MICROENVIRONMENT-DERIVED STEM CELL PLASTICITY

EDITED BY: Jelena Krstic, Marietta Herrmann, Ivana Gadjanski and Slavko Mojsilovic

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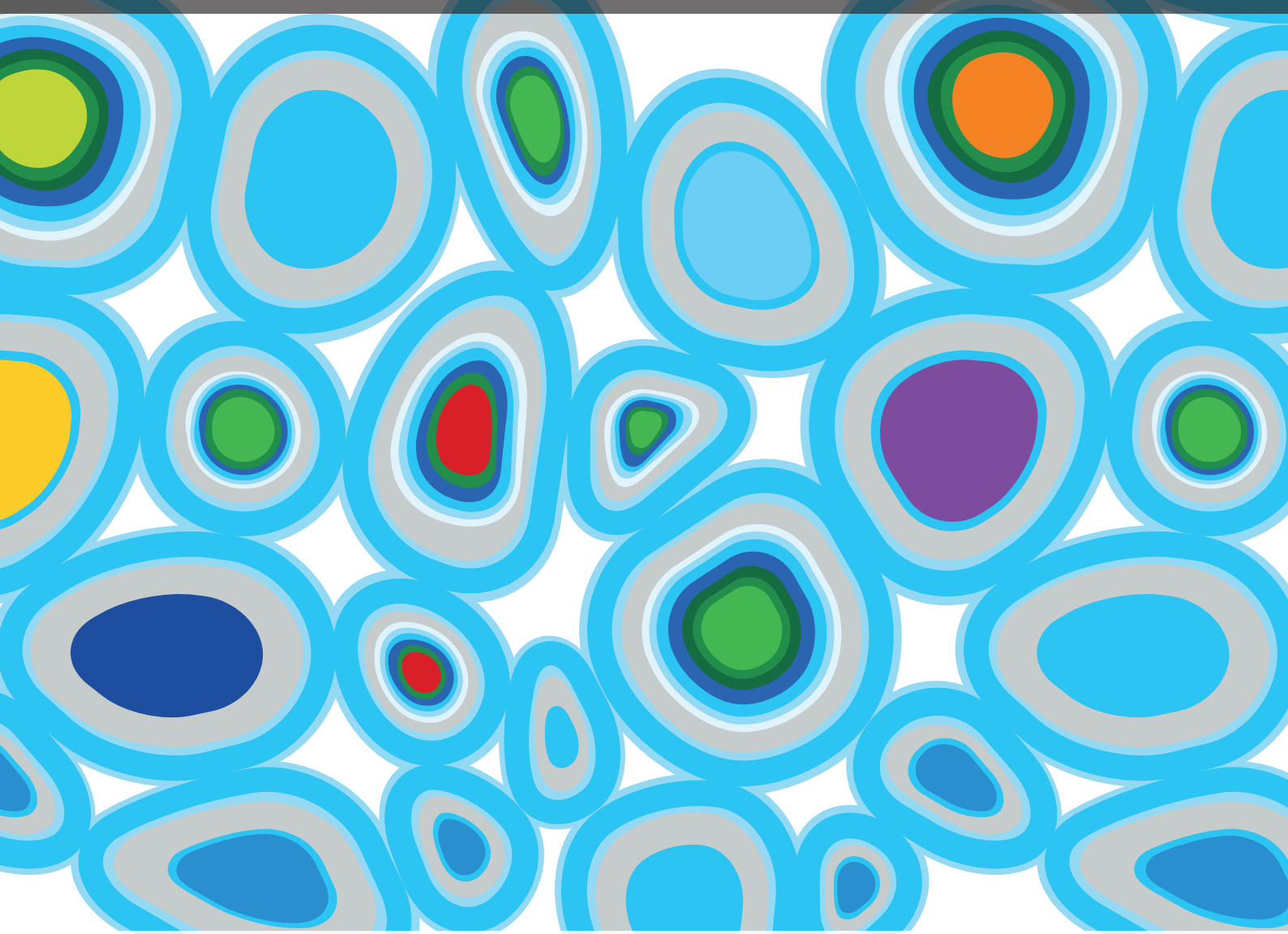

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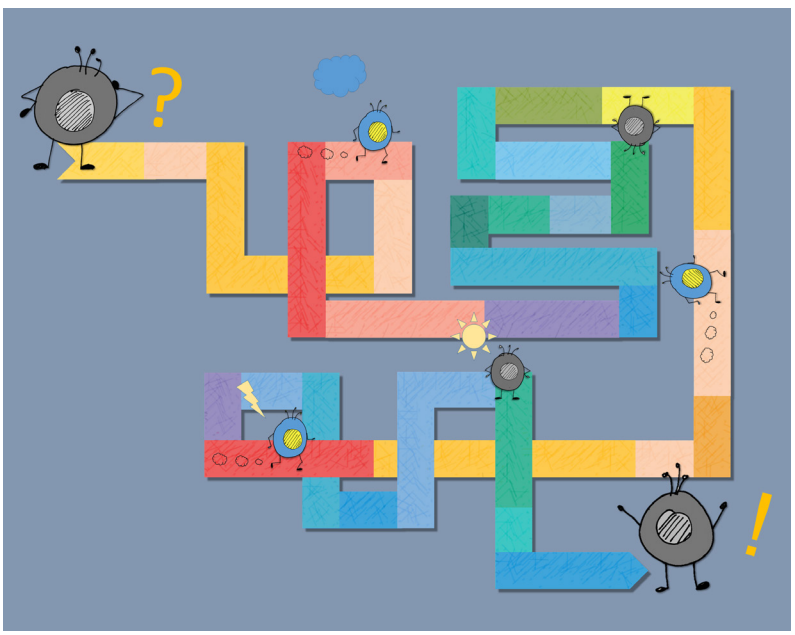
MICROENVIRONMENT-DERIVED STEM CELL PLASTICITY

Topic Editors:
Jelena Krstic, University of Belgrade, Serbia; Medical University Graz, Austria
Marietta Herrmann, AO Research Institute, Switzerland; University of Würzburg, Germany
Ivana Gadjanski, Belgrade Metropolitan University, University of Novi Sad, Serbia
Slavko Mojsilovic, University of Belgrade, Serbia



Stem cell plasticity is determined by their dynamic microenvironment.

Image: Dr. Jelena Krstic.

Plasticity is the hallmark of stem cells. At the same time, stem cells, like any other cell type, are influenced by their microenvironment and respond to it accordingly. A specific microenvironment is defined by a variety of factors, including biological and chemical factors, cell-cell interactions, but also metabolic and mechanical cues. Such dynamic and specialized microenvironment where the stem cells reside is considered a stem cell niche. Tissue injury as well as malignant tissue alterations lead to changes in the niche influencing the plasticity and biology of residing stem cells. Similarly, the niche changes upon tissue damage, which eventually induces differentiation of stem cells and ultimately regeneration of the tissue.

