun to be unveiled. Several signaling pathways have been implicated in this regulation relaying density signals to induce cell-cycle arrest in response to cell contact (Polyak et al., 1994a; Wieser et al., 1999; Heit et al., 2001; Faust et al., 2005; Zhao et al., 2008; Barry and Camargo, 2013; Gumbiner and Kim, 2014). The Hippo-YAP/TAZ pathway has been found to play important roles in contact inhibition through mechanical cues provided by the microenvironment (Zeng and Hong, 2008; Chen et al., 2012; Halder et al., 2012; Schroeder and Halder, 2012; Gumbiner and Kim, 2014; Mao et al., 2017). Discovered in Drosophila, Hippo-YAP/TAZ signaling is a conserved pathway involved in contact inhibition, mechanotransduction, proliferation, and organ size determination (Piccolo et al., 2014). Alterations in different components of the Hippo pathway have been implicated in cancer (Zeng and Hong, 2008; Zhao et al., 2008; Ma et al., 2014; Piccolo et al., 2014). The Hippo kinases set off a cascade of phosphorylation that culminates in the inactivation of YAP/TAZ, a transcriptional coactivator of cell proliferation and survival genes such as Ki67, c-Myc, Sox4, H19, AFP, BIRC5/survivin, and BIRC2/cIAP1 (Zeng and Hong, 2008; Pan, 2010). The subcellular localization of YAP depends on cell density. YAP is primarily present in the nuclei of cells cultured at low densities, whereas at confluence, YAP is phosphorylated as a consequence of Hippo kinase activity and accumulates in the cytoplasm, where it can no longer act as a transcriptional coactivator (Dong et al., 2007; Zeng and Hong, 2008; Zhao et al., 2010). In addition, formation and stability of adherens junctions and the cadherin–catenin complex in response to cell contact have been shown to stimulate Hippo signaling pathway and induce cell quiescence (Varelas et al., 2010; Schlegelmilch et al., 2011; Barry and Camargo, 2013; Gumbiner and Kim, 2014). Moreover, proteins involved in the regulation of apical–basolateral polarity in epithelia have also been implicated in Hippo-mediated inhibition of YAP/TAZ (Genevet and Tapon, 2011; Boggiano and Fehon, 2012; Richardson and Portela, 2017).

The correct establishment of a quiescent state involves an active process that is controlled by a complex set of signaling cascades including activation of Hippo signaling and attenuation of growth factor stimulated pathways like PI3K-AKT. These are controlled by microenvironmental cues originating from the ECM, tissue architecture, and neighboring cells, and occur despite adequate energy sources and growth factors. In an abnormal microenvironment, these signaling pathways are perturbed resulting in unrestrained cell growth (**Figure 2**).

PERSPECTIVES AND CONCLUDING REMARKS

From the above account, loss of quiescence is a central aspect of tumorigenesis, and it can be seen that the tissue microenvironment plays an essential role in quiescence regulation. Therefore, we argue that in a context-dependent manner the microenvironment can work as either a gas or brake pedal, similar to the analogy proposed to explain the essential roles of oncogenes and tumor suppressors in cell proliferation (Hinds and Weinberg, 1994). A healthy microenvironment may stop cells from re-entering the cell cycle, whereas an aberrant ECM, disruption of tissue and cell architecture, inflammation, and altered metabolism may permit cells to escape quiescence and proliferate uncontrollably. Biochemical and structural properties of the tissue microenvironment and the integration of growth factor and ECM-receptor signaling should be considered when studying cellular quiescence and proliferation and also in cancer diagnostics and treatment.

Looking ahead, it is important for the field of cancer biology to view loss of quiescence as an essential feature of neoplasia and make efforts toward understanding the molecular mechanisms of how quiescence is achieved in normal tissues and how it may be disrupted in cancer. In addition, it is essential to study cellular quiescence in assays that approximate the context that cells experience *in vivo*. Currently, studies designed to identify molecular players in quiescence are based on 2D cell culture models such as contact inhibition, serum deprivation, and cell synchronization that do not include the milieu by which cells are surrounded *in vivo*. Most of these experiments are done by forcing cell cycle synchronization using cytotoxic drugs that affect crucial biochemical pathways such as nucleic acid synthesis and cytoskeleton dynamics during cell division (Spencer et al., 2013). Because these strategies do not reproduce the establishment of quiescence *in vivo* and use strong cell stressors, the biological events of the cell cycle may be masked by assay-intrinsic artifacts. Furthermore, the majority of studies of the cell cycle have been performed by means of bulk assays that conceal the heterogeneous responses displayed by single cells in a population (Spencer et al., 2013). We believe that the appropriate assays for studying the regulation of the proliferation-quiescence decision are those where cells are embedded in 3D and are able to form morpho-functional tissue-like units. For instance, in the mammary acinogenesis assay, non-malignant cells are cultured in a 3D gel of a reconstituted basement membrane and are found to display a program of proliferation and morphogenesis followed by growth arrest and epithelial polarization (Weigelt et al., 2014).

A recent advance in the cell cycle field is the development of live-cell imaging using fluorescent probes for the cycle such as FUCCI and CDK-activity sensors (Spencer et al., 2013; Zielke and Edgar, 2015), which should be considered when planning experiments of quiescence acquisition in physiological contexts. These approaches are overcoming the need for artificially-induced cell cycle arrest and bulk biochemistry, allowing long-term, and real-time tracking of cell cycle dynamics at the single-cell level in asynchronous populations and are, in fact, redefining what we know about the molecular biology of how a cell adopts a proliferative or quiescent state (Spencer et al., 2013; Arora et al., 2017; Barr et al., 2017; Kedziora and Purvis, 2017). Moreover, so far, most attempts to identify molecules involved in the acquisition of quiescence have relied on gene expression profiling methods, such as DNA microarrays and RNA-sequencing, that are unable to discriminate between genes which are a consequence of cell cycle exit and those which play active roles in quiescence induction. The advent of highly robust forward genetic screening strategies such as short-hairpin RNA and CRISPR/Cas9 libraries may greatly contribute to the

discovery of intracellular molecules, which relay quiescence-inducing extracellular cues. Indeed, our laboratory is currently performing experiments designed to understand the dynamics and molecular regulation of the proliferation-quiescence decision at the single-cell level in the context of healthy and aberrant tissue microenvironments.

Although essential for tumorigenesis, mutations in proto-oncogenes, and in tumor suppressors and cumulative genetic instability have proved insufficient to explain malignant behaviors, including hyperproliferative phenotypes (Dolberg and Bissell, 1984; Olumi et al., 1999; Bissell and Hines, 2011; Palumbo et al., 2015; Harper et al., 2016; Hosseini et al., 2016). In this review, we showed robust evidence indicating that changes in the cell's surroundings must also occur to affect the proliferation-quiescence homeostasis. Therefore, studies toward understanding cell cycle deregulation in tumor cells should contain aspects of the tissue microenvironment. Furthermore, the development of new therapies to kill fast growing cells in tumors requires an integrative approach taking in account both the cancer cell genetics and the tumor microenvironment.

AUTHOR CONTRIBUTIONS

AB-C conceived the idea of the review, co-wrote, helped draw the figure panels, and edited the manuscript. AF and PR co-wrote the manuscript and drew the figure panels.

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REVIEW
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Underlying Causes and Therapeutic Targeting of the Inflammatory Tumor Microenvironment

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Historically, the link between chronic inflammation and cancer has long been speculated. Only more recently, pre-clinical and epidemiologic data as well as clinical evidence all point to the role of the tumor microenvironment as inextricably connected to the neoplastic process. The tumor microenvironment (TME), a complex mix of vasculature, inflammatory cells, and stromal cells is the essential "soil" helping to modulate tumor potential. Increasingly, evidence suggests that chronic inflammation modifies the tumor microenvironment, via a host of mechanisms, including the production of cytokines, pro-inflammatory mediators, angiogenesis, and tissue remodeling. Inflammation can be triggered by a variety of different pressures, such as carcinogen exposure, immune dysfunction, dietary habits, and obesity, as well as genetic alterations leading to oncogene activation or loss of tumor suppressors. In this review, we examine the concept of the tumor microenvironment as related to both extrinsic and intrinsic stimuli that promote chronic inflammation and in turn tumorigenesis. Understanding the common pathways inherent in an inflammatory response and the tumor microenvironment may shed light on new therapies for both primary and metastatic disease. The concept of personalized medicine has pushed the field of oncology to drill down on the genetic changes of a cancer, in the hopes of identifying individually targeted agents. Given the complexities of the tumor microenvironment, it is clear that effective oncologic therapies will necessitate targeting not only the cancer cells, but their dynamic relationship to the tumor microenvironment as well.

Keywords: chronic inflammation, clonal hematopoiesis, stroma, microenvironment, tumor suppressors, oncogenes, anti-inflammatory drugs

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INTRODUCTION

Chronic inflammation is a hallmark of cancer and many factors can trigger an inflammatory response in the microenvironment, including infectious pathogens, imbalanced immune regulation, carcinogen exposure, dietary habits and obesity, and genetic alterations leading to oncogene activation or loss of tumor suppressors (Elinav et al., 2013). A growing understanding of the relationship between chronic inflammation and the tumor microenvironment (TME) has dramatically altered our understanding of cancer. Evidence suggests that chronic inflammation creates a pro-tumorigenic environment via the production of pro-inflammatory mediators, angiogenesis, and tissue remodeling (Coussens and Werb, 2002). Some of the most critical

external factors that can promote chronic inflammation and increase cancer risk include tobacco, obesity, a sedentary lifestyle, and select infectious agents. Evolving insight into the mechanisms by which chronic inflammation supports a pro-tumorigenic environment has led to new (immuno)-therapies for cancer as well as lifestyle recommendations which may decrease cancer incidence.

In this review, we discuss the current knowledge of how epithelium cancer-initiating events cross talk to inflammatory cells during cancer initiation and progression. Specifically, we review the concept of the TME and both the extrinsic and intrinsic mechanisms that tie an inflammatory response to pro-tumorigenic events (**Figure 1**).

CELLULAR CONSTITUENTS OF AN INFLAMMATORY MICROENVIRONMENT

While the exact composition of the TME will differ from tissue to tissue, as well as between various tumors of the same tissue, key cellular players are recurrently found. In addition to malignant tumor cells, the TME is composed of a number of different cell types including fibroblasts, endothelial cells, pericytes, and various cells associated with the immune system (Quail and Joyce, 2013). In addition to B cells, natural killer (NK) cells and T lymphocytes, the myeloid cells within the TME include tumor-associated macrophages (TAMs), dendritic cells, neutrophils, and monocytes, the latter two of which have often been misclassified as myeloid-derived suppressor cells (MDSCs) on the basis of immunophenotypic markers. In mice, these myeloid cells are identified by immunophenotype ($Cd11b^+$, $Gr.1^+$) with Ly6C and Ly6G to further differentiate a monocytic-MDSC from a granulocytic-MDSC respectively (reviewed here Talmadge and Gabrilovich, 2013). Human MDSCs are defined through a combination of CD11B, CD14, CD15, and CD66 (Bronte et al., 2016). While studies have identified bona fide T cell suppressor function within this compartment, the label of MDSC is often applied even without functional demonstration leading to murky interpretations of their role within the TME. Understanding the molecular functions of these cells within the tumor may provide avenues for disrupting pro-tumorigenic signaling and tailored TME-targeted therapy. Furthermore, identifying markers capable of distinguishing functional MDSC from the immunophenotypically similar monocyte and neutrophil counterparts will be critical to elucidating their role in the TME (Bronte et al., 2016). In this section, we will review the functions of three of the most well studied constituents of the TME, TAMs, cancer-associated fibroblasts (CAFs) and endothelial cells comprising the tumor vasculature.

Abbreviations: AML, acute myeloid leukemia; APC, adenomatous polyposis coli; ASCO, American Society of Clinical Oncology; BMI, body mass index; CAFs, cancer-associated fibroblasts; CANTOS, Canakinumab Anti-inflammatory Thrombosis Outcome Study; CH, clonal hematopoiesis; CH-PD, CH with a presumptive driver mutation; COPD, chronic obstructive airway disease; ER, estrogen receptor; FDA, Food and Drug Administration; GCs, glucocorticoids; GI, gastrointestinal; GvHD, graft-versus-host disease; NOS, nitrogen species; HPV, human papillomavirus; HSCT, allogeneic hematopoietic stem cell transplantation; IECs, intestinal epithelial cells; IFN, interferons; JAKs, janus family of kinases; LOH, loss of heterozygosity; LPS, lipopolysaccharides; MDSCs, macrophage-derives suppressor cells; MDR, multi-drug resistance; MM, multiple myeloma; MPNs, myeloproliferative neoplasms; NSAIDs, non-selective non-steroidal anti-inflammatory drugs; PDAC, pancreatic ductal adenocarcinoma; Pten, phosphatase and tensin homolog; PV, polycythemia vera; RA, rheumatoid arthritis; ROS, reactive oxygen species; TAMs, tumor associated macrophages; TF, transcription factor; TKs, tyrosine kinases; TKIs, tyrosine kinase inhibitors.

Tumor-Associated Macrophages

TAMs have emerged as one of the most well studied components of the TME. These cells have been shown to interact with nearly every feature of tumor progression including mediating tumor cell proliferation, migration, invasion, angiogenesis, metastasis, and chemotherapeutic resistance (Noy and Pollard, 2014). The multifaceted role of TAMs in the TME is a direct reflection of the diverse responsibilities of macrophages in normal development, tissue homeostasis, and tissue repair (Okabe and Medzhitov, 2016). Indeed many of the developmental processes mediated by macrophages, including extracellular matrix remodeling, phagocytosis of apoptotic debris and angiogenesis, are critical hallmarks of the TME. As such, in some instances the inflammatory state of the tumor resembles an unresolved wound healing response (Dvorak, 1986; Schäfer and Werner, 2008). Perhaps the most well described function of TAMs involves their role in tumor cell migration and invasion. TAMs have been shown to engage in a CSF1-EGF paracrine signaling loop capable of leading tumor cells to the invasive edge of a tumor (Wyckoff et al., 2004). Additionally, TAMs have been shown to engage in a multicellular interaction with endothelial cells through the release of VEGFA to facilitate the intravasation of tumor cells from the primary site into circulation (Harney et al., 2015). In addition to the production of these growth factors, TAMs are the major source of matrix metalloproteinases and cathepsin proteases (Olson and Joyce, 2015; Varol and Sagi, 2017). In order to execute the diverse functions described above, macrophages employ immense transcriptional plasticity that falls along a continuum of activation (Xue et al., 2014; Glass and Natoli, 2016). On one extreme end of the spectrum lays classically activated macrophages (often termed M1-macrophages mirroring that of Th1 immunity). This activation state is canonically associated with IFNγ stimulation resulting in a STAT1 transcriptional program. Functionally, these cells are capable of perpetuating type I inflammatory responses through the secretion of chemokines such as CCL3, CCL4, and CCL5, as well as the production of nitric oxide and TNFα. Furthermore, IFNγ stimulation can boost antigen presentation capacity through the upregulation of *Ciita* a master transcriptional regulator of MHC II molecules. On the other extreme, alternatively activated macrophages (or M2 macrophages) are associated with an anti-inflammatory state. Here, the critical molecular regulators of alternative activation are the Th2 cytokines IL-4, IL-13, IL-6, and IL-10. Together these cytokines lead to the activation of STAT6 (IL-4 and IL-13) as well as STAT3 (IL-6 and IL-10). These programs, as well as other transcriptional regulators, drive the upregulation of Arginase 1 leading to decreased nitric oxide signaling, and increased expression of the wound healing associated chemokines CCL17 and CCL22. Functionally, this activation state is associated with inflammation resolution and

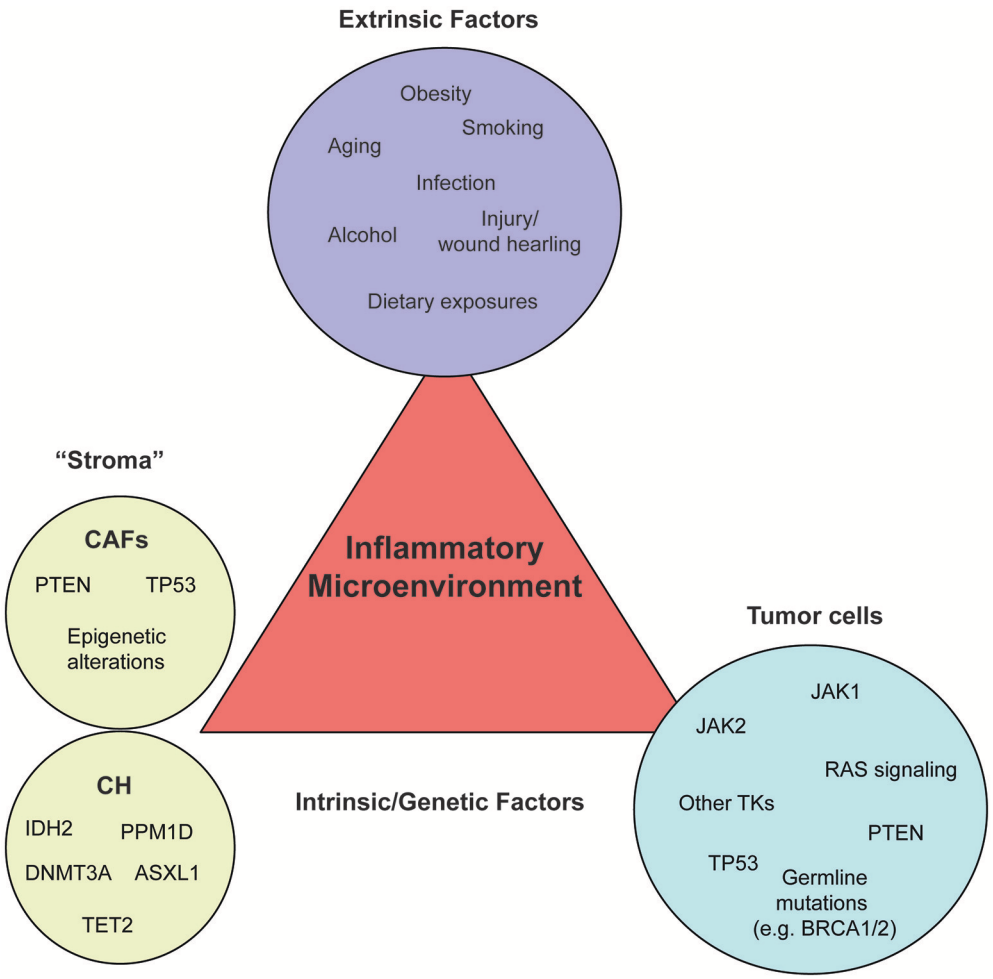

FIGURE 1 | External and intrinsic factors fueling an inflammatory microenvironment. Inflammation can be triggered by a variety of different pressures, such as carcinogen exposure, immune dysfunction, dietary habits, and obesity (extrinsic factors, purple circle), as well as genetic alterations (intrinsic factors) leading to oncogene activation or loss of tumor suppressors in the microenvironment (stroma, green circle) or the tumor cells themselves (tumor, blue circle). Each circle contains a number of examples which belong to the respective category. Most genetic and epigenetic alterations of the stroma have been identified in cancer-associated fibroblasts (CAFs) and white blood cells of healthy individuals or solid cancer patients without signs of hematological malignancies (CH).

extracellular matrix remodeling. In addition to the cytokines described above, TAM activation can be influenced by hypoxia and local metabolite concentrations such as lactate (Casazza et al., 2013; Colegio et al., 2014; Carmona-Fontaine et al., 2017). Despite the widespread description of TAMs as either M1 or M2, this dichotomy is clearly an oversimplification of the diverse states in which these cells are capable of existing (Murray et al., 2014). Elucidating the functional capacities of TAMs within the TME and the mechanism that regulate these processes will provide a clearer picture of these cells. Another more recently appreciated factor influencing TAM activation involves the origin of the cells. While TAMs were long thought to derive from circulating monocytes (Qian et al., 2011; Franklin et al., 2014), recent work suggests that TAMs are also derived from local tissue-resident macrophages (Bowman et al., 2016; Zhu et al., 2017). These are important to consider in the setting of therapeutic strategies aimed at reducing TAM accumulation through recruitment blockade, either through CCR2 or CXCR4 inhibition (Kioi et al., 2010; Qian et al., 2011). Further, in most cases, tissue-resident macrophages possess distinct developmental origins from their monocyte-derived counterparts, as they seed the tissue during embryogenesis developing via an erythro-myeloid precursor as opposed to a hematopoietic stem cell (Gomez Perdiguero et al., 2015). This distinct ontogeny appears to imprint a sort of epigenetic memory on the subsequent TAM, eliciting distinct gene expression profiles within the TME (Bowman et al., 2016; Zhu et al., 2017). While clodronate liposome-based depletion strategies have been used to preferentially deplete tissue resident macrophages in the pancreas (Zhu et al., 2017), more selective genetic ablation strategies will be of interest to translate the differences seen in gene expression studies into functional capacities. Translation of these studies from the mouse to human disease will require identification of markers capable

of distinguishing the ontogenetically defined TAM populations. One such marker, CD49D, has been found to be absent on brain-resident microglia and present on recruited bone marrow-derived macrophages in multiple brain malignancies (Bowman et al., 2016). Markers such as CD49D will likely be found in many distinct tissues, and may serve as biomarkers for future TME-targeted therapy.

Cancer-Associated Fibroblasts (CAFs)

In addition to the immune components of the TME described above, CAFs are an abundant, heterogeneous pool of cells that play multifactorial roles in cancer progression. CAFs are sometimes referred to as mesenchymal stem cells or tumor-associated fibroblasts (Paunescu et al., 2011). Regardless of the nomenclature, these cells are non-hematopoietic, non-epithelial cells resident to a tissue. These cells can be identified microscopically based on the spindle-like shape and large singular presence within the stroma of a tissue. During tissue homeostasis, these cells are responsible for deposition of type I collagen, laminin, perlecan, nidogen, and fibronectin, but are generally considered quiescent with limited migration and proliferation (Kalluri, 2016). Much like macrophage activation paradigms described above, fibroblasts undergo a similar activation process upon stimulation with factors such as TGFβ, PDGF, and FGF2 (Elenbaas and Weinberg, 2001). Like the TAMs described above, CAFs are distinct from their non tumor-associated counter parts and possess a unique activation state. Upon activation these cells change morphologically, increasing in size and with additional spindle-like processes. Functionally these cells possess increased capacity for migration, collagen crosslinking and secretion of cytokines and chemokines such as VEGFA, TGFβ, HGF, FGF, EGF, CXCL10, CCL5, IL-6, TNFα, and IFNγ (Kalluri, 2016). Through these phenotypic alterations, activated fibroblasts can orchestrate a wound healing response concomitant with extracellular matrix repair, recruitment of immune cells to eliminate pathogens, and regrowth of damaged epithelial tissue (Öhlund et al., 2014). Critically, fibroblast activation is a reversible process and as such wound healing responses are able to resolve and quiescence can be restored. If however, this process is not resolved tissue fibrosis can occur. In cancer, this fibrotic phenotype is widespread with even premalignant lesions are often associated with fibrosis or desmoplastic reactions (Rønnov-Jessen et al., 1996), however the causality of desmoplasia and malignant transformation remain an open discussion in human disease. In developed tumors, pancreatic adenocarcinoma (PDAC) presents an extreme example of unrestrained desmoplasia, driven in part through sonic hedgehog signaling in the stroma (Tian et al., 2009). The fibrotic stroma of PDAC presents a challenge for effective delivery of chemotherapy into tumors, and as such reducing the stromal component of the TME presents an interesting chemo sensitizing therapeutic option (Olive et al., 2009). A more full understanding of how CAFs are activated, and the results of inhibiting these activation states are necessary. While most studies have been completed *in vitro*, one study utilized genetic mouse models of squamous cell carcinoma. CAFs isolated from these early neosplasms possess a pro-inflammatory gene expression signature driven by NF-κB (Erez et al., 2010). Interestingly, the authors demonstrated that normal dermal fibroblasts could be "educated" to resemble CAFs through co-culture with carcinoma cells. This activation state has since been shown to be regulated through promoter hypermethylation (Zeisberg and Zeisberg, 2013; Li et al., 2015; Xiao et al., 2016). Upon activation CAFs interface with the tumor through many of the same mechanisms as an activated fibroblast during wound healing, yet many differences remain. *In vitro* proteomic studies identified an altered secretory phenotype in CAFs compared to non-malignant activated fibroblasts (De Boeck et al., 2013). In this study, CAFs were found to secrete higher levels of tenascin and the CXCR4 ligand SDF-1, both of which have been shown to be important in different stages of the metastatic cascade (Oskarsson et al., 2011; Vanharanta et al., 2013). Additionally, while one of the primary functions of fibroblasts in wound healing is to recruit immune cells, CAFs have been shown to negatively regulate immune responses in a TNFα and IFNγ dependent manner (Kraman et al., 2010). As such, targeting CAF-derived cytokines has been shown to enhance CSF1R targeted therapy (Kumar et al., 2017) as well as immune checkpoint blockade (Feig et al., 2013). Clinical efficacy of these combinations remains to be determined.

Angiogenesis and Tumor Vasculature

The formation of new blood vessels, termed angiogenesis, is one of the hallmarks of the tumor microenvironment (Hanahan and Weinberg, 2011). The newly formed vascular network serves as a means to deliver nutrients, cytokines, and oxygen into the tumor. Thus engaging in angiogenesis is a major step in disease development, with earlier stage, smaller tumors possessing fewer vessels than later, more aggressive tumors, which can be highly vascularized (Bergers and Benjamin, 2003). This is, of course, a broad generalization as the vascular content also varies by tissue. For example, while glioblastoma multiforme is one of the most vascularized tumor types (Das and Marsden, 2013), pancreatic ductal adenocarcinoma has low microvascular density and is instead entrenched with dense desmoplastic stroma (Longo et al., 2016). Unlike its' normal tissue counterpart, tumor vasculature often possesses aberrant morphology associated with increased branching and an overall disrupted network of endothelial cells. These abnormal structural findings are often caused by poor pericyte coverage and disruption of a supportive basement membrane (De Palma et al., 2017). Such a distorted network can lead to poor diffusion of oxygen and other small molecules in the tumor resulting in spatial heterogeneity.

While a critical process in malignant development, angiogenesis is not unique to tumors, but rather reminiscent of a wound healing response initiated by inflammation. Indeed, recruited monocytes, eosinophils, and neutrophils are capable of secreting pro-angiogenic factors (De Palma et al., 2017). One of the most potent inducers of angiogenesis is hypoxia, which activates hypoxia inducible factor 1 (HIF1) in both tumor cells and surrounding stromal cells leading to the production of vascular endothelial growth factor (VEGF), a potent mediator of new vessel growth (Krock et al., 2011). In addition, TAMs

and CAFs are also capable of stimulating angiogenesis through the secretion of VEGFA as well as the lymphangiogenic factor VEGFC and VEGFD (Quail and Joyce, 2013). Given its critical role in angiogenesis, VEGF-targeted agents have emerged as a major class of therapeutics with broad applicability in cancer (Ferrara and Adamis, 2016). Despite success in some tumor types (renal cell carcinoma), understanding which patients are most likely to benefit from anti-angiogenic therapy remains a challenge. Angiogenic signaling is further complicated in the context of inflammation where the paralogous factors angiopoietin-1 and angiopoietin-2 play a role in dampening and amplifying sensitivity to the inflammatory factor TNFα during wound healing (Fiedler et al., 2006). Two recent studies demonstrated that dual targeting of angiopoietin-2 and VEGF resulted in vascular normalization and extended survival in murine models of glioblastoma (Kloepper et al., 2016; Peterson et al., 2016). This combination was also found to potentiate immunotherapy via PD-1 blockage (Schmittnaegel et al., 2017). Understanding the interplay between these molecules and the tumor's dependency will be critical for maximizing future anti-angiogenic therapeutic approaches. While the vasculature plays a clear role in delivering nutrients to a tumor, several reports have provided evidence for an additional role in promoting cancer stem cells. In colorectal cancer (CRC) endothelial cells have been shown to support cancer stem cells through soluble Jagged-1 mediated activation of the Notch signaling (Lu et al., 2013). Similar results were found for Jagged-1, as well as DLL4, in glioblastoma models (Zhu et al., 2011). These studies suggest that targeting of endothelial cells may provide an avenue for modulating cancer stem cells; however, previous reports have demonstrated that anti-angiogenic therapies can actually lead to an increase in cancer stem cells due to increased hypoxia (Conley et al., 2012). Given the multifaceted role of the tumor vasculature, careful preclinical studies will be necessary to understand the consequences of endothelial-targeted therapy.

In addition to supporting primary tumor growth, endothelial cells are also involved in the metastatic cascade from the earliest step of intravasation to vascular cooption at the metastatic site. Histological interrogation identified a tri-cellular signaling hub known as TMEM structure, composed of a tumor cell, a Tie2^{high} TAM and an endothelial cell (Robinson and Jones, 2009). Further intravital imaging studies revealed that at this site local VEGF signaling leads to increased vascular permeability followed by a release of tumor cells into the blood stream (Harney et al., 2015). Later in the metastatic cascade, during extravasation, endothelial cells can serve as a barrier between the circulating tumor cells and host tissue. In brain metastasis for instance, cleavage of the endothelial adhesion molecule, JAM-B, is necessary for tumor cells to efficiently extravasate (Sevenich et al., 2014). Upon extravasation, metastatic cells once again rely upon close association with endothelial cells to support their survival in a process known as vascular cooption. These studies collectively highlight that endothelial cells play critical roles in both tumor development through increased vascularization, but are also intricately involved in many steps of disease progression.

CAUSES OF AN INFLAMMATORY MICROENVIRONMENT

External Factors and Exposures Cause Acute and Chronic Inflammation

Evidence indicates that chronic inflammation not only increases the risk of a multitude of cancers, including colon, liver, pancreatic, lung, bladder, gastric, and breast, but may also increase the risk of tumor progression and metastasis (Iyengar et al., 2015). Moreover, it is well known that a variety of external factors and exposures can cause both acute and chronic inflammation. Because many of these factors/exposures, such as diet, are modifiable, there is a growing scientific and public health interest in understanding the relationship between extrinsic pressures that promote chronic inflammation and the TME.