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EDITORIAL
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Editorial: Microenvironment-Derived Stem Cell Plasticity

Jelena Krstic[1,2]*, *Marietta Herrmann*[3,4], *Ivana Gadjanski*[5,6] *and Slavko Mojsilovic*[2]

[1] *Institute of Cell Biology, Histology and Embryology, Medical University Graz, Graz, Austria,* [2] *Institute for Medical Research, University of Belgrade, Belgrade, Serbia,* [3] *AO Research Institute, Davos, Switzerland,* [4] *IZKF Group Tissue Regeneration in Musculoskeletal Diseases, Orthopedic Center for Musculoskeletal Research, University of Würzburg, Würzburg, Germany,* [5] *Belgrade Metropolitan University, Belgrade, Serbia,* [6] *Biosense Institute, University of Novi Sad, Novi Sad, Serbia*

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Editorial on the Research Topic

Microenvironment-Derived Stem Cell Plasticity

Plasticity is the hallmark of stem cells. At the same time, stem cells, like any other cell type, are influenced by their microenvironment and respond to it accordingly. A specific microenvironment is defined by a variety of factors, including biological and chemical factors, cell-cell interactions, but also metabolic and mechanical cues. Such dynamic and specialized microenvironment where the stem cells reside is considered a stem cell niche. Tissue injury as well as malignant tissue alterations lead to changes in the niche influencing the plasticity and biology of residing stem cells. Similarly, the niche changes upon tissue damage, which eventually induces differentiation of stem cells and ultimately regeneration of the tissue.

Within this research topic, different aspects of microenvironment-derived stem cell plasticity are discussed including techniques to detect niche-residing stem cells, cell communication within the niche and the influence of oxygen tension and inflammation on stem cell plasticity. Furthermore, disease-associated microenvironments are addressed. Finally, two contributions focus on the use of micro environmental cues for therapeutic application of stem cells.

Interactions of hematopoietic stem/progenitor cells with their microenvironment is central for regulation of hematopoiesis, both in steady state and in disease. Development of imaging techniques brings about powerful new methodologies to enlighten these very complex and dynamic processes. Morikawa and Takubo concisely summarize methods used for visualization, tracking and localization of hematopoietic stem cells within their microenvironment, and review recent advances in our understanding of stem cell/niche biology through examples of imaging-based studies of various physiological and pathological hematopoietic activities.

An overview of the communication between mesenchymal stem cells (MSCs) and other cells in their microenvironment via extracellular vesicles (EVs) is provided by Dostert et al. The two-way interaction has been considered, describing MSCs as donors and recipients of EVs. A number of factors discovered in EVs have been distinguished as protein effectors and micro RNAs involved in cell-cell communication, specifically focusing on EV-mediated tissue regeneration, immunomodulation and cross talk with cancer cells.

Oxygen tension is an important aspect of the tissue microenvironment. Miclau et al. have summarized the current literature on how oxygen directs MSC fate in the context of fracture healing. There is clear evidence from preclinical and clinical studies, that a disrupted vasculature and/or ischemic conditions due to co-morbidities have a negative impact on fracture healing. However, on a cellular level, it is yet not clear how the tissue oxygen level is associated with stem cell differentiation; unexpected results have also been reported in the context of tissue revascularization indicating the need to better understand the regulation of oxygenation in fracture healing.

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Edited by:
Erdal Karaoz,
Liv Hospital, Turkey

Reviewed by:
Hakan Darici,
Istinye University, Turkey
Vasiliy Galat,
Ann and Robert H. Lurie Children's Hospital of Chicago, United States

***Correspondence:**
Jelena Krstic
j_kocic@yahoo.com;
jelena.krstic@medunigraz.at

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It has been shown that MSCs have immunomodulatory properties and the ability to adapt to the tissue environment. Tumor necrosis factor α (TNF-α) and transforming growth factor β (TGF-β) are two cytokines present in different niches, which may have opposing roles (pro- and anti-inflammatory). Lerrer et al. demonstrate in their study that these cytokines cooperatively induce a pro-inflammatory fate in MSCs. This implies that the complex composition of the niche-specific secretome is of critical importance for the immunomodulatory fate of stem cells. Michael et al. provide in their mini review very interesting aspects on the specific outcomes of inflammatory incidents affected by direct regulation of stem cells. During tissue damage caused by either pathogen infection or physical/chemical damage (sterile induced inflammation), the inflammatory response acts as a regulator of tissue stemness either by directly affecting tissue stem cells or by shifting differentiated cells toward a stem-like cell character. Menendez and Alarcón present a threshold model for aging and cancer, based on regulation of cellular reprogramming by senescence-inflammatory signaling, and offer new therapeutic strategies for these ailments. According to this model, a transient cellular reprogramming induced by activation of innate immunity or senescence-associated inflammatory components brings cells to a state of epigenetic plasticity, enabling adaptive phenotypic adjustments in order to promote damage repair and tissue regeneration. However, if this de-differentiation is not accompanied by re-acquisition of the original or alternative differentiated cell fate, the resulting tissue plasticity impairs the replacement of damaged cells and leads to chronically unresolved tissue damage.

Malignant tissue alterations such as cancer are associated with drastic changes in the tissue environment and its metabolome. In this context, the metabolic plasticity of cancer cells has been reported by Johnson et al. who analyzed the expression of the monocarboxylate transporter 1 (MCT1) in a significant number of breast cancer tissue samples with variable characteristics in terms of hormone-dependence. They showed that MCT1 level of expression varies between cancer subtypes and that it can be correlated to breast cancer recurrence, thus suggesting MCT1 as a prognostic biomarker as well as a target worth exploring for cancer therapy.

Stem cell plasticity can also be used as a tool to improve the regenerative capacity of cells. Such approach is presented by Ghosh et al., who investigated how TGF-β pretreatment affects MSC-mediated wound healing. In the mouse model, TGF-β-pretreated MSCs distribute more evenly in the wound and improve wound closure. Based on *in vitro* findings, the authors propose a time-dependent model of TGF-β-stimulated bone marrow MSCs that helps the wound healing process. They suggest that increased MSC adhesion and migration properties are regulated via integrin/focal adhesion kinase activation which is essential for both SMAD3-dependent and -independent signal transduction after TGF-β treatment.

Finally, Ottoboni et al. review the literature on neural stem cell (NSC) plasticity and methods for manipulation of their plasticity for treatment of central nervous system (CNS) diseases. They particularly emphasize the double-side effect that plasticity can have, even threatening the NSC therapeutic capacity, because manipulation of NSCs or of their trophic microenvironment might induce unwanted side effects, such as senescence. The authors conclude that it is necessary to increase the knowledge on (i) the influence of cell metabolism on NSC behavior during CNS diseases; (ii) the *ex-vivo* maintenance of NSCs; (iii) the most suitable window time for transplant to best leverage graft survival and disease specific environmental conditions. While this review was focused on neural stem cells, the mentioned aspects similarly present a challenge in other research fields.

AUTHOR CONTRIBUTIONS

All authors JK, MH, IG, and SM contributed equally in writing the Editorial.

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MINI REVIEW
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How Do Mesenchymal Stem Cells Influence or Are Influenced by Microenvironment through Extracellular Vesicles Communication?

Gabriel Dostert[1], *Benjamin Mesure*[1], *Patrick Menu*[1,2] *and Émilie Velot*[1,2]*

[1] *Laboratoire d'Ingénierie Moléculaire et Physiopathologie Articulaire, UMR 7365 Centre National de la Recherche Scientifique – Université de Lorraine, Biopôle de l'Université de Lorraine, Vandœuvre-lès-Nancy, France,* [2] *Faculté de Pharmacie, Université de Lorraine, Vandœuvre-lès-Nancy, France*

Mesenchymal stem cells (MSCs) are widely used in cell therapy and tissue engineering thanks to their self-renewal, their multipotency, and their immunomodulatory properties that make them an attractive tool for regenerative medicine. A large part of MSCs positive effects is due to their secretion products which participate in creating a favorable microenvironment and closely relate these cells to other cell types. Extracellular vesicles (EVs) belong to cellular secretions. They are produced by cells continuously or after stimulation (e.g., calcium flux, cellular stress) and act in tissue homeostasis and intercellular communication. The understanding of the role of EVs is growing, more particularly their impact on cell migration, differentiation, or immunomodulation. EVs derived from MSCs show these interesting properties that may be considered in therapeutics, although they can have adverse effects by facilitating cancer propagation. Moreover, MSC behavior may also be influenced (proliferation, differentiation) by EVs derived from other donor cells. The aim of this mini review is to summarize the two-way communication between MSCs and other cell types, and how they can affect each other with their microenvironment through EVs. On the one hand, the manuscript presents the influence of MSC-derived EVs on diverse recipient cells and on the other hand, the effects of EVs derived from various donor cells on MSCs. The discrepancies between cancer cells and MSCs communication according to the sources of MSCs but also the tumor origins are also mentioned.

Keywords: mesenchymal stem cells (MSCs), extracellular vesicles (EVs), recipient/donor cells, intercellular communication, microenvironment

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Edited by:
Jelena Krstić,
Institute for Medical Research, Serbia

Reviewed by:
Drenka Trivanović,
Institute for Medical Research, Serbia
Etienne Moussay,
Luxembourg Institute of Health, Luxembourg

***Correspondence:**
Émilie Velot
emilie.velot@univ-lorraine.fr

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INTRODUCTION

At physiological level, the cells composing a tissue, an organ, or even an entire organism are constantly trading information either by physical contact or by long distance communication. This phenomenon allows their maintenance but can also lead to variations in the cellular behavior. The study of these interactions has permitted to develop new therapeutic strategies such as cell therapies.

Thanks to their self-renewal, their multipotency, and their immunomodulatory properties, mesenchymal stem cells (MSCs) are an attractive tool for regenerative medicine. For example, MSCs are used in clinical trials to treat pathologies such as graft vs. host disease (GvHD) (Le Blanc et al., 2008; Zhou et al., 2010; Introna et al., 2014), although concerns remain regarding efficacy, safety, and feasibility of such treatments (Si et al., 2011; Mendicino et al., 2014). The most common sources of MSCs are from adult origin like bone marrow or adipose tissue, but their collects request an invasive procedure. Perinatal sources like Wharton's jelly from umbilical cord offer more accessibility and larger quantities of MSCs with higher proliferation rate and greater immunomodulatory properties (El Omar et al., 2014).

Numerous studies have shown the regenerative potential of MSCs to counteract organ failures (Xing et al., 2014; Desando et al., 2016; Sattayaprasert et al., 2016). It has also been demonstrated that injecting MSC conditioned medium and not the cells themselves can induce the same effects. This is due to the composition of MSC secretions, which are of two types, soluble factors [e.g., soluble tumor necrosis factor receptor 1, transforming growth factor (TGF-β) 1; (Melief et al., 2013; Ke et al., 2016)] or extracellular vesicles (EVs). EVs are involved in tissue reparation, immunomodulation, and proliferation. A miscellaneous EV population can be found in biological fluids. Three kinds of EVs are mostly described by the scientific community according to their size and biogenesis (Höög and Lötvall, 2015; Yáñez-Mó et al., 2015; Kowal et al., 2016). The biggest vesicles are secreted after cell apoptosis and are large EVs from 1 to 5 μm called apoptotic bodies (Atkin-Smith et al., 2015). EVs from 0.1 to 1 μm are termed microparticles, ectosomes, or microvesicles. They are generally produced by cells during stress or metabolic changes and result from the budding of the plasma membrane (Ratajczak et al., 2006). Endosome-derived EVs named exosomes are small EVs—with a size varying from 30 to 150 nm depending on the literature—which are secreted continuously whatever cellular state (stress or physiological conditions) (Valadi et al., 2007; György et al., 2011; Vlassov et al., 2012; Crescitelli et al., 2013). The size similarity between EVs can impair their identification if size is used as the only parameter for characterization. Lately, the difficulties to purify a homogeneous EV population and to select appropriate EV markers to classify them have been discussed and remain. All vesicle populations will be referred as EVs in this mini review and the attention will be highlighted on their biological effects.

MSCs are commonly known as donor cells by providing EVs to other cells types, nevertheless their part as recipient cells is less described (**Figure 1**). A recent report shows that human bone marrow (BM)-MSCs can be both donor and recipient cells. Osteogenically induced BM-MSCs are donors of EVs which are able to guide osteogenic differentiation. The stimulation

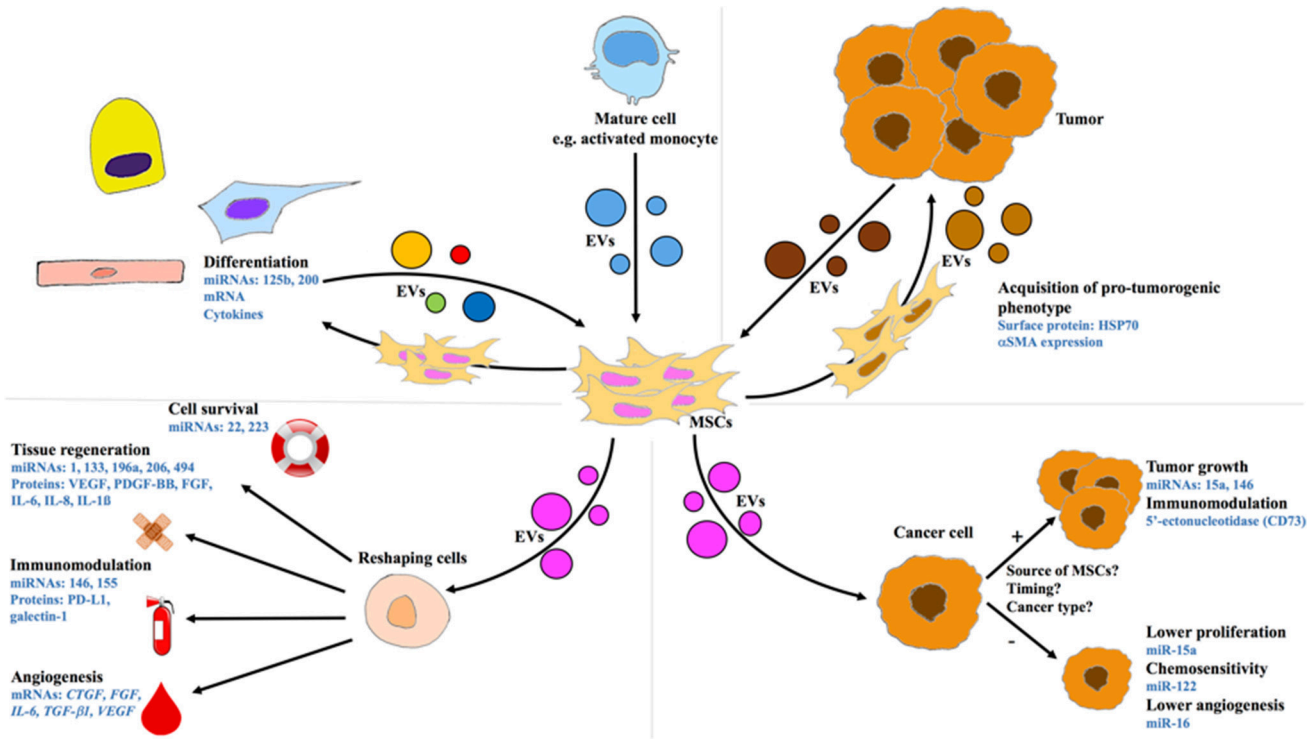

FIGURE 1 | Intercellular communication between MSCs and other cell types through EVs. Upper panels: MSCs as recipient cells. **Lower panels:** MSCs as donor cells. **Left Panels:** MSCs exchanging with differentiated cells. **Right Panels:** Cross-talk between MSCs and cancer cells. CTGF, connective tissue growth factor; EV, extracellular vesicle; FGF, fibroblast growth factor; HSP, heat shock protein; IL, interleukin; mRNA, messenger RNA; miRNA or miR, microRNA; MSC, mesenchymal stem cell; PDGF, platelet-derived growth factor; PD-L1, programmed death-ligand 1; SMA, smooth muscle actin; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