Tobacco

Historically, tobacco has been the most notorious carcinogen. At present, tobacco exposure is the leading preventable cause of death. Roughly 85% of all lung cancers are secondary to smoking with additional cancers attributable to secondary smoke (Warren and Cummings, 2013). In developed countries, tobacco is associated with roughly 30% of all malignancies (McGuire, 2016). Tobacco use is associated with numerous other cancers as well, including but not limited to, head and neck, pancreatic, gastric, esophageal, acute myeloid leukemia (AML), bladder and renal cell cancers (United States Public Health Service, and Office of the Surgeon General, 2010). For example, actively smoking triples the risk of bladder cancer (Freedman et al., 2011; McGuire, 2016). Tobacco increases cancer risk not only by causing direct genetic changes to the epithelium (particularly the lung), but also through altering epigenetic events, eliciting epithelial to mesenchymal cell transition, and inducing a chronic inflammatory and hypoxic microenvironment. Ongoing inflammation promotes apoptotic arrest, angiogenesis and in turn cell proliferation (Milara and Cortijo, 2012). Tobacco inherently contains a multitude of direct carcinogens, including polycyclic aromatic hydrocarbons, *N*-nitrosamines, aromatic amines, aldehydes, volatile organic hydrocarbons, and metals among others (Pfeifer et al., 2002). The activation of these carcinogens in the host can result in the formation of DNA adducts, a form of DNA damage and source of mutagenesis. In addition to directly affecting DNA function of the cell, tobacco can modify the immune function of the host (Sopori, 2002). For example, tobacco can increase the abundance of alveolar macrophages in the lung, which may increase oxidative stress and oxygen radicals, thereby promoting tumor growth. In a study of 1,819 individuals, systemic levels of 78 makers of inflammation and immunity were measured. The study included 548 never smokers, 857 former smokers, and 414 current smokers. Significant differences in several immune markers were noted between active and never smokers. These include but are not limited to differences in CCL17/TARC, CCL11/EOTAXIN, IL-15, IL-1B, IL-1Rα, CRP, SVEGFR3, IL-16, sIL-6R, and SCF. Interestingly, the authors note that many of these makers are critical to immune function as well as cell growth. Overall,

they report that on average smokers appear to have an immune profile consistent with an overall immunosuppressive function of tobacco (Shiels et al., 2014). Indeed multiple studies have demonstrated that tobacco has immunosuppressive functions (Cui and Li, 2010). One mechanism by which nicotine may be immunosuppressive is by impairing macrophage and neutrophil function (Milara and Cortijo, 2012). In tobacco smokers with chronic obstructive airway disease (COPD) the lung epithelium undergoes repeated injury and repair thereby inducing transformation of the normal epithelium to a more malignant phenotype. Tobacco smokers who continue to smoke also have poorer outcomes from a multitude of cancers, perhaps as a result of suppression of NK cell activation (Lu et al., 2007). Despite the significant link between tobacco and lung cancer, some non-smokers still develop lung cancer. Toward this end, understanding the mechanisms by which smokers as well as non-smokers may share common markers related to chronic airway inflammation may shed important light into molecular targets for the diagnosis and treatment of lung cancer. Alternatively, sequencing of non-small cell lung cancers in tobacco smokers vs. non-smokers identifies distinct genetic signatures. Tobacco induced tumors have a greater mutational burden than those found in non-smokers, which may in turn predict response to immunotherapy (Godwin et al., 2013; Hellmann et al., 2016).

Alcohol

Alcohol can act in concert with tobacco to significantly increase the risk of cancer. Historically, alcohol was long associated with liver as well as head and neck cancers (LoConte et al., 2018). However, alcohol alone is now classified as a carcinogen, increasing the risk of several cancers in a linear dose dependent fashion, including breast, pancreas, liver, colon, esophagus and head and neck cancers. In hepatocellular cancers specifically, alcohol is known to induce chronic liver inflammation and fibrosis, which is itself a pre-cursor to malignancy (also discussed above). In addition, there is also evidence suggesting that patients with hepatocellular cancers and increased alcohol consumption show poorer outcomes once diagnosed with cancer (Barbara et al., 1992). More recently, alcohol has been associated with a host of other cancers. According to the American Society of Clinical Oncology (ASCO), between 5 and 6% of new cancers and cancer deaths globally can be "directly attributable to alcohol" (LoConte et al., 2018). Alcohol can cause direct DNA damage as a result of ethanol conversion to acetaldehyde as well as disrupt folate metabolic pathways (Seitz and Becker, 2007). With respect to the TME, evolving preclinical data suggests that ethanol can directly disrupt immune surveillance and innate immune response as well as induce reactive oxygen species (ROS) production and oxidative stress in CAFs (Sanchez-Alvarez et al., 2013). Alcohol may also disrupt the vascular endothelium thereby creating a microenvironment more permissive to metastasis and tumor migration (Xu et al., 2012). In breast cancer patients, particularly those with estrogen receptor (ER) positive breast cancers, Sanchez-Alvarez and colleagues suggest that ethanol induces ketone production in CAFs (Sanchez-Alvarez et al., 2013). In preclinical models, CAFs have also been shown to fuel tumor growth via oxidative mitochondrial metabolism and promote a more aggressive breast cancer phenotype (Donnarumma et al., 2017).

Obesity

Historically, public health campaigns for cancer prevention have focused on tobacco's obvious link to cancer risk. More recently, however, ASCO suggests that obesity may soon outweigh tobacco as the leading modifiable risk factor for cancer (Ligibel et al., 2014). Not only does obesity increase the risk for a multitude of cancers but it may also decrease treatment delivery and worsen outcomes for those newly diagnosed. Frighteningly, over two thirds of the adult US population are overweight or obese and the number is growing (Flegal et al., 2002). Evolving data suggests that excess fat or "hyperadiposity" drives chronic inflammation, which in turn engenders a pro-tumorigenic milieu (Iyengar et al., 2016). White adipose tissue from obese patients is infiltrated by leukocytes, specifically macrophages, and T lymphoctyes (Underhill and Goodridge, 2012). When enlarged fat cells die, they release cytokines that recruit additional macrophages (Cinti et al., 2005). These macrophages then form a "crown" around the dying adipocytes, setting off an inflammatory cascade. The occurrence of these crown- like structures are commonly observed in obese patients with both breast and tongue cancers (Morris et al., 2011). Notably, Iyengar and colleagues have shown that even patients with a normal body mass index (BMI) but a relatively increased amount of body fat can also have inflammation in their breast tissue, indicating that BMI alone is not sufficient to predict for the influence of fat on body composition (Iyengar et al., 2017). Specifically, BMI is the ratio of a person's weight to height. This weight/height measurement alone does not reflect a more nuanced understanding of a person's body composition, or their relative percentages of fat and muscle. A seemingly lean person may be in fact "skinny fat," wherein they have a relatively low BMI but their body composition is predominantly fat. Much of the work connecting obesity to cancer has been studied in the breast cancer population, because both fat and normal tissue in the breast can be readily evaluated as well as because of the relationship between fat and steroid/hormonal production. In breast cancer studies, inflamed fat tissue within the breast itself can increase local cytokine production, as well as expression of aromatase and ER gene expression (Cleary and Grossmann, 2009). Not surprisingly, obesity increases the risk of death among postmenopausal women with ER-positive breast cancer (Fuentes-Mattei et al., 2014). Obese mouse models suggest that activation of the AKT-mTOR pathway in the breast itself may specifically promote worse outcomes. Obesity has also been linked to poor outcomes for other types of cancers (Ligibel et al., 2014). In squamous cell cancer of the tongue for example, a diagnosis of obesity prior to tongue cancer diagnosis was associated with a five-fold increase of death (Iyengar et al., 2014).

Dietary Exposures and Exercise

Given the relationship of obesity and cancer, diet and exercise modifications have gained increasing interest. Much of the work to date on the role of exercise, cancer, and inflammation has been done in the preclinical setting. Preclinical studies suggest

that exercise can decrease inflammatory markers and modulate the TME (Koelwyn et al., 2015). Though the mechanisms by which exercise directly reduces inflammation are not entirely clear, possible hypotheses include decreasing IL-6, reduction in adipose tissue, and inhibition of TNFα (Koelwyn et al., 2015). Exercise may also increase the cytotoxicity and number of NK cells (Bigley and Simpson, 2015). Much like pharmacologic interventions, ongoing clinical trials are evaluating whether exercise as part of ongoing therapy may modulate tumorigenesis. Early data suggests that exercise leads to a reduction in visceral fat mass, decreased low grade chronic inflammation, and a subsequent reduction in pro-inflammatory adipokine secretion, as well as a reduction in macrophage infiltration into adipose tissue (Klionsky et al., 2016). In preclinical cancer models, exercise may decrease TAM and neutrophil infiltration and increase intratumoral cytotoxic T cell infiltration (Koelwyn et al., 2017). While the majority of the work in this space has been preclinical, a more recent randomized controlled trial evaluated the relationship of exercise and outcomes in breast cancer survivors. Specifically, this randomized controlled trial assessed the effects of a 16-week combined aerobic and resistance exercise intervention on metabolic syndrome, sarcopenic obesity, and serum biomarkers among sedentary, overweight, or obese survivors of breast cancer (Dieli-Conwright et al., 2018). Serum biomarkers included IGH1, insulin, IL-6, IL-8, TNFα, and steroid hormones (estrogen and testosterone). Sarcopenic obesity, BMI, and circulating biomarkers, including insulin, IGF-1, leptin, and adiponectin were significantly improved after exercise intervention (Dieli-Conwright et al., 2018). In addition to exercise, although data have not been entirely conclusive, some epidemiology studies have linked a higher fiber, lower fat diet to a decrease in some cancers. In breast cancer patients, for example, a high fiber, low fat diet results in lower circulating levels of estradiol among patients with a history of breast cancer, even in the absence of weight loss (Rock et al., 2004). Traditionally, designing and interpreting clinical trials to assess diet and cancer intervention have been challenging.

Gut Microbiota

More recently, gut bacteria have emerged as a possible link between metabolites in food and the TME. The immune system is dependent in part on exposure via the gut to microbiota as part of immunosurveillance (Kroemer and Zitvogel, 2018). Preclinical data suggests that diet can modify gut bacteria, specifically altering toll-like receptors on macrophages and dendritic cells as well as adipose inflammation (Garrett, 2015). Interestingly, in animal models, modification of gut bacteria affects CRC incidence and natural history (Song and Chan, 2017). Moreover, as reviewed by Song and Chan, a more "Western" diet, high in processed foods, red meat, processed sugars, and refined grains can lead to a dysregulated immune response and increased levels of inflammatory markers that is associated with a higher risk of colon cancer. Multiple, epidemiologic cross-cultural studies of diet and fecal bacteria indicate that diets high in fiber and low in fat alter gut bacteria and in turn are associated with a lower colon cancer risk. Alternatively, a diet high in vegetables, fruits, and whole grains lowers colon cancer risk. It is also well known that antibiotics can modify gut flora and recent data suggests that the use of repeated antibiotics may increase the incidence of lung, prostate, bladder and breast cancer possibly by altering gut flora (Velicer et al., 2004; Iida et al., 2013; Boursi et al., 2015). Evolving evidence suggests that gut bacteria may even influence response to immunotherapy. In melanoma patients, analysis of patient fecal microbiome samples indicated a higher diversity in bacteria and amount of Ruminococcaceae bacteria among those who responded to anti-PD-1 immunotherapy (Gopalakrishnan et al., 2018). Chaput and colleagues also demonstrated that gut microbiota, and in particular *Faecalibacterium* and other *Firmicutes* improved response to the CTLA-4 blockade by ipilimumab (Chaput et al., 2017). It is also hypothesized that some chemotherapies may uniquely alter gut bacteria and modulate immune response (Viaud et al., 2013).

Infectious Agents

In addition to modifiable behaviors associated with an inflammatory pro-tumorigenic cascade, several infectious agents have also been linked to cancer incidence (Moore and Chang, 2010). Globally, it is estimated that roughly 15% of all cancers are associated with infections (Parkin, 2006). Microbes (including bacteria and viruses) have been implicated in cancer in a number of different mechanisms, sometimes in combination. Possible mechanisms include direct DNA damage, via oncogenes or tumor suppressor inhibition, as well as via the promotion of chronic inflammation and in some instances, such as HIV, immunosuppression (Kuper et al., 2000). Common microbial associations with cancer include helicobacter pylori (gastric cancer and gastric MALT lymphoma), schistosoma haematobium (bladder cancer), HPV (human papillomavirus) (cervical and oral cancers), *clonorchis sinensis* (cholangiocarcinoma) and hepatitis B and C viruses (hepatobilliary cancers) to name a few (Kuper et al., 2000). However, the mechanisms by which each infection promotes cancer are not entirely linear. Hepatitis B and C viruses for example do not neatly fit into a category of direct or indirect carcinogens. Rather, it is likely that they contribute to carcinogenesis via a host of mechanisms including the introduction of viral products to the cancer cell as well as by inducing chronic inflammation (Tsai and Chung, 2010). As it is beyond the scope of this article, Moore and Chang provide an extensive review of the relationship between viruses and cancer (Moore and Chang, 2010). Similarly, the relationship between *H. Pylori* and gastric cancer is also multifactorial. *H. Pylori* both promotes inflammation of gastric epithelial cells and also induces specific protein changes and gene mutations (Chiba et al., 2008). Notably, not all patients with select infections go on to develop cancer. The ways in which infections promote cancer either by directly inducing changes to host cells or inducing a chronic inflammatory response varies significantly. In addition to efforts to eradicate infections with known cancer associations, such as with the HPV vaccine or *H. Pylori* treatment, it will be equally important to understand why some people are protected while others progress to infection-associated cancers.

Intrinsic Mechanisms Leading to an Inflammatory Microenvironment

The mechanisms by which extrinsic factors can promote a pro-tumorigenic environment are inextricably linked to the ways in which genetic and epigenetic alterations can aide a cancer cell escape host defense mechanisms. Importantly, oncogenic mechanisms require a tight bidirectional cross talk of cancer cells with their microenvironment mediated by the production of chemokines, cytokines, growth factors, prostaglandins, ROS and nitrogen oxygen species (NOS), as well as recruitment of inflammatory cells into the tumor tissue. Many of the powerful oncogenes possess the ability to initiate a signaling cascade resulting in an inflammatory response in the proximity of the cells that harbor those oncogenes. The discovery that many oncogenic drivers are deeply involved in the modulation of a pro-oncogenic microenvironment and inflammatory processes suggested possible paracrine effects where altered expression or activity of the same genes in a stromal and/or immune cell may dictate epithelial fate and vice versa.

Tumor-Elicited Inflammation, Oncogenes and Tumor Suppressors

The advent of high-throughput sequencing techniques has led to the identification of hundreds of genetic and epigenetic alterations in genes associated with signaling pathways involved in cancer. Historically, genetic and functional studies have focused on a better understanding of the consequences of oncogenic activation in the context of the tumor cell itself and thus far only a limited number of studies has assessed their contribution beyond the cell-intrinsic effects. Here, we review common oncogenic and tumor suppressor pathways that contribute to tumor-associated inflammation (**Figure 1**). These include receptor and non-receptor tyrosine kinases (TKs), RAS signaling, TP53, APC, and PTEN.

Tyrosine Kinases

The activity of (receptor) TKs is central to many cellular processes. TKs also play cardinal roles in cytokine function, and are crucial for the signal transduction of various pro- and anti-inflammatory cytokines such as TNFα, IL-6, and IL-10. Due to their central status, TKs have received heightened attention as therapeutic targets, partially due to their potential to combat the chronic inflammatory state associated with many malignancies such as rheumatoid arthritis (RA), cardiovascular diseases, and cancer. In solid and blood cancers, TKs are frequently mutated leading to ligand-independent constitutive activation of downstream signaling pathways. For example, more than 90% of the non-leukemic classical myeloproliferative neoplasms (MPNs) are clearly driven by abnormal JAK2 activation, especially the cytokine receptor/JAK2 pathways and their downstream effectors (Passamonti et al., 2011). In line with a crucial role of JAK2 in cytokine signal transduction, MPN patients are characterized by high levels of pro-inflammatory cytokines in their circulation, which can be reduced by JAK inhibitor therapy (Verstovsek et al., 2010; Geyer et al., 2015; Mondet et al., 2015). Indeed, it is believed that the impressive clinical activity of ruxolitinib is a result of its anti-inflammatory effects. This suggests that aberrant JAK-STAT pathway activation is important for the induction and maintenance of the inflammatory state in MPN patients. Notably, whether *JAK2* is mutated or not, the efficacy of ruxolitinib is comparable (Deininger et al., 2015). We have recently shown that both mutant and non-mutant hematopoietic cells are the source of pro-inflammatory cytokines in MPN mouse models and patients and that JAK-STAT signaling in mutant and non-mutant cells has to be inhibited in order to achieve therapeutic response (Kleppe et al., 2015b). This data suggests that sequential, interlinked, and selective steps, which bear clear resemblance to tumor-cell-organ microenvironment interactions commonly found in solid cancer and metastasis, also drive aberrant cytokine production in hematological malignancies. Other TKs which may be involved in the induction of an inflammatory state include c-Kit, EGFR, PDGFR, RET, VEGFR, c-Fms, and FGF (extensively reviewed in Yang and Karin, 2014).

RAS Signaling

The RAS superfamily of small GTPases comprises a group of more than 150 small G proteins. RAS proteins are signal transduction molecules central to many cellular processes. Mutations in one of the three canonical *RAS* genes, H-*RAS,* N-*RAS,* and K-*RAS,* are among the most common genetic abnormalities in human cancers. It is well established that aberrant RAS activation drives neoplastic transformation by influencing diverse aspects of the malignant phenotype in a cell autonomous manner, most importantly cell proliferation, survival, and mobility. Interestingly, more recent reports suggest that the role of oncogenic RAS extends beyond the effects on the tumor cell itself. Oncogenic Ras causes genotoxic stress and senescence in cells (Coppé et al., 2008). Intriguingly, senescent cells are known to secrete a myriad of inflammatory factors. In line, oncogenic Ras has been shown to accelerate and amplify a senescence-associated secretory phenotype that largely depends on IL-8 and IL-6 secretion thereby promoting tumorigenesis through effects on non-transformed cells during the process of inducing senescence (Coppé et al., 2008). Interestingly, high Ras activity in pancreatic acinar cells leads to cellular senescence and is sufficient to induce an inflammatory phenotype that is similar to the histological features of chronic pancreatitis suggesting that mutant K-*ras* is a cause rather than a secondary effect of chronic pancreatitis (Ji et al., 2009). Notably, patients with chronic pancreatitis have an increased risk of developing pancreatic cancer and K-*RAS* mutations are commonly found in chronic pancreatitis (Lüttges et al., 2000), but also observed in hyperplastic ducts within normal pancreas (Tada et al., 1996). Indeed, a large proportion of the adult human population possesses *RAS* mutations in tissues besides the pancreas, including colon and lung. Ras-mediated cytokine production has been repeatedly linked to activation of the inflammatory regulator NF-κB. It has been shown that in presence of mutant Ras, inflammatory stimuli initiate a NF-κB-dependent positive feedback loop involving Cox-2 resulting in prolonged Ras signaling and chronic inflammation and precancerous lesions in mice (Daniluk et al., 2012). In keratinocytes, expression of oncogenic Ras instigates an autocrine loop through IL-1α,

IL-1R, and MyD88 leading to phosphorylation of IκBα and NF-κB activation (Cataisson et al., 2012). Moreover, activation of oncogenic Ras has been shown to enhance expression of squamous cell carcinoma antigens 1 and 2 and IL-6 via the NF-κB pathway (Catanzaro et al., 2014). K-*RAS* mutations mediate therapeutic resistance and are associated with poor prognosis, and until now, no effective anti-RAS inhibitor has reached the clinic (Cox et al., 2014). Given the growing body of evidence linking aberrant RAS and NF-κB it is intriguing to speculate that the NF-κB pathway could be exploited as potential preventive and therapeutic target in cancers harboring mutant *RAS*.

TP53

TP53 is a stress-responsive transcription factor (TF) and acts as a major tumor suppressor inhibiting neoplastic transformation by preventing the escalation of chronic tissue imbalance (Cooks et al., 2014). TP53 is a central hub for diverse stress signals, including ROS and NOS, cytokines, and infectious reagents (Cooks et al., 2014). TP53 also participates in the control of multiple cell cycle checkpoints. Mutations disabling TP53 tumor suppressor functions are the most frequent events in human cancer. For example, molecular alterations of *TP53* are a defining feature of ovarian high-grade serous carcinomas (Cancer Genome Atlas Research, 2011; Vang et al., 2016). In addition to the cell autonomous effects of TP53 inactivation/dysfunction, compelling evidence suggests that *TP53* missense mutants may not merely lose their tumor suppressive functions, but can also acquire new oncogenic properties through the activation of cell non-autonomous pathways. Specifically, multiple studies have linked mutant TP53 (such as *TP53* p.R273H) and chronic inflammation to tumorigenic progression through different molecular interactions, including NF-κB (Cooks et al., 2013; Di Minin et al., 2014; Cui and Guo, 2016). In line, it has been demonstrated that *TP53* mutants interact directly with NF-κB and that both factors impact the other's binding at diverse sets of active enhancers thus promoting a unique enhancer landscape of cancer cells in response to chronic inflammation (Rahnamoun et al., 2017). Moreover, clinical studies in primary breast carcinoma, head and neck squamous cell carcinoma, and CRC suggest that *TP53* inactivation or deletion induces inflammation (Yin et al., 1993; Brentnall et al., 1994; Hussain et al., 2000; Linderholm et al., 2000; Lee et al., 2007). The effects of Tp53 loss have also been studied in diverse mouse models. For example, in a mouse model of prostate cancer, Tp53 loss resulted in enhanced transcription of cytokines and chemokines, accumulation of ROS and protein oxidation products, enhanced macrophage activation and neutrophil clearance, hypersensitivity to LPS, and high expression of metabolic markers (Komarova et al., 2005). Further, Lujambio and colleagues showed that Tp53-deficient hepatic stellate cells secrete factors that stimulate polarization of macrophages into a tumor-promoting M2 state leading to increased liver fibrosis and accelerated transformation of adjacent hepatocytes (Lujambio et al., 2013). Collectively, inhibition of tumor-associated inflammation is likely another important tumor suppressive function of TP53.

APC

CRC represents a paradigm for the link between inflammation and cancer (Lasry et al., 2016). Patients with inflammatory bowel disease, such as ulcerative colitis, are more likely to develop CRC and non-steroidal anti-inflammatory drugs show strong preventive effects (Chia et al., 2012). In mouse models of colitis, genetic, and functional studies have shown that inflammation alone suffices for tumor development and that inflammation-induced DNA damage can link chronic colitis and tumor initiation. Moreover, colorectal tumors exhibit tumor-elicited inflammation and upregulation of inflammatory signature genes (Wang and Karin, 2015). In fact, the type, density, and location of immune cells within human colorectal tumors have proven to be a reliable measure of patient outcome (Galon et al., 2006; Grivennikov et al., 2010; Norton et al., 2015). Inactivating mutations in *APC,* resulting in aberrant β-catenin activation, are found in 80% of all human colon cancers. In addition, *APC* loss predisposes humans to familial adenomatous polyposis, an autosomal dominant syndrome, in which patients develop numerous colorectal polyps (Groden et al., 1991). The tumor suppressor activity of APC has been extensively studied in the setting of epithelial transformation. In mice, the presence of an autosomal dominant *Apc* mutation in intestinal epithelial cells (IECs) leads to tumor development upon inactivation of the other allele due to spontaneous loss of heterozygosity (LOH) (Moser et al., 1990, 1993; Jackstadt and Sansom, 2016). Colon-specific deletion of *Apc* leads to formation of colorectal tumors with upregulation of pro-inflammatory cytokines. Interestingly, this work suggests that epithelial barrier defects and microbial invasion into the TME leads to an activation of IL-23 producing myeloid cells, which, in turn, drive IL-17 mediated tumor growth (Grivennikov et al., 2012). Chronic NF-κB activation in IECs has been shown to lead to the development of intestinal adenomas linking inflammation and tumorigenesis (Greten and Karin, 2004; Vlantis et al., 2011). In line with a pro-tumorigenic function of NF-κB in CRC, crossing transgenic mice with chronic epithelial NF-κB activation to $Apc^{Min/+}$ mice leads to accelerated LOH and intestinal tumor initiation through iNOS up-regulation (Shaked et al., 2012). Intriguingly, little is known about the consequences of APC inactivation in immune cells. While it has been shown that *Apc* mutant mice are characterized by an altered intestinal immune homeostasis and impaired control of inflammation by regulatory T lymphocytes (Gounaris et al., 2009; Akeus et al., 2014; Chae and Bothwell, 2015), only a recent study demonstrated that Apc inactivation in T-cells renders the immune system unable to tackle gut inflammation due to deficient T-cell activation (Agüera-González et al., 2017).

PTEN

The phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) functions as tumor suppressor by inhibiting PI3K-dependent cellular proliferation, survival, growth, and motility. PTEN function is frequently disrupted in human cancer, but has also been shown to play a role in other diseases (Leslie and Downes, 2004). The phosphatase has originally been identified as tumor suppressor through its mutation leading to abolished or greatly decreased phosphatase

activity. More recently, it has been shown that PTEN protein expression is lost in a greater number of patients as originally expected based on mutational frequency. Both genetic and epigenetic mechanisms are discussed as possible mechanisms causing loss of PTEN protein expression in the absence of coding sequence mutations (Leslie and Downes, 2004). PTEN represents another tumor suppressor that has been shown to exhibit its oncogenic functions, at least in part, through manipulation of the microenvironment by triggering the release of inflammatory mediators from tumor cells. For example, PTEN dysfunction has been shown to increase the expression and signaling of pro-inflammatory chemokine CXCL8 in prostate cancer cells that resulted in a coordinated response of both tumor and stromal cells. Increased release of Cxcl8 from *Pten*-deleted tumor cells augmented the sensitivity and responsiveness of tumor cells to stromal chemokines by concurrently inducing the upregulating of chemokine receptors on tumor cells and inducing stromal chemokine production (Maxwell et al., 2014). In one study, progression of *Kras* mutant PDAC was associated with deletion and loss of expression of *Pten*. Interestingly, Pten loss and activation of K-Ras cooperated and accelerated pancreatic cancer development by promoting NF-κB activation and its cytokine network, which in turn promoted stromal activation and immune cell infiltration (Ying et al., 2011). Similarly, Kim and colleagues showed that knockdown of tumor suppressor *Pten* and *Tp53* in breast cancer cells synergized to activate a pro-inflammatory Il-6/Stat3/NF-κB signaling axis (Kim et al., 2015). In addition, loss of *Pten* has been shown to prevent anti-tumor immunity (Spranger et al., 2015; Peng et al., 2016). In conclusion, available data suggests that loss of *PTEN* leads to activation of an inflammatory loop that contributes to malignant transformation.

Genetic Studies of the Tumor Microenvironment

About two decades ago, Fattaneh Tavassoli and his team were the first to report genetic alterations, specifically LOH at microsatellite markers, in the stroma of mammary carcinomas (Moinfar et al., 2000). Since then a number of human studies have analyzed the mutational spectrum of selected tumor suppressors and LOH/allelic imbalances of specific markers. In 2001, Kurose and colleagues analyzed invasive LCM-procured epithelium and stroma from adenocarcinoma samples of the breast and reported that both epithelial and stromal cells harbor LOH of specific markers including those at 10q23 (in the vicinity of *PTEN*), 17p13–p15 (in the vicinity of *TP53*) and 16q24 with a higher frequency in the neoplastic epithelial compartment (Kurose et al., 2001). Around the same time, a different group reported LOH on chromosome 17p13, 3p25-26, and 9q32-33 in the stroma of invasive urothelial carcinoma (Paterson et al., 2003). In 2002, Kurose et al. reported that mutations in the tumor suppressors *PTEN* and *TP53* occur at a high frequency in the neoplastic breast epithelium and/or stroma (Kurose et al., 2002). Charis Eng and her team followed then up on their work with a larger study analyzing the mutational status of *TP53* and LOH in 218 invasive breast cancers patients (Patocs et al., 2007). They found a high frequency of *TP53* mutations in hereditary (49%) and sporadic tumor (27.4%) stroma. Similarly, 60% (hereditary) and 51% (sporadic) of the patients carried LOH or allelic imbalances in the stroma. In addition to previous studies, the authors related their genetic findings to clinical and pathological features of the disease. Interestingly, in the sporadic group, the presence of *TP53* mutations in the stroma was associated with lymph node status and nodal metastasis. Those observations suggest that genetic alteration of *TP53* in the stroma may accelerate tumor growth. Overall, numerous independent investigators have described a variety of genetic, epigenetic, and genomic alterations in the stroma of a broad variety of solid tumors and inflammatory conditions (Man et al., 2001; Tuhkanen et al., 2004; Hu et al., 2006; Ishiguro et al., 2006; Kim et al., 2006; Bian et al., 2007; Joshua et al., 2007; Weber et al., 2007; Yagishita et al., 2008). While such findings still remain controversial (Allinen et al., 2004), it challenges the current paradigm that the microenvironment, albeit aberrant, would not be targeted by genetic alterations and highlights the necessity to further our mutational understanding of this crucial component. Importantly, the finding of genetic and epigenetic changes in the stroma raises a number of questions, regarding the mechanisms leading to these genetic lesions, the populations in which they are found, their functional importance to tumor development, and clinical implications for patients with mutations in the stroma. Using a variety of different approaches, multiple groups have tried to model how modulation of known tumor suppressors and/or oncogenes in stromal cell types may affect malignant transformation using mouse models. Early work by the group of Terry van Dyke suggested that oncogenic stress mediated by an initial driver event in the epithelium would create pressure in the microenvironment that leads to loss and selection of a Tp53-deficient stromal compartment (Hill et al., 2005). This is in line with the knowledge that stromal fibroblasts with intact Tp53 can render the microenvironment less supportive of the survival and spread of adjacent tumor cells by secretion of a spectrum of growth inhibitors (Komarova et al., 1998; Moskovits et al., 2006). Intriguingly, Hill and colleagues further showed that loss of *Tp53* in the stromal compartment disrupts the homeostasis between the epithelial and stromal tissues ultimately leading to loss of *Tp53* also in the tumor suggesting that stromal loss may actually precede epithelial *Tp53* loss (Hill et al., 2005; Palumbo et al., 2015). About a decade later, Farmaki and colleagues also showed that the TME induces strong selective pressure onto stromal cells, selecting specific subpopulations of stromal fibroblasts that can survive and expand more efficiently within the TME (Farmaki et al., 2012). Notably, higher numbers of cancer cells were associated with a stronger proliferative advantage of *Tp53*-deficient fibroblasts as compared to wild-type cells in line with the concept that loss of *Tp53* heightens the sensitivity of mutant fibroblasts to epithelial-derived growth factors. Mechanistic studies revealed that the oncogenic effect of *Tp53*-deficient microenvironment is mediated by enhancing the levels of inflammatory cytokines/chemokines and immunosuppressive molecules, which disturbed immune cell composition and exacerbated immunosuppressive function within the microenvironment (Guo et al., 2013). Further, ablation of Tp53 in fibroblasts has been shown to promote tumor growth

in a murine prostate cancer model (Addadi et al., 2010). While TP53 has been the major focus of most mechanistic studies, it is conceivable that mutational inactivation of other tumor suppressors as well as epigenetic alterations such as histone modifications and DNA methylation may be responsible for the generation of stromal cells with pro-tumorigenic properties (Hu et al., 2005; Peng et al., 2005; Bar et al., 2009). It will be important to uncover the identity and regulation of secreted factors that are responsible for the tumor cell-induced inhibition of stromal TP53 induction and other potential tumor suppressors.