of recipient BM-MSCs by these EVs induces expression of bone morphogenic protein (*BMP*)*-2*, *osterix*, osteopontin (*OPN*), osteocalcin (*OCN*), and alkaline phosphatase (*ALP*). Thus, the recipient BM-MSCs may be employed for therapeutic use to improve bone regeneration (Martins et al., 2016). Each cell produces its own secretions which allow the formation of its own microenvironment affecting surrounding cells including MSCs. In this mini review, we focus on the effects of EV exchange between MSCs and other cell types in both ways.

MSCs AS DONOR CELLS

MSC-EVs and Tissue Regeneration

MSC microenvironment acts differently depending on the target cells (**Figure 1**). MSC-EVs can produce modifications in gene expression and mediate cell maturation or differentiation. For example, osteoblasts undergoing stimulation with EVs originated from BM-MSCs show clear calcium deposits *in vitro* in the same way as osteogenic medium stimulation. This is supported by *ALP*, *OCN, OPN,* and runt-related transcription factor (*RUNX*)*2* gene overexpression. These *in vitro* results were confirmed *in vivo* by using hydrogels supplemented with EVs to improve bone regeneration through the expression of micro RNA miR-196a.

MSC-EVs induce angiogenesis stimulation of human umbilical vein endothelial cells by enhancing tube formation in Matrigel™-coated wells. This observation has been made with fresh or frozen EVs, showing their preservation potential under −80°C (Teng et al., 2015). Montemurro et al. showed that EVs derived from cord blood MSCs carry transcripts related to angiogenic and proliferative function such as connective tissue growth factor (*CTGF*), fibroblast growth factor (*FGF*), interleukin (*IL*)*-6* but also *TGF-ß1,* and vascular endothelial growth factor (*VEGF*) (Montemurro et al., 2016). Transcripts were not the only ones to participate in the pro-angiogenic properties of MSC-EVs. Pro-angiogenic properties beneficial for myocardial tissue repair were highlighted after MSC pre-conditioning by serum starvation plus low oxygen settings in order to mimic ischemic conditions after myocardial infarction. Modification of culture conditions leads to modulation of protein targeting toward MSC-EVs. Specific protein content during stimulation has an effect in angiogenesis and especially by nuclear factor (NF)-κB signaling pathway (Anderson et al., 2016). Intramyocardial injection of MSCs or MSC-EVs also reduces myocardial ischemia in rats 48 h after infarction. Compared to the control, MSCs or MSC-EVs showed similar effects with reduction of the infarct size 28 days after post-operation. Injection of EVs derived from MSCs also improved cardiac function and promoted the number of blood vessels in myocardium infarcted region with a higher increase than MSC injection (Bian et al., 2014). In skeletal muscle, MSC-EVs are able to improve tissue repair. The specific content in growth factors (VEGF, fibroblast growth factor, platelet-derived growth factor-BB), cytokines (IL-1β, IL-6, IL-8), and especially in non-coding RNA may act to maintain a healthy and functional tissue. Three important myogenic micro RNAs (miRNAs: miR-1, -133, and -206) that have been involved in muscle regeneration after injection in rat model appear inside MSC-EVs. Although miR-494 is not specific for muscle repair, it was also found in high concentration inside MSC-EVs. This miRNA participates in myogenesis, migration activity, and protects against ischemia/reperfusion injury in cardiac muscle (Nakamura et al., 2015; Hofer and Tuan, 2016). The potential of MSC-EVs to treat cardiovascular injuries is widely studied. During ischemia, tissue oxygenation becomes almost null, but during this time, MSCs secrete large amounts of EVs containing a high concentration of miR-22 compared to normoxic conditions. Interactions between EVs containing miR-22 and cardiomyocytes allows a reduction of infarcted zone volume and apoptosis in the ischemic myocardium by the down regulation of methyl CpG binding protein 2 (Mecp2) (Feng et al., 2014). Another study highlights the beneficial effect of MSC-EVs on cardiomyocytes survival, this effect being higher when MSCs overexpress transcription factor GATA-4 (Yu et al., 2015). Wang et al. have pointed miR-223 in MSC-EVs as a primordial effector in cardioprotective properties. During polymicrobial sepsis, miR-223 contained inside EVs contributes to the regulation of two proteins, semaphorin-3A (Sema3A) and signal transducer and activator of transcription 3 (Stat3), which are involved in apoptosis. The absence of miR-223 inside MSC-EVs has an impact on cardiac cells and may have deleterious effects. On the contrary, his presence will allow a better defense against sepsis (Wang et al., 2015). Furthermore, MSC-EV regenerative potential has been demonstrated in neurogenesis (Xin et al., 2013), liver fibrosis (Li et al., 2013), and cutaneous wound healing (Zhang et al., 2015).

MSC-EVs and Immunomodulation

MSC-EVs take place in immunomodulation to lower the immune system activation through the induction of anti-inflammatory cytokines and regulatory T cells (Treg) (**Figure 1**) (Del Fattore et al., 2015), but also by regulating macrophages polarization (Ti et al., 2015) and neutrophils mobilization (Zhu et al., 2014). More generally, MSC-EVs have been shown to balance expansion of myeloid progenitors (Goloviznina et al., 2016). MSC-EVs can activate monocytes by Toll-like receptor (TLR) signaling pathway. In contrast with activation by lipopolysaccharides (LPS), the surface receptor involved is unknown but will cause the same signaling cascade via myeloid differentiation primary response gene 88 (MYD88) and NF-κB (Zhang et al., 2014). Another notable difference is observed in cytokine production. Thus, when monocytes are stimulated by MSC-EVs, they differentiate into macrophages which secrete IL-10, leading to Treg expansion. During this study, test of skin allograft rejection by mice treated with MSC-EVs showed similar results with another experiment using cyclosporine A, an immunosuppressor (Zhang et al., 2014). MSC-EVs can induce the decrease of B lymphocyte and natural killer (NK) cell proliferation. Unlike MSCs, EV immunomodulation is not mediated by indoleamine-pyrrole 2,3-dioxygenase (IDO) pathway but by a molecule on their surface: programmed death-ligand (PD-L)1 (Di Trapani et al., 2016). To be activated, MSCs need to be stimulated by pro-inflammatory cytokines to trigger their immunosuppressive answer. Generally, an increase of IDO activity is a marker of activation when MSCs and T cells

are in contact, but during contact between MSC-EVs and T cells, IDO concentration remains stable without modifying the immunological potential. In addition to PD-L1, an endogenous leptin found on EV surface—galectin-1—is also involved in the immunomodulatory response (Del Fattore et al., 2015). Effects of 5′-ectonucleotidase (CD73) has been studied as well because this enzyme found on MSCs and MSC-EVs actively produces adenosine, a molecule known to be immunosuppressive (Kerkelä et al., 2016). Moreover, inflammatory priming induces the increase of miR-155 and -146 level inside MSC-EVs. These specific miRNAs intervene in the regulation of inflammatory reactions (Di Trapani et al., 2016). Clinical applications are also possible to reduce inflammation in some pathologies such as therapy refractory GvHD. Shortly after MSC-EV therapy, cutaneous, and mucosal GvHD showed a very promising response, allowing to reduce four times the administrated steroid doses. Such EV-based treatment have beneficial effects for the patient without side effects (Kordelas et al., 2014).