Clonal Hematopoiesis and Inflammation

Leukocytes represent a crucial component of the inflammatory TME. The various leukocyte subsets, including macrophages, neutrophils, basophils, and lymphocytes can interact with each other, but also with non-hematopoietic stromal cell types and epithelial tumor cells, thereby orchestrating tumor progression and invasiveness. To date, many groups have studied the prognostic value of infiltrating immune cells in solid tumors (reviewed by Barnes and Amir, 2017; Hammerl et al., 2017). With the increasing interest in harnessing the immune system to treat cancer with checkpoint inhibitors and other novel agents, a better understanding of the composition of the immune infiltrate as prognostic marker is of increasing importance. However, the simple presence of a specific immune cell type in the TME does not predict their function. For example, macrophages cover a continuum of functional states that allows them to fulfill different tasks depending on the microbial and cytokine milieu. Further complicating the scenario, recent data challenge the paradigm that the integrity of the genome of immune cells is intact in solid cancer patients. At first, a large body of genetic data emerged demonstrating that elderly individuals without signs of overt leukemia harbor somatic mutations in hematopoietic cells leading to expansion of mutant blood cells. Most of the mutations were identified in genes encoding for known leukemia drivers such as the chromatin modifiers *TET2, ASXL1,* and *DNMT3A* (Busque et al., 2012; Genovese et al., 2014; Jaiswal et al., 2014; Shlush et al., 2014; McKerrell et al., 2015; Young et al., 2016; Buscarlet et al., 2017). Not surprisingly, follow up studies showed that patients with clonal hematopoiesis (CH) are at an increased risk of developing hematological malignancies. However, the same study showed that patients with CH are also at an increased risk of atherosclerotic cardiovascular disease compared to individuals without CH (Jaiswal et al., 2014, 2017a,b). Atherosclerotic cardiovascular disease has long been thought of as an inflammatory disease; however, only recently, data from the CANTOS study was published reporting that selectively targeting inflammation by using a therapeutic monoclonal anti-IL-1β antibody can reduce cardiovascular risk (Ridker et al., 2017a). But is there a mechanistic link between the presence of mutant cells in the blood, the development of atherosclerotic cardiovascular diseases, and inflammation? Intriguingly, a growing body of evidence suggests a causal link between mutations in epigenetic modifiers seen in CH and inflammation. For example, *Tet2*-deficient macrophages exhibit an increase in inflammasome-mediated IL-1β secretion, which is associated with accelerated development of atherosclerosis in these mice (Fuster et al., 2017). *Tet2* also seems to exhibit a suppressive role in the regulation of immunity and inflammation, independent of its role in DNA methylation and hydroxymethylation, but by repression of transcription via histone deacetylation (Zhang et al., 2015). Specifically, loss of Tet2-mediated gene transcription resulted in increased expression of inflammatory mediators upon injection of the mice with the highly potent inflammatory stimulus LPS. Further, *Tet2*-deficient mice were more susceptible to experimental colitis and endotoxin shock (Zhang et al., 2015). *Dnmt3a*-deficient mast cells display an increased sensitivity to acute and chronic inflammatory stimulation (Leoni et al., 2017). Yet in another immune cell type, both Dnmt3a and Tet2 seem to be important for the regulation of macrophage activation, polarization and inflammation (Yang et al., 2014; Li et al., 2016). It is likely that this is just the tip of the iceberg and the coming years will provide a greater understanding of the functional and regulatory roles of these important epigenetic regulators in different immune compartments, myeloid malignancies, solid tumors, and non-malignant inflammatory diseases.

The accumulating body of evidence that older individuals have clinically unapparent CH, together with the increasing awareness of the importance of microenvironment in tumor progression, we hypothesized that immune cells infiltrating tumors might be characterized by clonally selected mutations. Targeted sequencing analysis of leukocytes isolated from a small number of tumors from treatment naïve breast cancer patients demonstrated that indeed infiltrating CD45-positive hematopoietic cells harbor somatic mutations in cancer genes, including *BCOR*, *TET2*, *DNMT3A*, in a subset of patients (Kleppe et al., 2015a). These mutations were not found in peripheral blood cells, admixed tumor cells, or epithelial germline samples. This finding suggests that mutant infiltrating leukocytes may interact with cancer cells, which has significant clinical implications for tumor development and response to treatment. Our data was partially corroborated by a recent report that hematopoietic cells are also genetically abnormal in a fraction of patients with advanced solid cancers even at a younger age (Coombs et al., 2017). Coombs and colleagues analyzed paired tumor and blood samples from ∼8,000 patients with advanced solid cancers. In total, one fourth of all patients carried at least one CH mutation in the blood sample, with 4.5% of the patients harboring presumptive driver mutations (CH-PD). CH was associated with increasing age, tobacco use, and prior radiation therapy. Further, patients with CH had an increased risk of hematologic cancers and CH-PD was associated with a shorter survival. Notably, the primary cause of death was progression of the primary non-hematopoietic tumor. While both studies support the intriguing idea that immune cells are genetically abnormal which could be of utmost importance from a diagnostic and therapeutic standpoint, there are fundamental differences in the study design and the conclusions. Regardless, neither study data discerns between the different leukocyte subsets. As such, it will be critical to assess the distribution and frequency of CH mutations within the different hematopoietic/immune compartments.

PREVENTION AND THERAPEUTIC INTERVENTION

Checkpoint Blockade Immunotherapy

Historically, there has been a longstanding clinical interest in the overlap between the immune system and cancer. Most famously, in the early nineteenth century, William Coley developed a "Coley's Vaccine," a concoction of bacteria, after noticing tumor regression among patients who developed high fevers from *Steptococcus pyogenes* infection. His treatments and the research therein largely fell out of favor with the advent of surgical advances, radiation, and chemotherapy. In the last few years, however, the growing understanding of the tumor microenvironment and interplay between the immune system and cancer has dramatically changed the landscape of immunotherapy options. In addition to investigational efforts into vaccines and oncolytic viruses, several immunotherapy treatments have recently been approved. Specifically, an understanding of immune response and activation in cancer, and in particular the role of immune blockade by CTLA-4 (cytotoxic T lymphocyte-associated protein 4) and PD-1/PD-L1 (programmed cell death protein 1/programmed cell death protein ligand 1) has revolutionized the treatment of several types of cancers, including but not limited to melanoma, non-small cell lung cancer, urothelial, head and neck, and renal cancers. Ipilimumab was the first anti- CTLA-4 antibody to be approved in advanced melanoma (Sharma and Allison, 2015). In determining which cancers benefit from checkpoint inhibition, evidence suggests that those with a higher mutational burden (such as in response to tobacco) and in some instances microsatellite instability-high (MSI-h) tumors may respond best (Pleasance et al., 2010; Snyder et al., 2014; Le et al., 2015). Not surprisingly, given the activation of the immune system with these therapies, oncologists have had to become increasingly facile with managing a host of immune related side effects (Postow and Hellmann, 2018; Postow et al., 2018). For a more extensive review of clinically relevant checkpoint inhibitors and immunotherapy please see: (Farkona et al., 2016).

Non-steroidal Anti-inflammatory Drugs (NSAIDs)

COX enzymes (COX1 and COX2) are the primary targets of non-selective non-steroidal anti-inflammatory drugs (NSAIDs), which include aspirin, indomethacin, piroxicam, sulindac, and ibuprofen. Inhibition of COX enzymes results in the inhibition of prostaglandins, which play important roles in many physiological processes. NSAIDs are commonly used for the treatment of fever, pain, and swelling. Slowly, albeit still somewhat controversial and largely based on epidemiologic studies, NSAIDs have emerged as drugs with potential anti-cancer activity which may decrease the incidence and mortality of colon, breast, stomach, and lung cancers (reviewed in Ulrich et al., 2006, **Figure 2**). For example, chronic use of aspirin has been suggested to reduce the risk of pro-inflammatory conditions such as inflammatory bowel disease and the risk to develop colorectal cancer (Chia et al., 2012). Similarly, different studies suggest that ibuprofen might also stop certain cancers from developing (Harris et al., 2003; Johnson et al., 2010). Overall, a large body of observational data regarding a protective effect of NSAIDs from developing certain cancers, specifically CRC, is strong. A recently performed meta-analysis investigated the relationship between NSAIDs and lymph nodes/distant metastasis. The study suggests that NSAIDs hold potential in the management of cancer metastasis, regardless of whether NSAIDs are used at pre-diagnosis or post-diagnosis (Zhao et al., 2017). At this point, it cannot be denied that anti-inflammatory compounds may represent novel, less toxic, agents for cancer therapy, nonetheless, carefully designed, controlled, blinded, and randomized trials are required to create a benefit-risk assessment and address many outstanding questions such as the lowest effective dose, the age at which to initiate therapy, the duration of treatment, and which population benefits of NSAIDs chemopreventive activity. Toward this end, the AspECT trial, a large phase III, randomized study is designed to assess the long-term chemoprevention effect of esomeprazole in combination with or without aspirin in patients with Barrett's metaplasia (NCT02070757).

Glucocorticoids

Glucocorticoids (GCs) have been used in the clinic for over half a century. Indeed, due to their strong anti-inflammatory and immunosuppressive properties, GCs are the most prescribed immune suppression medications worldwide. Orally inhaled GCs are commonly used to suppress various allergic, inflammatory, and autoimmune disorders. For example, they are by far the most effective drugs for the treatment of asthma, which is largely due to their efficacy to inhibit inflammatory cytokine gene expression (Barnes, 2011). GCs exhibit multiple modes of action and interfere with the function of basically all immune cell types. For example, GCs suppress cytokine release and cell migration, induce apoptosis, and change cell differentiation fates (Perretti and Ahluwalia, 2000). In oncology, GCs have only shown modest efficacy in breast and prostate cancer and not in other cancer types. In general complexity and controversial observations are associated with GC treatment in non-hematologic cancer types (Lin and Wang, 2016). Nonetheless, GR antagonists are currently tested in several clinic trials in combinations with chemotherapy, including breast, prostate, and lung cancer. In hematological cancers, GCs such as dexamethasone have proven astonishingly effective in the treatment of lymphoid neoplasms including acute lymphoblastic leukemia, chronic lymphocytic leukemia, MM, Hodgkin's lymphoma, and non-Hodgkin's lymphoma where GCs induce growth arrest and apoptosis. GC-induced apoptosis of lymphoid cells is sought to be induced via multiple signaling pathways including Bim, a member of the Bcl2 family, and suppression of cytokines via inhibition of the activity of different TFs such as NF-κB (Lin and Wang, 2016). In contrast, only little is known about the mode of action of GCs in solid cancers.

JAK Inhibitors

The Janus family of kinases (JAKs) comprises four members: JAK1, JAK2, JAK3, and TYK2. JAKs are critical for the signal transduction of about 60 different cytokines that rely on type I and II cytokine receptors. Many of these cytokines are central to the growth of malignant cells and drivers of immune-mediated

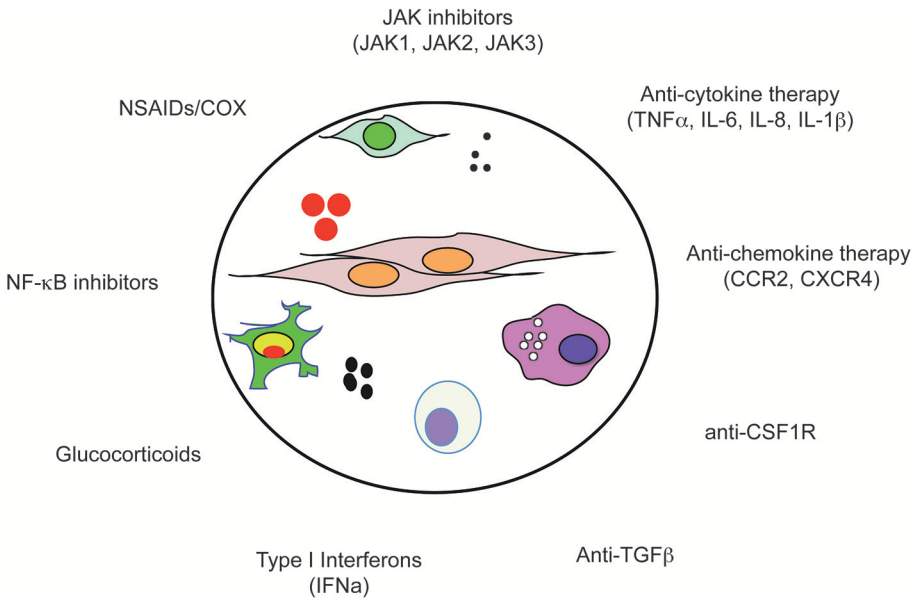

FIGURE 2 | Anti-inflammatory drugs. The tumor microenvironment (circle) is composed of tumor cells, fibroblasts, endothelial cells, pericytes, and various cells associated with the immune system such as macrophages and NK cells. Importantly, within the microenvironment a tight bidirectional cross talk of cancer cells with their microenvironment occurs which is mediated by the production of chemokines, cytokines, growth factors, prostaglandins, ROS, and NOS, as well as recruitment of inflammatory cells into the tumor tissue. Due to the complex nature of the microenvironment, a large number of druggable dependencies have been identified and are currently under investigation as therapeutic targets for anti-inflammatory drugs.

diseases. Consequently, JAKs have emerged as a new class of pharmacologic agents. The first selective JAK inhibitor to enter clinical trials was tofacitinib. Tofacitinib potently inhibits JAK3 and JAK1 and to a lesser extent JAK2 and shows a high degree of kinome selectivity (O'Shea et al., 2013). Tofacitinib is the first JAK inhibitor approved for the treatment of moderate to severe RA and active psoriatic arthritis. Mechanistically, as a JAK inhibitor, tofacitinib efficiently blocks the biological effects of common γchain cytokines including IL-2, IL-4, IL-15, and IL-21 and consequently suppresses allergic Th2 responses (Fukuyama et al., 2015). For example, in a Th2-dependent asthma mouse model, tofacitinib reduces pulmonary eosinophilia (Kudlacz et al., 2008). Further, tofacitinib has been shown suppress the differentiation of pathogenic Th1 and Th17 cells as well as innate immune signaling by limiting the production of pro-inflammatory cytokines in a LPS-induced sepsis model (Ghoreschi et al., 2011). Interestingly, tofacitinib and ruxolitinib, a JAK1/2 inhibitor, are currently being tested in patients with skin and hair disorders, including the autoimmune disease alopecia areata, and mild to moderate atopic dermatitis (Bissonnette et al., 2016; Mackay-Wiggan et al., 2016; Liu et al., 2017). Also other pharmacologic agents targeting different JAK family members have found increasing attention as anti-inflammatory targets in different disease contexts. Ruxolitinib (Jakafi) was the first FDA approved JAK inhibitor for the treatment of myelofibrosis (MF) and, more recently, for patients with polycythemia vera (PV) who have had an inadequate response to hydroxurea (Verstovsek et al., 2010; Raedler, 2015). Ongoing clinical trials also assess the efficacy of ruxolitinib in patients with post-MPN AML and CML with minimal residual disease, another form of MPN (Eghtedar et al., 2012; Pemmaraju et al., 2015; Assi et al., 2018). Interestingly, on a mechanistic level, recent work from Tarafdar and colleagues suggests that CML stem cells downregulate MHC-II, allowing them to evade the host immune response. They found that this deregulation can be reverted by JAK inhibition and IFNγ (Tarafdar et al., 2017). Besides ruxolitinib, other JAK2 inhibitors are also under clinical development for the treatment of MPNs (Kontzias et al., 2012) and their strong anti-inflammatory potential provides a rationale for repurposing these drugs as solid tumor therapeutics (Quintás-Cardama et al., 2011; Plimack et al., 2013; Buchert et al., 2016). However, clinical studies of different JAK inhibitors in solid tumors have been marked by lack of activity. For example, a phase 1 study of JAK1/2 inhibitor AZD-1480 in solid tumor patients was discontinued due to unusual adverse side effects and lack of clinical activity (Plimack et al., 2013). A comprehensive table listing clinical trials conducted with JAK inhibitors can be found in Buchert et al. (2016). Despite initial excitement, in 2016 Incyte discontinued several clinical trials due to insufficient level of efficacy including: the phase III study (JANUS 2) of ruxolitinib or placebo plus capecitabine in patients with advanced or metastatic pancreatic cancer, the phase II sub-study of ruxolitinib in patients with metastatic colorectal cancer and low CRP, and the phase II studies in breast and lung cancer. In addition, Incyte discontinued the investigation of INCB39110, a selective JAK1 inhibitor as first-line treatment for metastatic pancreatic cancer, however, preclinical studies still continue. The JAK/STAT pathway also presents new potential targets in graft-vs.-host disease (GvHD).

Despite major improvements in allogeneic hematopoietic stem cell transplantation (HSCT), GvHD still remains a matter of concern, especially if patients show no adequate response to systemic corticosteroid. Corticosteroid-refractory (SR) acute and chronic GvHD is associated with poor prognosis and therapeutic options for salvage therapy are needed. Due to its strong anti-inflammatory properties, multiple groups have performed retrospective studies to assess the potential of ruxolitinib as salvage therapy in steroid resistant GVHD patients (Zeiser et al., 2015; Mori et al., 2016; Khandelwal et al., 2017). The data suggests that while low-dose ruxolitinib shows potential in this setting, it will be important to determine in prospective studies the optimal dose to achieve the best-tolerated dose and an optimized tapering schedule to avoid withdrawal symptoms which are also observed in MF patients upon discontinuation of the drug. Overall, JAK inhibitors have a wide range of indications due to their central role in the regulation of cytokine signaling. It can be expected that the market for JAK inhibitors will continue to grow in the coming years and, similarly, the field will probably move toward using novel strategies to achieve more specific and versatile inhibition of this important family of kinases.

Anti-cytokine Therapy

Cytokine and cytokine receptors have been recognized as excellent drug targets for a variety of diseases characterized by chronic inflammation due to their important function as rate-limiting signaling molecules (Feldmann, 2008). TNFα blockade in patients with rheumatoid arthritic was the first major success demonstrating the beneficial effect of anti-cytokine therapy in a disease associated with chronic inflammation (Elliott et al., 1993; Feldmann and Maini, 2001). The role of TNFα and IL-6 as master regulators of tumor-associated inflammation and tumor-promoting functions makes them promising targets for adjuvant anti-cancer therapy (Yan et al., 2006; Grivennikov and Karin, 2011). Clinically, elevated TNFα serum levels have been detected in patients with a wide range of tumor types, although TNFα is not universally detectable. Notably, high levels of TNFα have been correlated with tumor stage, extent of para-neoplastic complications, and worse survival. Due to a prospective study showing that TNFα and IL-6 levels correlate with the degree of disease, and PSA progression in prostate cancer patients, TNFα and IL-6 are currently suggested as additional markers that reflect the activity level of this disease (Michalaki et al., 2004). Three biologics targeting TNFα are currently approved including etanercept, infliximab, and adalimumab and different small molecules that inhibit TNFα signaling or synthesis (for example thalidomide) are under development. TNFα blocking agents may not only find a place as anti-tumor agents, but may have a role in controlling the severe cancer pain associated with metastatic bone lesions. Similarly, anti-IL-6 therapy holds potential to alleviate cancer-related symptoms. Several early phase clinical studies with IL-6 targeting agents currently support the hypothesis that IL-6 may be indeed an effective anti-cancer target. Several humanized monoclonal antibodies against soluble and membrane bound IL-6R (tocilizumab, REGN88) or against IL-6 (siltuximab and sirukumab) are currently in clinical trials at various development stages for several types of cancer including MM, metastatic renal cell carcinoma, B-cell lymphoproliferative diseases (Heo et al., 2016). A fully human anti-IL-8 antibody, ABX-IL8, has shown promising therapeutic efficacy in preclinical models (Yang et al., 1999; Huang et al., 2002) however the therapeutic value of blocking IL-8 has yet to be assessed in cancer patients. Excitingly, recently published data from the CANTOS study, a randomized trial of the role of IL-1β inhibition in atherosclerosis, suggests that anti-inflammatory therapy targeting the IL-1β pathway could significantly reduce incidence of lung cancer and lung cancer mortality (Ridker et al., 2017b). CANTOS was not formally designed as a cancer detection or treatment trial and will need to be replicated; however, there is precedent for other cancer types (MAPp1, NCT01767857; Lust et al., 2009; Hong et al., 2014; Goel et al., 2016). In contrast to the success stories of anti-TNFα and anti-IL-6 targeting studies, little clinical success has been observed with therapeutics targeting single cytokines in other diseases such as COPD or asthma. One can speculate that the lack of clinical success of therapeutic targeting single cytokines or cytokine receptors in other inflammatory diseases may be due to the redundancy of the cytokine network and thus required to target more than one cytokine at a time. This may also be true for the treatment of different types of cancer; however, it is too early to say what place anti-cytokine therapy will claim in the field of oncology.

Interferons

IFNs present another group of cytokines which exhibit anti-tumor activity and can activate the immune system and are leveraged in the clinic (reviewed in Parker et al., 2016). Type I IFNs, IFNα and IFNβ, can be produced and released by tumor cells and by most cell types in the human body (Parker et al., 2016). In contrast, IFNγ, a type II IFN, is primarily expressed by T cells and NK cells. Regardless of their source, all IFNs can act via intrinsic and extrinsic mechanisms thereby exerting direct antitumor effects or indirect effects through antitumor immune responses. Tumor cell intrinsic effects of IFNs are a result of their capability to regulate the expression of genes involved in various cellular processes such as cell growth, proliferation, and survival. For example in patients with PV, IFNα has been shown to inhibit the growth of JAK2V617F-mutant hematopoietic stem and progenitor cells via activation of the p38 MAPK pathway (Lu et al., 2010). Tumor cell extrinsic effects of IFNs are mediated through their ability to influence the activity of nearly every immune cell type including T cells, NK cells, monocytes, and macrophages (Hervas-Stubbs et al., 2011). Recent work by Parker and colleagues showed that intact type I IFN signaling is required to induce anti-metastatic immunity by NK and CD8 T cells. Interestingly, their work further showed that the tumor cells themselves were a source of type I IFNs and that suppression of the tumor cells own IFN production functions as immune-evasion mechanism (Bidwell et al., 2012). IFNs exert their immunoregulatory effects on the cells of the immune system through the regulation of tumor antigens on tumor cells (Greiner et al., 1984) and antigen presentation by MHC complexes and ligands for receptors of immune checkpoints (Propper et al., 2003; Schreiner et al., 2004). Further, IFNs can regulate immunity by triggering the release of "downstream" chemokines, cytokines,

and interleukins such as IL-15 (Nguyen et al., 2002; Burkett et al., 2004). Besides their role in immunity, IFNs also promote angiogenesis and osteoclastogenesis which are important to tumor growth (Cheon et al., 2014). Unlike most cytokines which are targeted with antagonists, IFNα is used as therapeutic protein in the treatment of patients with some types of solid cancer, including Kaposi sarcoma, melanoma, and renal cell carcinoma, and different hematologic malignancies such as MPNs, and viral diseases (Kirkwood, 2002). The first type of IFN-based therapy showed striking results on the survival rates of patients with hairy cell leukemia and CML (Platanias, 2013; Stein and Tiu, 2013). While the discovery of the Philadelphia chromosome as genetic alteration in CML and the arrival of BCR-ABL targeting TKIs in the clinic have largely replaced the need for IFNα-based treatment in this disease, some recent clinical studies have shown that combination of imatinib and IFNα is superior to either therapy alone, perhaps due to the fact that IFNα targets preferentially CML stem cells (Talpaz et al., 2013). Notably, due to its success in numerous clinical trials, IFNα is used as first-line treatment choice for patients with high-risk PV (Barbui et al., 2011; Falchi et al., 2015). Regardless of the specific use, IFN-based therapies have their limitations due to dose-limiting side effects. As a direct consequence, various other strategies such as the use of IFNα conjugated with polyethylene glycol moieties or pattern recognition receptor agonists to stimulate type I IFN production in patients are under development aimed at increasing efficacy over toxicity (Parker et al., 2016).

CSF1R Inhibition

In contrast to IFN-based therapy, colony-stimulating factor 1 receptor (CSF1R) has only recently emerged as a cancer drug target. CSF1 is essential for the development and maintenance of macrophages, and as such provides a critical target for depleting these cells within the TME. The efficacy of CSF1R inhibition, and its capacity to deplete TAMs seems to vary depending upon tumor type and tissue. Administration of BLZ945, a small molecule inhibitor of CSF1R, in a murine model of glioma led to regression and long term increases in survival (Pyonteck et al., 2013). Surprisingly, this reduction in tumor burden was not associated with TAM depletion, but rather TAMs underwent "re-education," an alteration in gene expression profile with decreased expression of M2-associated anti-inflammatory markers. Meanwhile breast cancer models utilizing either BLZ945 or CSF1R neutralizing antibodies led to depletion of TAMs with limited therapeutic efficacy as a monotherapy (DeNardo et al., 2011; Shiao et al., 2015; Olson et al., 2017). Understanding why an environment is permissive to maintaining TAMs in the face of CSF1R inhibition will be critical for clinical implementation. One possibility is the expression of GM-CSF, IFNγ, and CXCL10, factors that were shown to rescue macrophage from BLZ945-mediated killing (Pyonteck et al., 2013). Whether these factors are also causatively involved in "re-education" remains to be determined. While CSF1R inhibition as a monotherapy has shown various model dependent results, combination therapy with cytotoxic agents has shown broader applicability. In murine breast tumor models, combinations of CSF1R inhibitors such as PLX3397 or BLZ945 with radiotherapy or paclitaxel displayed greater efficacy than with either single agent alone (DeNardo et al., 2011; Shiao et al., 2015; Olson et al., 2017). This combined efficacy does not appear to be limited to cytotoxic therapy as in a murine model of pancreatic adenocarcinoma PLX3397 treatment synergized with immune checkpoint blockade (Zhu et al., 2014). In this setting, treatment with PLX3397 led to a reduction in macrophages, where the remaining cells underwent a reprogramming not dissimilar from that seen in gliomas with BLZ945 treatment (Pyonteck et al., 2013). Similar combined efficacy was also seen in glioma models combining CSF1R inhibition with multiple TKIs (Yan et al., 2017). In another study, prolonged CSF1R inhibition led resistance through macrophage-derived IGF1 signaling and PI3K activation in tumor cells (Quail et al., 2016). In this setting, combination CSF1R and PI3K/IGF1R inhibition was capable of preventing relapse. Collectively, these studies demonstrate that CSF1R inhibition shows promise in chemo sensitizing combination therapies and in combination with TKIs. Multiple clinical trials are currently ongoing evaluating CSF1R inhibition with checkpoint immunotherapy, radiation therapy, and targeted small molecules (Cannarile et al., 2017). In addition to identifying the optimal tumor types for CSF1R inhibition, proper dosing schedules will also need to be determined. While the goal of many Phase I trials is to identify the maximum tolerable dose of the drug, care should be taken to understand the different outcomes of high dose and low dose CSF1R inhibition. It is possible, that at high doses, CSF1R inhibitors may deplete macrophages even in "protective" environments, and that lower doses may offer a capacity to "re-educate" macrophages without depleting them in the TME. Proper biomarkers, potentially derived from gene expression studies following CSF1R inhibition, will be necessary to determine the optimal dose based on the desired outcome of depletion vs. reprogramming.