MSC-EVs in Cross-Talk with Cancer Cells

Despite the numerous studies about pro-angiogenic effects of MSC-EVs, these vesicles may have a reverse effect on cancer cells. A study on mouse breast cancer cell line (4T1) headed by Lee et al. showed that miR-16 level in EVs derived from mouse BM-MSCs contributes to decrease the secretion of VEGF by cells. These modifications lead to a suppression of angiogenesis *in vitro* and thus a reduction in tumor spread (Lee et al., 2013). In the case of cancers therapy with MSC-EVs, there are a lot of divergences between studies because of the source of MSCs, the tumor origin, but also the timing of stimulation with EVs (**Figure 1**). Depending on the papers, MSC-EVs can promote tumor progression (Roccaro et al., 2013; Shi et al., 2016), decrease it (Bruno et al., 2013), or have no effect (Hendijani et al., 2015). In all cases, the content in cytokines and miRNAs seems to be the key factor. The effect of nucleic acids contained in EVs in the cross-talk between tumor cells and MSCs has been well-described by Lopatina et al. (2016). They came to the conclusion that these cells exchange either oncogenic and anti-tumoral RNAs. For example, EVs secreted by multiple myeloma BM-MSCs display a lower tumor suppressive miR-15a content (Roccaro et al., 2013), associated with more cytokines regulating cell adhesion and migration as well as oncogenic proteins. When stimulated with MSC-EVs, cells from nasopharyngeal carcinoma (CNE2) undergo toward a mesenchymal transition, with a decrease of epithelial markers like epithelial cadherin (Shi et al., 2016). MSC-EVs used on different cancer cell lines can also stimulate metalloproteinase (MMP)-2 expression, thus helping tumor migration (Yang et al., 2015). These vesicles have also the ability to transfer CD73 on tumor cells, giving them the ability to metabolize AMP into adenosine, which reduces NK and T cell activation. MSC-EVs can also confer drug resistance to gastric cancer cells by stimulating multidrug resistance protein expression and reducing chemo-induced apoptosis (Ji et al., 2015). However, liver cancer cell lines like HepG2 stimulated with MSC-EVs have difficulties in the cell cycle progression and are subject to apoptosis (Bruno et al., 2013). *In vivo* growth of glioma xenografts is also reduced by miR-146 which is present in MSC-EVs (Katakowski et al., 2013). The chemosensitivity of hepatocellular carcinoma cells was also raised by miR-122 contained in adipose-tissue MSC-EVs. Some cell lines remain unaffected by MSC-EVs, neither in a pro- nor an anti-tumor way. For example, when the lung cancer cell line A549 is exposed to umbilical cord MSCs conditioned medium, it does not lose or gain proliferation rate, even if MSCs are stimulated with interferon γ. The association of conditioned medium with the therapeutic agent doxorubicin does not modify its native effect (Lou et al., 2015). Even if the direct use of MSC-EVs in cancer therapies is still not fully understood and should be carefully controlled, it appears that they could be an interesting vector to address therapeutic cargos to tumors (Chen et al., 2011; Johnsen et al., 2014; Yang et al., 2015).

MSCs AS RECIPIENT CELLS

EVs from Differentiated Cells

EVs derived from differentiated cells are able to modulate MSC phenotype (**Figure 1**). An *in vitro* study has highlighted the potential of EVs derived from neuronal cells to mediate MSC neuronal induction. Indeed, miR-125b—which is known to act in neuronal differentiation—is expressed by MSCs after 1 week of stimulation by these EVs (Takeda and Xu, 2015). When derived from endothelial cells, EVs can influence MSC proliferation, migration, and secretion of soluble factors such as matrix MMP-1, MMP-3, chemokine ligand 2 (CCL-2), and IL-6 (Lozito and Tuan, 2014). In the case of renal tubular morphogenesis and kidney structure, cells known as mesenchymal-epithelial cells are required. The origin of these cells can be explained by the migration and transition of MSCs from bone marrow induced by EVs derived from human renal proximal tubular epithelial cells. The presence of the miR-200 family (miR-200a, -200b, and -200c) has been highlighted in these EVs. Their uptake by BM-MSCs induces a MSC phenotype modification with a mesenchymal to epithelial transition, characterized by the acquisition of polarized epithelial cell properties by BM-MSCs. This is a physiological process involved in kidney formation (Chiabotto et al., 2016). Immune cells such as monocytes communicate also with MSCs via EVs. LPS activated monocytes secrete a lot of soluble factors as well as EVs. The conditioned medium of these cells has the property to modulate MSC phenotype by upregulating osteogenic gene expression (Omar et al., 2011). *RUNX2*, *BMP-2,* and *OCN* expression were evaluated after 72 h of MSC stimulation by conditioned medium or EVs derived from activated monocytes compared to control. During EVs stimulation, *RUNX2* and *BMP-2* were significantly increased compared to control in the same way as conditioned medium but *OCN* was only over expressed with EVs. This indicates that EVs derived from activated monocyte promote osteogenic differentiation in MSCs (Ekström et al., 2013). In a rat model of calvarial bone defect, it has been shown that EVs obtained from induced pluripotent stem cell-derived MSCs associated to a tricalcium phosphate scaffold are able to stimulate bone regeneration by recruiting BM-MSCs at the defect site. The rat's BM-MSCs are activated through the phosphatidylinositol 3

kinase (PI3K)/Akt pathway leading to osteogenic differentiation (Zhang et al., 2016).

EVs from Cancer Cells

Genetic modifications of MSCs are also possible via EV transfer from cancer cells (**Figure 1**). When EVs are originated from healthy cells, their effects seem beneficial. However, when EVs come from cancer cells, their influence on MSCs may be harmful. Lindoso et al. have demonstrated that EVs derived from renal cancer stem cells can induce epigenetic changes in recipient cells. MSCs are attracted to the tumor region and change their phenotype, becoming pro-tumorigenic. This correlates with the overexpression of genes involved in cell migration: *chemokine receptor type (CXCR)4* and *CXCR7*; matrix remodeling: *collagen type IV alpha 3 chain (COL4A3)*; as well as angiogenesis and tumor growth: *IL-8*, *OPN,* and *myeloperoxidase*. EVs secreted by cancer stem cells allow a better chemoattraction of MSCs, which promote tumor development and spread (Lindoso et al., 2015). There are also differences in the evolution of MSC phenotype which is reliant on the origin of EVs. Thus, MSCs can differentiate into myofibroblasts under action of EVs from prostate cancer. The myofibroblastic marker alpha-smooth muscle actin α-SMA) is expressed by more than 50% of MSCs exposed to prostate cancer derived EVs vs. only 5% of cells under TGF-ß1 stimulation, which is known to induce α-SMA expression. There is also a correlation between the quantity of EVs and α-SMA acquisition (Chowdhury et al., 2015). This differentiation has also been demonstrated with EVs from breast cancer cells or chronic lymphocytic leukemia (Cho et al., 2011; Paggetti et al., 2015). Another example of MSC phenotype modification has been highlighted by Li et al. during a study about lung tumor EVs. *In vitro,* EVs from the lung cancer line A549 stimulate the production and secretion of inflammatory cytokines in MSCs. Three cytokines: IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 were released by MSCs when they are triggered by A549 cell-derived EVs. The priming of MSCs by lung cancer cell-derived EVs would occur through the activation of TLR2/NF-κB signaling by the interaction of EVs surface heat shock protein 70 (HSP70) with cells (Li et al., 2016). In a different experiment, EVs from KMBC (human cholangiocarcinoma cells) enhance modifications of MSCs toward a fibroblastic phenotype. Such transformation is not the only change: MSCs brought into contact with KMBC-EVs subsequently secrete IL-6 which causes an increase in cancer cells proliferation (Haga et al., 2015). Chronic myelogenous leukemia (CML) uses the same pathway to spread. CML-EVs interact with BM-MSCs and this stimulation initiates IL-8 production by MSCs which permits CML cells proliferation (Corrado et al., 2014). The cross-talk between MSCs and cancer cells leads to a specific sequence. First, cancer cells interact with MSCs via their EVs to modulate them and in response, the modified MSCs participate in cancer progression trough their own EVs (Corrado et al., 2014; Haga et al., 2015).

CONCLUSION

Cellular communication by EVs relies on the nature of donor cells and recipient cells (**Figure 1**). Research on MSC-EVs is prolific and shows their potential in regenerative medicine, whereas there is less literature about EVs originated from healthy differentiated cells. For these last, the difficulties may originate from a low proliferation and a poor EV production *in vitro*. In the case of cancers, the mechanisms of the cross-talk between the tumor and MSCs are yet not fully unraveled, but some works on EVs as therapeutic cargo show promising results.

AUTHOR CONTRIBUTIONS

GD discussed the work and wrote the manuscript. BM helped to write the manuscript and designed the figure. PM helped to discuss the work and to edit the manuscript. EV drafted the manuscript, discussed the work, edited the manuscript, and gave the final approval of the version to be published.

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Inflammation Shapes Stem Cells and Stemness during Infection and Beyond

*Stella Michael †, Charis Achilleos †, Theofano Panayiotou † and Katerina Strati **

Department of Biological Sciences, University of Cyprus, Nicosia, Cyprus

The outcome of an inflammatory incident can hang in the balance between restoring health and tissue integrity on the one hand, and promoting aberrant tissue homeostasis and adverse outcomes on the other. Both microbial-related and sterile inflammation is a complex response characterized by a range of innate immune cell types, which produce and respond to cytokine mediators and other inflammatory signals. In turn, cells native to the tissue in question can sense these mediators and respond by migrating, proliferating and regenerating the tissue. In this review we will discuss how the specific outcomes of inflammatory incidents are affected by the direct regulation of stem cells and cellular plasticity. While less well appreciated than the effects of inflammatory signals on immune cells and other differentiated cells, the effects are crucial in understanding inflammation and appropriately managing therapeutic interventions.

Keywords: inflammation, stem cells, stemness, stem cells and sterile inflammation, stem cells and microbes

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University of Belgrade, Serbia

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Beklem Bostancioglu,
Karolinska Institutet, Sweden
Shree Ram Singh,
National Cancer Institute, USA

***Correspondence:**
Katerina Strati
strati@ucy.ac.cy

†These authors have contributed equally to this work.

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INTRODUCTION

Inflammation has a well-established role in the defense of organisms against microbial invasion. The presence of commensal and pathogenic microbes, both intracellular and extracellular, is usually detected by receptors residing mostly in the surface of innate immune cells known as pattern recognition receptors (PRRs). These receptors are the first line of the surveillance system which ultimately recognizes pathogen associated molecular patterns (PAMPs) and triggers the inflammatory response. Acute and chronic inflammation in a number of diseases associated with pathogens and the interplay between infection and inflammation is of paramount importance to clinical outcomes (Apidianakis and Ferrandon, 2014).

While inflammation is a defense against pathogens it can also be triggered during processes unrelated to microbial insult. This process, termed sterile inflammation, is typically linked to chemical or physical triggers. Inflammatory cells present at the site of the damage recognize danger-associated molecular patterns (DAMPs) and secrete molecules which prime the tissue restoration via the proliferation of quiescent adult stem cells (Nagaoka et al., 2000; Koh and DiPietro, 2011; Petrie et al., 2014; Kizil et al., 2015). Sterile inflammation can have profound effects on tissue homeostasis and repair, for example during wound healing, or during the onset and initiation of inflammatory diseases.

We summarize here evidence for the direct crosstalk between the inflammatory response and stem cells both in cases of microbial and sterile induced inflammation (**Figure 1**). Inflammation is emerging as an important regulator of stem cells and plays an intricate role in health and disease.

REGULATION OF STEM CELLS IN RESPONSE TO MICROBIAL MOTIFS

Recently PRRs were shown to be expressed in the surface of tissue stem cells suggesting that there is the potential for direct effects of PAMPs on stem cell behavior (Boiko and Borghesi, 2012). For example, hematopoietic stem cells express toll-like receptors (TLRs) whose activation leads to the differentiation of myeloid progenitors intomonocytes and macrophages immune cells (Nagai et al., 2006) as seen in the presence of the vaccinia virus in the bone marrow (Singh et al., 2008). Stem cells of solid tissues, more prominently the gut, have also been shown to express PRRs integrating inflammation to immune clearance and subsequent tissue regeneration. Intestinal stem cells (ISCs) expressing TLR4 show increased proliferation and expansion of the stem cell population in the intestinal epithelium (Santaolalla et al., 2013). ISCs have also been shown to express Nod2, a general sensor for peptidoglycan (Girardin et al., 2003). The constitutive expression of Nod2, in ISCs, provides protection against stress (Nigro et al., 2014). The ability of gut stem cells to respond