Inflammation and Drug Resistance

Diverse resistance mechanisms to targeted cancer therapy have emerged and present one of the foremost challenges in cancer today. Drug resistance may pre-exist (intrinsic, primary drug resistance) or may be acquired under the strong selective pressure during the course of treatment (acquired resistance) (Zahreddine and Borden, 2013). Importantly, some of these resistance pathways lead to multi-drug resistance (MDR), generating an even more difficult clinical problem to overcome. Alterations in the level of cytokines, chemokines, and growth factors have emerged as yet another mechanism conferring resistance to chemotherapeutic treatments (Jones et al., 2016). For example, mounting evidence suggests a crosstalk between IL-6 and MDR in cancer and potential therapeutic opportunities arising from this role of IL-6 (Ghandadi and Sahebkar, 2016). Since the 1990s, elevated serum levels of IL-6 have been associated with worse survival in breast cancer patients (Zhang and Adachi, 1999; Salgado et al., 2003; Knüpfer and Preiss, 2007). Subsequently, functional studies revealed that autocrine production of IL-6 by tumor cells confers resistance to several chemotherapeutic agents, suggesting that acquisition of the ability of tumor cells to produce IL-6 represents another self-protective mechanism

(Conze et al., 2001). Further, increased secretion of IL-6 has been linked to resistance to bortezomib in MM and to etoposide and cisplatin in hormone-independent prostate carcinomas (Borsellino et al., 1995; Frassanito et al., 2001; Voorhees et al., 2007). IL-6-induced STAT3 feedback activation in response to the EGFR inhibitor erlotinib has been associated with the development of resistance and poor prognosis in lung adenocarcinoma (Lee et al., 2014). Work published by Korkaya and colleagues suggests that trastuzumab resistance may be mediated by an IL-6 inflammatory loop (Korkaya et al., 2012). Importantly, soluble factors mediating drug resistance such as IL-6 can be released by the tumor cell itself or the TME. For example, Ara and colleagues demonstrated that in neuroblastoma the bone marrow microenvironment is a source of IL-6 and sIL-6R, thereby allowing cancer cells to escape the cytotoxic effects of multiple chemotherapeutics (Ara et al., 2013). Regardless of the source of the cytokine IL-6, IL-6 targeting strategies promise to be effective in combination with cytotoxic agents, TKIs, or targeted antibody therapy to prevent drug resistance. Besides IL-6, IL-8 has emerged as another resistance cytokine. Similarly to IL-6, clinical studies have linked high serum levels of IL-8 to disease progression of various cancer types (Xie, 2001). In line, by using cytokine antibody arrays to identify cytokines associated with drug resistance, two independent groups concordantly found that IL-6 and IL-8 are key markers for the development of drug resistance (He et al., 2011; Shi et al., 2012). The list of "resistance cytokines" is long including AMF (autocrine mobility factor), AM (adrenomedullin), IL-4, and IL-10 (Jones et al., 2016). In addition, a recent study showed that patients with increased levels of the inflammatory biomarkers ferritin and C-reactive protein (CRP) had a markedly poorer response to trastuzumab-containing therapy (Alkhateeb et al., 2012). Another important factor to consider when discussing the relation between cytokines and cancer drug resistance is the fact that the inflammatory master regulator NF-κB is activated by a variety of cytotoxic chemotherapy agents including cisplatin, paclitaxel, docetaxel, and doxorubicin (Nakanishi and Toi, 2005; Li and Sethi, 2010). Anticancer therapeutic induced NF-κB activation often leads to the activation of pro-survival signaling pathways. In addition, NF-κB is often constitutively active in response to a variety of cancer-promoting agents. Both the constitutive and therapy-induced NF-κB activation eventuates in drug-resistant tumors, at least in part, by the induction of inflammatory cytokine secretion. Consequently, NF-κB signaling has emerged as an attractive molecular target for pharmacological intervention and its inhibitors as potential sensitizer to anticancer drugs; however, despite the clinical success in newly diagnosed and relapsed/refractory multiple myeloma (MM) and mantle cell lymphoma patients, the NF-κB inhibitor bortezomib (Valcade) has fallen short of original expectations (Lin et al., 2010; Godwin et al., 2013). Paradoxically, in some instances bortezomib has been shown to activate NF-κB signaling and stimulate cytokine secretion. For example, exposure to bortezomib of prostate cells led increased expression and release of IL-8. Mechanistically, bortezomib increased the accumulation of IκB kinase β (IKKβ) in the nucleus and increased recruitment of nuclear IKKβ, phosphorylated p65, and transcription factor early growth response-1 (EGR1) to the IL-8 promoter (Singha et al., 2014). Given that IL-8 is another resistance-associated cytokine this may explain why bortezomib is less effective in certain tumor types. Overall, there is a strong link between inflammation and drug resistance and beyond their ability to prevent or decrease chemotoxicity, anti-inflammatory agents may also have therapeutic effects when combined with conventional agents, acting additively or synergistically, or sensitizing cancer cells to treatment with conventional cancer therapies. In order to optimize the therapeutic window and regimens of anti-inflammatory cancer therapy, clinical oncologists, and experimental cancer researchers will need to identify for each drug the appropriate molecular target, cancer type, disease stage, and treatment duration. Based on the current state of knowledge, it can be expected that anti-inflammatory agents will be most effective in combination with anti-angiogenic, cytotoxic, and cytostatic agents.

Future Direction

Herein we discuss the constituents of the TME, the causes of inflammation within, and therapeutic strategies aimed at disrupting the signaling pathways critical to bolstering cancer development. Given the evolutionary nature of cancer, it is unlikely that any single therapeutic option will lead to durable cures or eradication of complex tumors. Indeed combinations therapies targeting tumor cell intrinsic oncogenic pathways, and TME-derived support pathways will offer the best means to effectively eradicate disease. These combinations are not trivial, as the TME itself is an evolving system with relatively understudied kinetics in the clinical setting. Effective therapeutic intervention may require new paradigms of dosing and schedule to identify therapeutically optimal schedules that may be non-linear and independent of maximum tolerable doses. These arduous studies may benefit from preclinical animal model development, but will inevitably require clinical testing. To expedite studies, the development of biomarkers sensitive to TME-targeted is of the utmost importance. Without these tools, potentially viable compounds and strategies may be left behind due to suboptimal dosing or rather suboptimal understanding of the correct dosing paradigm. While the molecules and pathways described here may provide sound mechanistic and molecular targets for clinical development, unveiling their full potential will require an integrated understanding of the systems-level effects of inflammation. Instead of strict molecular pathways, inflammatory microenvironments can be thought of as ecological landscapes and causes of inflammation as selective pressures sculpting tumor evolution along these landscapes. This ecological landscape is both dynamic and systemic, extending beyond the simple genetics within malignant tumor cells and beyond invasive tumor edge.

AUTHOR CONTRIBUTIONS

MK has written most of the part of this review dealing with prevention and therapeutic invention as

well as intrinsic mechanisms. RB has written most of the part on cellular constituents of an inflammatory microenvironment. EC has written most of the part of this review dealing with external factors and exposures causing inflammation. All authors have corrected the whole manuscript.

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Roles of PLODs in Collagen Synthesis and Cancer Progression

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Collagen is the major component of extracellular matrix. Collagen cross-link and deposition depend on lysyl hydroxylation, which is catalyzed by procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD). Aberrant lysyl hydroxylation and collagen cross-link contributes to the progression of many collagen-related diseases, such as fibrosis and cancer. Three lysyl hydroxylases (LH1, LH2, and LH3) are identified, encoded by *PLOD1, PLOD2*, and *PLOD3* genes. Expression of PLODs is regulated by multiple cytokines, transcription factors and microRNAs. Dysregulation of PLODs promotes cancer progression and metastasis, suggesting that targeting PLODs is potential strategy for cancer treatment. Here, we summarize the recent progress in the investigation of function and regulation of PLODs in normal tissue development and disease progression, especially in cancer.

Keywords: collagen, extracellular matrix, lysyl hydroxylation, procollagen-lysine 2-oxoglutarate 5-dioxygenase, cancer progression

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INTRODUCTION

Collagen is one of the major components of extracellular matrix. The collagen-cell interaction induces biochemical and biophysical signals, which is essentially for normal tissue function and cancer progression (Egeblad et al., 2010; Xiong and Xu, 2016). The collagen family contains 28 members (Heino, 2007) and can be divided into two groups: fibrillar collagen (type I, II, III, V, XI) and non-fibrillar collagen (type IV, VIII, X, IX, XII, XIV, XV, XVIII, XIX, XXI). Collagen is the most abundant protein in our body, and presents in both normal tissues and cancer. Type I collagen, the most common type fibrillar, has been identified in many tissues, including skin, tendon, vascular ligature and bone; while type II collagen is the main collagenous component of cartilage. Non-fibrillar type IV collagen is required for basement membrane formation (Paulsson, 1992). Cell-collagen interaction induces cellular signaling via integrin [included α1β1 (Tulla et al., 2001; Hamaia et al., 2012), α2β1 (Tulla et al., 2001; Carafoli et al., 2013), α10β1 (Camper et al., 1998) and α11β1 (Tiger et al., 2001; Hamaia et al., 2012)], discoidin domain receptors (Leitinger, 2003, 2011) and Leukocyte-Associated Immunoglobulin-Like Receptor-1 (Rygiel et al., 2011; Kim et al., 2017). Collagen regulates tumor progression by modulating cancer cell migration, invasion (Xiong et al., 2014), proliferation (Pollard, 2004), survival (Cheon et al., 2014) and metastasis (Oudin et al., 2016; Sun et al., 2016).

All collagen is composed of a triple helix, and the most common motif of the triple helix sequence is Gly-X-Y (X and Y represent proline or hydroxyproline) (Albaugh et al., 2017). Collagen is synthesized in the rough endoplasmic reticulum (ER) as a precursor (Nimni, 1983). After peptide bond formation, proline and lysyl hydroxylation is catalyzed by prolyl 4-hydroxylase (P4H) and procollagen-lysine,2-oxoglutarate 5-dioxygenase (PLOD). The hydroxylation of lysyl residues is

one of the critical steps of collagens biosynthesis. It usually occurs in the Y position of the repeating Gly-X-Y motif (Barnes et al., 1974; Valtavaara et al., 1998). Three PLODs (PLOD1, PLOD2 and PLOD3) has been identified, catalyzing the lysyl hydroxylation to hydroxylysine (Hausmann, 1967; Rhoads and Udenfriend, 1968; Kivirikko Ki, 1998; Rautavuoma et al., 2004).

Hydroxylysine residue is critical for the formation of covalent cross-links and collagen glycosylation (Valtavaara et al., 1998). PLODs catalyze hydroxylation of lysine (Lys) intracellularly before collagen is secreted, and then lysyl oxidase (LOX) binds to hydroxylysine (Hyl) residues in the extracellular collagen fibers and induces the cross-link formation (Saito and Marumo, 2010). This enzyme dependent collagen crosslinking stabilizes newly formed collagen fibers and enhances the stiffness of the matrix. During collagen maturation, the hydroxylysine residues in the helix region are often modified by the O-linked glycosylation. These reactions are catalyzed by hydroxylysine galactosyltransferase (GT) and galactosylhydroxylysine -glucosyltransferase (GGT) (Shinkai and Yonemasu, 1979; Yamauchi and Sricholpech, 2012). The enzymatic activities of GT and GGT are found in multifunctional PLOD3, but not in PLOD1 and PLOD2 (Heikkinen et al., 2000). Mutations of the human *PLOD3* gene lead to congenital disorders affecting connective tissues of various organs (Salo et al., 2008), suggesting that GGT activity of PLOD3 is crucial for the normal function of collagen.

The mutation or overexpression of PLODs has been detected in many human diseases. The kyphoscoliotic type of Ehlers-Danlos syndrome (EDS type VIA) is due to a mutation in the *PLOD1* gene (Rohrbach et al., 2011; Zahed-Cheikh et al., 2017). The reduction of PLOD3 protein at the basement membrane is associated with recessive dystrophic epidermolysis bullosa (RDEB) progression (Watt et al., 2015). The overexpression of PLOD2 is detected in many types of cancer. Therefore, investigating the function and the regulation of PLODs in normal organ development and disease progression may identify potential targets for the treatment of collagen-related diseases.

Structure of PLODs

Proteins in the PLOD family are highly homologous; the overall identity in protein sequences among PLOD1, 2 and 3 is 47% (Valtavaara et al., 1998). PLOD protein has binding sites for cofactor Fe^{2+} and L-ascorbate. It also contains 26 amino acid signal peptide and a Prolyl 4-hydroxylase alpha subunit homologs domain (**Figure 1**). *PLOD1* gene locates on chromosome 1p36 (Tasker et al., 2006) and is composed by 19 exons (Giunta et al., 2005). Collagen hydroxylation catalyzed by PLOD1 is crucial for bone mineral density (BMD) and bone quality (Tasker et al., 2006). *PLOD2* gene is at chromosome 3q23-q24 (Szpirer et al., 1997) and also contains 19 exons. Two splice variants (LH2a and LH2b) have been identified in the *PLOD2* gene; LH2b differs from LH2a by incorporating the small exon 13A (Valtavaara, 1999). PLOD2 plays a key role in formation of stabilized collagen cross-links (Gilkes et al., 2013a). *PLOD3* gene is localized to chromosome 7q36 (Hautala et al., 1992; Szpirer et al., 1997; Valtavaara et al., 1998), and PLOD3 activity is important for the biosynthesis of type IV and VI collagen (Rautavuoma et al., 2004; Sipilä et al., 2007). PLOD1 and PLOD3 hydroxylate lysyl residues in the collagen triple helix, whereas PLOD2 (LH2b) hydroxylate lysyl residues in the telopeptides of collagen (Valtavaara, 1999). PLOD3 has glycosylation activity that induces either monosaccharide or disaccharide attaching to collagen hydroxylysines (Valtavaara, 1999).

Regulation of PLOD Expression

PLOD expression is mainly regulated at the transcription level. A number of cytokines, signaling pathways, and microRNAs have been identified to be involved in transcriptional regulation of PLODs (**Table 1**). PLOD2 is induced by hypoxia-inducible factor-1α (HIF-1α) under hypoxia condition, which in turn enhance hypoxia-induced Epithelial-Mesenchymal Transition (EMT) phenotypes in glioma cells (Song et al., 2017) and breast cancer cells (Gilkes et al., 2013b). In addition, hypoxia-inducible factor 1 (HIF-1) also activates transcription of PLOD1 in breast cancer cells; however, function PLOD2 is more important for HIF-1-induced cancer progression (Gilkes et al., 2013b). PLOD2 is also directly regulated by miR-26a-5p and miR-26b-5p, and PLOD2 expression is a potential prognostic marker for patients with bladder cancer (Miyamoto et al., 2016) and renal cell carcinoma (Kurozumi et al., 2016). TGF-β signaling is another important regulator of PLOD2 expression (Remst et al., 2014). SP1 and SMAD3, as downstream targets of TGF-β signaling, recruit histone modifying enzymes to the *PLOD2* promoter region and induced PLOD2 transcription (Gjaltema et al., 2015). In addition, transcription factor E2Fs (Hollern et al., 2014) and FOXA1 (Du et al., 2017) have been identified as regulators of PLOD2 during cancer progression (**Figure 2**).

Regulation of PLOD1 and PLOD3 expression is not well-investigated compared to PLOD2. Differential display analysis identified *PLOD1* as a potential target gene of TNFα in human chondrocyte-like cells (Ah-Kim et al., 2000). Using chromatin immunoprecipitation and luciferase reporter assay, Hjalt showed that PITX2 directly regulates PLOD1 expression by binding to the promoter region. Inactivation of this pathway may cause the Rieger syndrome (Hjalt et al., 2001). One report show that miR-663a reduces PLOD3 expression by targeting to 3'-UTR of PLOD3 mRNA, subsequently reducing extracellular accumulation of type IV collagen (Amodio et al., 2016).