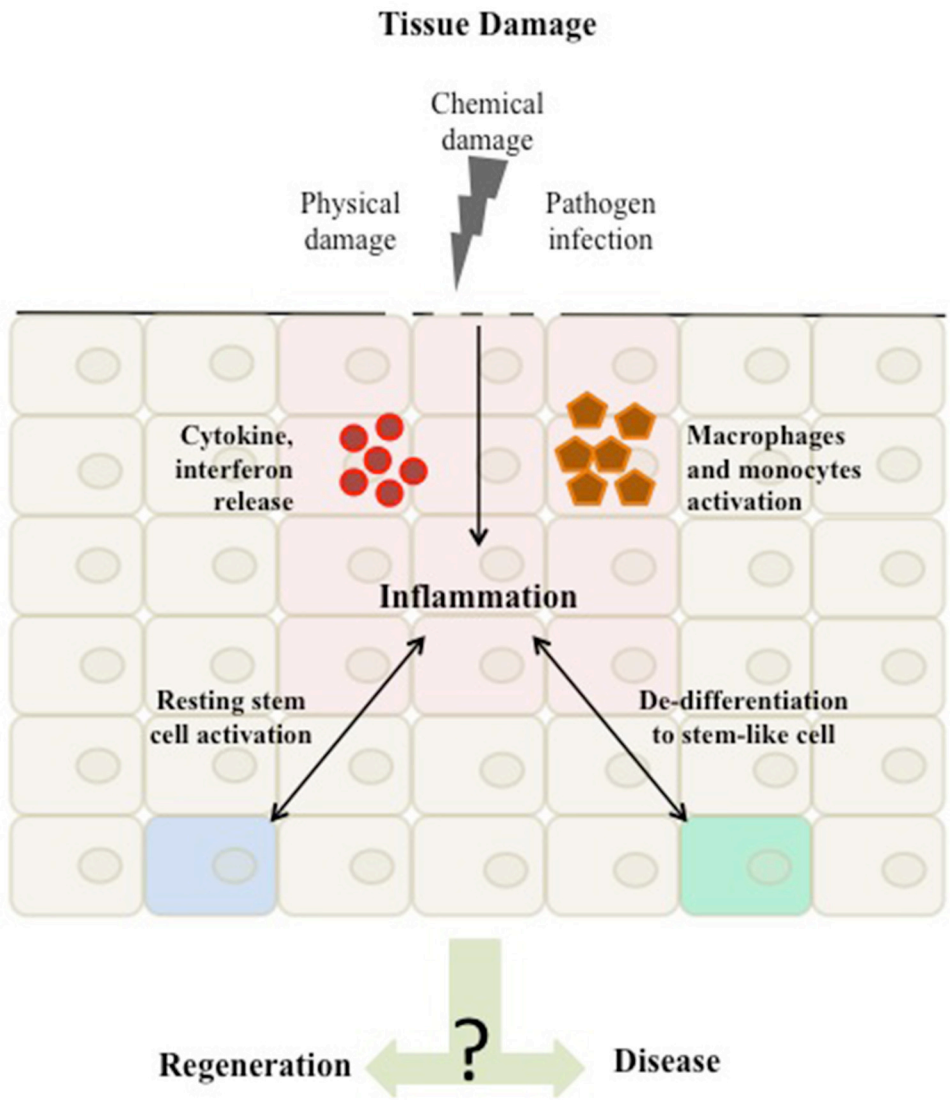

FIGURE 1 | Tissue damage can arise as a result of physical damage, chemical damage or pathogen infection. Once the damage is detected a homeostatic inflammatory response is activated to regenerate the tissue. This inflammation is characterized by the activation of immune cells, such as macrophages and monocytes and the release of inflammatory mediators, such as cytokines and interferons to the site of damage. These mediators in turn can affect native tissue cells to respond by migrating and proliferating resulting in tissue repair. Evidence suggest that the inflammatory response acts as a regulator of tissue stemness either by directly affecting tissue stem cells or by shifting differentiated cells toward a stem-like cell character. The balance in this inflammatory response and its mediated stemness is a critical driver of either maintaining tissue integrity or promoting aberrant homeostasis and disease.

directly to patterns, such as LPS (via TLR4) and peptidoglycan (via Nod2), may underlie mechanisms of tissue response not only to pathogenic bacteria but also commensals and is likely important to general tissue homeostasis via the interaction with intestinal microbiota. The findings in mammalian systems are corroborated by extensive literature in other experimental systems, such as Drosophila (Panayidou and Apidianakis, 2013).

Once an inflammatory program has already been initiated the production of cytokines, interferons etc. by local immune populations can further impact the behavior of stem cells. In the gut, innate lymphoid cells produce interleukin-22, a potent survival factor, which can directly act on ISCs promoting growth and epithelial regeneration (Lindemans et al., 2015). Chronic HBV infection also stimulates release of interleukin-22 by inflammatory cells, inducing proliferation of liver stem cells (Feng et al., 2012). In addition, the mediator of inflammation TNF-α is activated as a result of brain inflammation in neural stem cells seen in conditions of trauma, multiple sclerosis and pathogen infections. TNF-α activation ultimately brings about proliferation of the neural stem cells (Widera et al., 2006). Neural stem cells in the hippocampus have also been shown by *in vivo* studies to proliferate upon the presence of bacterial enterotoxins (Wolf et al., 2009). In the urinary tract, upon *E. coli* infection, the uroepithelial stem cells are activated for epithelial renewal in response to the inflammatory response (Mysorekar et al., 2009).

The presence of pathogenic burden in the hematopoietic system rapidly depletes immune cells stimulating intermediate blood progenitors to maintain blood cell balance (Hawkins et al., 2006). Inflammation-induced myelopoiesis, due to pathogen presence, results in the release of interleukin-27 causing activation and differentiation of hematopoietic stem cells (HSCs) (Furusawa et al., 2016). Chronic infection, as seen in the presence of the *Mycobacterium avium,* results in the activation of quiescent HSCs through the release of the inflammatory mediator interferon-γ (Baldridge et al., 2010).

Inflammation is proposed to promote tissue recovery via its effects on differentiated cells to regenerate the tissue (Karin and Clevers, 2016). In some cases the differentiated cells de-differentiate in response to inflammation, acquiring stem-like characteristics and increased cellular plasticity. In support of this idea, the induction of immunity was found to be required for efficient nuclear reprogramming *in vitro* (Lee et al., 2012).

Tissue reprogramming is achieved through the upregulation of growth factors and cytokines in the inflammatory microenvironment (Grivennikov et al., 2010). This could be attributed to changes in the expression of specific genes/pathways which shift a differentiated cell closer toward a stem cell character. Alternatively, the effects could impart tissue stem cells or progenitors with increased/altered capabilities.

There are a number of examples to support the idea that inflammation caused by infections leads to tissue regeneration and/or cellular stemness. Such a response has been observed in viral infections of HBV and HCV where inflammation in the liver induced expression of stemness markers (Karakasiliotis and Mavromara, 2015). Specifically, the secretion of interleukin 6 (IL6) by the inflammatory cells during HBV infection regulates the expression of Oct4 and Nanog pluripotency factors (Chang et al., 2015). Furthermore, the hypoxia factor HIF-1α produced in the HCV virally-infected cells confers an epithelial-mesenchymal transition (EMT) character (Wilson et al., 2012). It is important to note that in this case, the EMT is accompanied by enhanced viral replication.

In fact, inflammation-mediated changes on the differentiation status of the tissue are a factor in the pathology which accompanies disease. Persistent induction of stemness in the infected tissue in the presence of chronic inflammation, as seen in infections of the gut, can likely contribute to carcinogenesis (Apidianakis and Ferrandon, 2014; Kuo et al., 2016). However, emerging concept suggests that it may also be beneficial to the pathogen. Several groups have shown that for some infectious agents it can play a role during their replication (as in the case of HBV and HCV), their dissemination, and protection (Masaki et al., 2013; Nigro et al., 2014; Karakasiliotis and Mavromara, 2015). A more prominent example, the leprosy bacterium infects preferentially Schwann cells of the nervous system and induces their reprogramming into stem-like cells. The infected stem-like cells then migrate to the mesenchyme where they re-differentiate to mesenchyme tissue allowing for expansion of the infection (Masaki et al., 2013). The innate immune response has been shown to precede this reprogramming (Masaki et al., 2014). For efficient dissemination, the cells need to evade the host immunity and they do so by inducing an inflammatory response achieved through the release of factors from the stem-like cells. This subsequently recruits macrophages that form granulomas able to bypass immunity and migrate.

The inflammatory response is important to the host organism as a protective mechanism against pathogen invasion as well as tissue regeneration through the induction of stemness. In some cases however, through inflammation, pathogens are able to escape immune surveillance for their protection and dissemination with possible consequences to their lifecycle and replicative potential, as we have seen in the cases of the HBV and HCV viruses or of bacteria in the intestine or the nervous system.