ACHP (2-Amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)−3 pyridinecarbonitrile), a selective inhibitor of IκB kinase, suppresses expression of all three *PLOD* genes in dermal fibroblasts, but not in lung

Abbreviations: PLOD, procollagen-lysine,2-oxoglutarate 5-dioxygenase; LH, lysyl hydroxylases; EDS, Ehlers-Danlos syndrome; RDEB, recessive dystrophic epidermolysis bullosa; BMD, bone mineral density; GT, galactosyltransferase; GGT, galactosylhydroxylysine -glucosyltransferase; BS, Bruck Syndrome; bAVM, brain arteriovenous malformations; HIF-1α, hypoxia-inducible factor-1α; EMT, Epithelial-Mesenchymal Transition; HIF-1, hypoxia-inducible factor 1; ACHP, 2-Amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)−3 pyridinecarbonitrile; BMP-2, bone morphogenetic protein-2; AT-MSCs, adipose tissue-derived mesenchymal stem cells; ESCC, esophageal squamous-cell carcinoma; HCC, hepatocellular carcinoma; HLCCs, hydroxylysine aldehyde–derived collagen cross-links; NSCLC, non-small-cell lung cancer; ER, endoplasmic reticulum; CAFs, Cancer associated fibroblasts; ECM, extracellular matrix; 3D, three-dimensional.

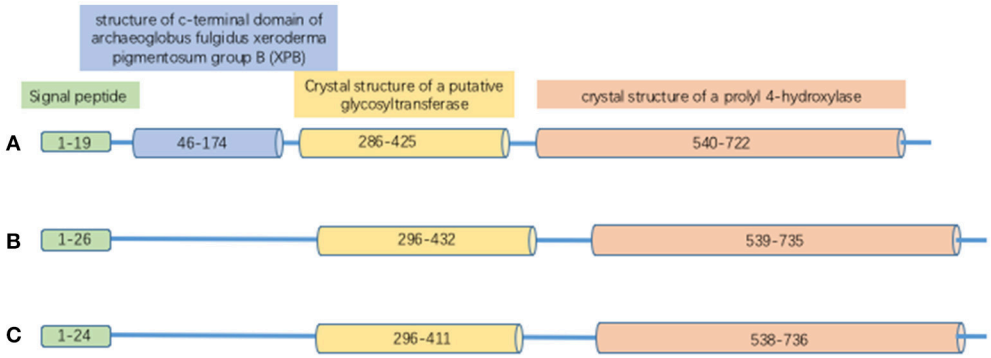

FIGURE 1 | The structure of PLOD proteins. **(A)** PLOD1; **(B)** PLOD2; **(C)** PLOD3.

TABLE 1 | The regulation of PLODs.

PLODs	Regulated by	Tissues or cell lines	Results
PLOD1	ACHP	Dermal fibroblasts	Suppression
	BMP-2	AT-MSCs	Early upregulated, later downregulated
	TGF-β	AT-MSCs	Early upregulated, later downregulated
	HIF-1α	Hypoxic breast cancer cells	Upregulated
	PITX2	A variety of tissues	Upregulated
PLOD2	ACHP	Dermal fibroblasts	Suppression
	HIF-1α	Glioma cell, hypoxic breast cancer cells	Upregulated
	miR-26a-5p and miR-26b-5p	Bladder cancer, renal cell carcinoma	Upregulated
	TGF-β	Human synovial fibroblasts	Upregulated
	E2Fs	NSCLC, Mouse Model of Metastatic Breast Cancer	Upregulated
	FOXA1	NSCLC	Upregulated
	ER complex of resident chaperones	Dermal fibroblast	Upregulated the activity
PLOD3	ACHP	Dermal fibroblasts	Suppression
	BMP-2	AT-MSCs	Downregulated
	TGF-beta1	AT-MSCs	Downregulated
	miR-663a	Human hepatoma Huh7 cells, Hek 293 cells and Hela cells	Downregulated

fibroblasts (Mia and Bank, 2015). Therefore, activation of NF-kB pathway may induce PLOD expression in certain types of cells. Treatment with bone morphogenetic protein-2 (BMP-2) and TGF-β1 induces PLOD1 expression in adipose tissue-derived mesenchymal stem cells (AT-MSCs). Interestingly, neither BMP-2 nor TGF-β1 can induce PLOD2 expression (Knippenberg et al., 2009). Given the crucial function of PLODs in collagen synthesis, further defining the molecular mechanisms by which PLOD expression is regulated may significantly expand our understanding of collagen-related disease progression.

PHYSIOLOGICAL FUNCTIONS OF PLODs

Collagen is the major component of connective tissues and maintains the structural integrity and the stability of tissues and organs (Patino et al., 2002). The hydroxylysine residues provide attachment sites for the carbohydrates and tensile strength and mechanical stability for the collagen fibrils (Rautavuoma et al., 2004). The abnormal expression or mutation of PLODs is associated with collagen-related diseases, such as Kyphoscoliotic type of EDS VIA (Pousi et al., 1994; Giunta et al., 2005; Abdalla et al., 2015; van Dijk et al., 2017; Zahed-Cheikh et al., 2017), Bruck Syndrome (BS) (Gistelinck et al., 2016) and RDEB (Watt et al., 2015) (**Table 2**). PLOD1 regulates the hydroxylation of lysyl residues on collagen type V. The duplication of the exon 10 to exon 16 region of *PLOD1* (p.Glu326_Lys585dup) gene (Pousi et al., 1994; Giunta et al., 2005) and two mutations on Gln208 and Tyr675 cause the loss function of PLOD1, which may lead to EDS VIA (Abdalla et al., 2015; van Dijk et al., 2017). In addition, *PLOD1* has been identified as a susceptibility gene for reduced BMD (Tasker et al., 2006; Yamada and Shimokata, 2007).

Dysregulation of PLOD2 is associated with brain arteriovenous malformations and cancer progression. PLOD2 is overexpressed in brain arteriovenous malformations (bAVM), and the levels of PLOD2 expression correlated with bAVM size (Neyazi et al., 2017). PLOD2 mutant zebrafish display

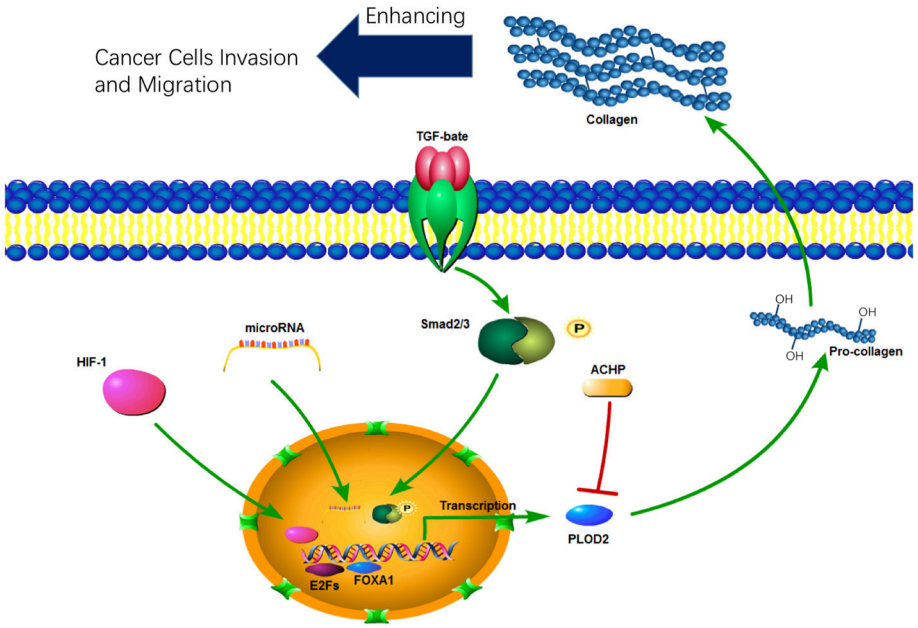

FIGURE 2 | The function and regulation of PLOD2 in cancer progression.

TABLE 2 | The association of PLODs with human diseases.

PLOD protein family	Collagen substrate	Human disease
PLOD1	Type V	EDS VIA (Pousi et al., 1994; Giunta et al., 2005; Abdalla et al., 2015; van Dijk et al., 2017; Zahed-Cheikh et al., 2017) BMD (Tasker et al., 2006; Yamada and Shimokata, 2007) Early Alzheimer's disease (Chong et al., 2013)
PLOD2	Type I (Gistelinck et al., 2016)	bAVM (Neyazi et al., 2017) BS (Gistelinck et al., 2016) Carcinoma (Conklin et al., 2011; Rajkumar et al., 2011; Noda et al., 2012; Gilkes et al., 2013b; Li et al., 2017; Song et al., 2017)
PLOD3	Type IV and VI (Sipilä et al., 2007) type I (Sricholpech et al., 2011)	Recessive dystrophic epidermolysis bullosa (RDEB) (Watt et al., 2015)

molecular and tissue abnormalities in the musculoskeletal system that are concordant with clinical findings in BS patients (Gistelinck et al., 2016). There is evidence that the levels of mature hydroxylysine aldehyde-derived cross-links typical for skeletal tissues is increased in vein graft disease, this is accompanied by upregulation of PLOD2 (Kahle et al., 2016). Furthermore, increased PLOD2 expression has been detected in the macroscopically injured region of the capsule, and upregulation of TGF-β1, TGFβR1, and PLOD2 is likely related to the disease progression (Belangero et al., 2016).

It has been shown that PLOD3 mutations are associated with the connective tissue disorder (Salo et al., 2008). Analysis of PLOD3 knock-out embryos and cells indicate that loss of PLOD2 reduces glycosylated hydroxylysines on type IV and VI collagen with abnormal distribution (Sipilä et al., 2007). Reduced glycosylation may inhibit the tetramerization and secretion of type VI collagen. Another function of PLOD3 is to glucosylate galactosylhydroxylysine residues in type I collagen in osteoblasts. The G-Hyl glucosylation induced by PLOD3 is crucial for collagen fibrillogenesis *in vitro* (Sricholpech et al., 2011).

PLODs in Cancer Progression and Metastasis

Increased collagen deposition and cross-linking promote cancer development and progression by enhancing cancer cell migration, invasion and proliferation (Provenzano et al., 2006, 2008; Levental et al., 2009; Zhu et al., 2015). Therefore, PLODs may contribute to cancer progression by modulating collagen cross-link and maturation.

Increased PLOD expression has been detected in many types of cancer. The PLOD2 expression level is significantly upregulated in breast cancer compared to normal mammary tissue, and the upregulation correlates with short disease-related survival (Gjaltema et al., 2015). In esophageal squamous-cell carcinoma (ESCC), expression of the tumor suppressor gene esophageal cancer-related gene 4 has a negative association with PLOD1 and PLOD2 (Li et al., 2017). The PLOD2 expression is significantly correlated disease-free survival and tumor size in hepatocellular carcinoma (HCC) (Noda et al., 2012). PLOD3 is overexpressed in HCC (Elsemman et al., 2016; Shen et al., 2018) and is a potential diagnosis marker for early-stage HCC (Shen et al., 2018). Knockdown of PLOD3 suppresses liver

tumor incidence as well as tumor growth rates in spontaneous mouse HCC model (Shen et al., 2018). Nicastri used a quantitative proteomic technique and identified 54 up-regulated glycoproteins in colorectal cancer samples, including PLOD2 and PLOD3 (Nicastri et al., 2014).

Increased PLOD2 expression is crucial for tumor invasion and metastasis (**Figure 2**). For instance, silencing PLOD2 expression in the breast cancer cell line MDA-MB 231 reduces cancer metastasis and collagen deposition in the primary tumor tissue; interestingly, PLOD2 expression has little effect on the primary tumor growth (Gilkes et al., 2013b). Hypoxia- and TGF-β1-induced PLOD2 expression promotes the migratory, invasive and adhesive capacities of cervical cancer cells by promoting EMT and the formation of focal adhesion (Remst et al., 2014; Xu et al., 2017). In HIF-1α-deficient tumors, ectopic PLOD2 expression restores the migration and metastatic potential, and inhibition of PLOD2 activity suppresses the tumor metastases (Eisinger-Mathason et al., 2013). Although HIF-1 induces expression of PLOD1 and PLOD2, PLOD2 expression in breast cancer cells is more important for fibrillary collagen formation, tumor stiffness and cancer metastasis to lymph nodes and lungs (Gilkes et al., 2013b).

Function of PLOD2 in lung cancer progression differs slightly from breast cancer; ectopic expression of PLOD2 enhances both primary cancer growth and metastasis (Chen et al., 2015). PLOD2 hydroxylates telopeptidyl lysine residues on collagen, subsequently increasing the level of hydroxylysine aldehyde–derived collagen cross-links (HLCCs) and lowering levels of lysine aldehyde–derived cross-links in lung cancer tissue (Chen et al., 2015). Recent study also reveal that PLOD2 expression induces PI3K/AKT signaling in glioma (Song et al., 2017) and non-small-cell lung cancer (NSCLC) (Du et al., 2017); activation of the PI3K pathway may contribute to increased cell proliferation, migration and invasion.

It is well established that PLOD2 protein locates in ER (Liefhebber et al., 2010). However, a recent study shows that PLOD2 protein can be secreted by lung cancer cells and induce collagen remodeling (Chen et al., 2016). Addition of recombinant PLOD2 to the extracellular space promotes HLCC formation in the extracellular matrix, suggesting that secreted PLOD2 is functional (Chen et al., 2016). However, function of secreted PLOD2 in cancer development and progression remains to be determined.

Cancer associated fibroblasts (CAFs) and stellate cells, as the major source of ECM production in the tumor microenvironment, promote tumor cell invasion and migration through the PLOD2-induced collagen cross-link (Bozóky et al., 2013; Pankova et al., 2016). PLOD2 is highly expressed in CAFs; silencing PLOD2 expression in CAFs significantly reduced the tumor invasion and metastasis (Pankova et al., 2016). Knockdown of PLOD2 in pancreatic stellate cells inhibits directional migration of cancer cells within the matrices by constructing an insensitive microenvironment of three-dimensional (3D) matrices to tumor migration (Sada et al., 2016). These results indicate that PLOD2 expressed in stromal cells is crucial for cancer progression.

FUTURE DIRECTION

Loss of function mutations and abnormal PLOD expression are involved in many collagen-related diseases. Impairment of PLOD1 function contributes the development of Kyphoscoliotic type of EDS. Mutations of PLOD3 cause the connective tissue disorder (Salo et al., 2008). Many studies demonstrate that increased PLOD2 and PLOD3 expression is required for cancer progression and metastasis. Therefore, targeting PLOD is a potential therapeutic strategy for cancer and other collagen-related diseases. Unfortunately, there is no potent PLOD inhibitor available. Since the client protein and function of PLOD1, PLOD2, and PLOD3 in collagen synthesis are different, it is important to develop specific inhibitors for PLOD to halt cancer progression. Another strategy to inhibit PLOD activity in cancer tissue is to reduce PLOD expression; therefore, further understanding how PLOD is regulated during cancer development may identify signaling pathways to target PLOD.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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