REGULATION OF STEM CELLS DURING TISSUE (RE)GENERATION

Sterile inflammation has also been shown to lead to profound changes in the differentiation status of the tissue. An example is the regenerative process which takes place in response to wound healing. Wound healing requires an ordered sequence of events ranging from acute inflammation, tissue organization, and remodeling (Gurtner et al., 2008; Karin and Clevers, 2016). However, the period of tissue repair varies between the extent of the damage and the site of the damaged tissue (Meyer et al., 1992; Gordon et al., 2003; Pitsouli et al., 2009).

Tissue repair following an insult restores health in the tissue and preserves the state of homeostasis. Regeneration in the tissue is achieved via the priming of resident slow-cycling stem cells to adopt a proliferative state and yield transit-amplifying cells which will differentiate to restore tissue architecture. Sterile inflammation plays an important role in this process in

ways which are likely distinct to those seen during infection (Bezbradica et al., 2016).

The intestine can regenerate very rapidly. Tissue restoration is mediated via neutrophil infiltration at the site of the damage. These are responsible for JNK activation and the priming and proliferation of slow–cycling ISCs (Karin and Clevers, 2016). Work from Riehl et al. (2000) further supported the role of inflammatory signals, as administration of inflammatory cytokines and growth factors following radiation exposure rescued damaged epithelium via the proliferation and differentiation of intestinal stem cells (Riehl et al., 2000). However, homeostasis is sustained via the activation of developmental and inflammatory pathways which activate dormant ISCs. For instance, JNK signaling pathway is responsible for the secretion of IL6 which activates the JAK-STAT pathways leading to ISC proliferation (Jiang et al., 2009; Liu et al., 2010; Kuhn et al., 2014).

In the case of the skin epithelium, which also encounters frequent damage, it is proposed that keratinocytes trigger the inflammatory phase of the wound healing by secreting molecules like IL6 and TNFα (Wang et al., 2004; Ryser et al., 2014; Rittié, 2016). These inflammatory molecules along with other developmental mediators, such as extracellular matrix components (Kurbet et al., 2016) signal to the niche of slow-cycling adult stem cells in order to train them toward proliferation and ultimate differentiation (Cotsarelis et al., 1990; Taylor et al., 2000).

Another organ that has a fast-paced regeneration with the ability to recover its mass even after substantial loss is the liver (Michalopoulos, 2007). While it remains controversial whether the adult liver depends on a stem cell population during homeostatic conditions, various stem cell or progenitor populations have been described to participate in its regeneration following injury (Yimlamai et al., 2014; Wang et al., 2015). Soon after partial hepatectomy, the inflammatory cytokines TNF-α and IL6 are upregulated at the site of regeneration guiding hepatocytes to enter mitosis and restore lost tissue. Beyond the involvement of unipotent mature hepatocytes, which assist in the repair process of the liver particularly in response to acute inflammation, liver progenitor cells are also present to mediate liver repair. This is typically the case in the context of chronic inflammation, when mature hepatocytes have reached their replicative limit (Viebahn and Yeoh, 2008; Español-Suñer et al., 2012). While some controversy exists with regard to the exact characteristics of liver progenitors (Dollé et al., 2010), they respond to inflammatory signals following injury to generate differentiated cells essential for liver regeneration.

Our understanding of the effects of inflammatory signaling on stem cells, stems mostly from model tissue systems, such as the intestine, skin and liver. However, the inflammatory signaling to resident tissue stem cells is corroborated as crucial to regeneration in more poorly understood tissues and non-mammalian model systems as well (Apidianakis and Rahme, 2011). In mice, the recently described dclk1 progenitors have been shown to respond to inflammation during pancreatic regeneration (Westphalen et al., 2016). There is also extensive work implicating crosstalk between neural stem cells and inflammation in mammals and zebrafish (Kizil et al., 2015). Specifically, during zebrafish brain regeneration, inflammation is the trigger which initiates neural stem cell proliferation (Kyritsis et al., 2012).

While inflammation can prime stem cell responses it is becoming increasingly clear that in certain contexts stem cells possess immunomodulatory potential. Mesenchymal stem cells and neural stem cells are the two cell types most often ascribed with immunomodulatory potential (Kizil et al., 2015; Le Blanc and Davies, 2015). In both these examples stem cells have been shown to dampen or alter inflammation with beneficial outcomes in inflammation-associated disease. Despite the fact that the mechanisms are still poorly understood there is justified excitement for the potential application of these properties in therapeutics.

The crosstalk between sterile inflammation and stem cell plasticity within a tissue during the wound healing response is a critical step in the regenerative process. Priming stem cells that reside in the circulation, in addition to the stem cells of the regenerating tissue, may also contribute to this process. HSCs are likely directed to liver and skin following physical damage (Rennert et al., 2012). It was further suggested that inflammatory cytokines and growth factors released due to tissue injuries can stimulate a signaling wave toward bone marrow-residing stem cells to enter the circulation and inhabit the injured site. These bone-marrow stem cells and locally residing tissue stem cells hold the capacity of tissue regeneration. Perhaps more surprisingly, sterile inflammatory signaling, such as that initiated by IFNγ and TLR4, plays a role not only in the regeneration of adult tissue but is a well-conserved regulator in their production during development (Li et al., 2014; He et al., 2015). These findings certainly reframe currently held ideas about the evolutionary function of inflammation.

INFLAMMATORY SIGNALING DURING DISEASE

In some cases inflammation, particularly where it is chronic, can lead to the development of disease. The continuous and often aberrant response of stem cells as a result of this signaling has been shown to play an important part in this process. Intestinal stem cells express TLR4 the activation of which can lead to ER-stress, a trigger for stem cell apoptosis during necrotizing enterocolitis (Afrazi et al., 2014). In Barrett's esophagus $Lgr5^+$ gastric cardia stem cells can migrate in response to the inflammatory signaling and are the likely source of the metaplastic and dysplastic cells observed in the course of the disease (Quante et al., 2012).

The disease most commonly associated with inflammation is of course cancer. Multiple studies have produced substantial evidence suggesting that cancer and inflammation are in many cases connected, interdependent biological processes (Coussens and Werb, 2002; Balkwill et al., 2005; Karin, 2006). This is true in cases where the cancer is associated with microbial or viral causes, but also in cases where no pathogen is directly linked. For example, in addition to their mutagenic effects, carcinogens in tobacco smoke cause damage and chronic inflammation to the lungs and increase the risk of cancer

development (Punturieri et al., 2009). Autoimmunity is also associated with increased tumor development. The chance of developing colitis-associated cancer or lymphoma is increased in people suffering from inflammatory bowel or celiac disease, respectively (Kraus and Arber, 2009; Waldner and Neurath, 2009).

The important bidirectional link between inflammation and stem cells has direct implications on cancer development. Studies have suggested that HSC recruitment and differentiation is directly linked to increased inflammation. For example, CD34+ progenitor cells are recruited to sites with increased inflammation, probably using the same adhesion and chemokine receptors used for stem cell homing to the bone marrow (PSGL-1, CXCL12, α4β1 integrin, CD44, and others) (Blanchet and McNagny, 2009). Inflammatory mediators seem to have a vital role in inducing expression of stemness-related genes. The expression of stemness-related genes in cancer is likely linked to the generation and evolution of the compartment of cells able to regenerate tumor diversity, the cancer stem cells (CSCs) (Kuo et al., 2016; Uthaya Kumar et al., 2016). Upregulation of OCT4 has been shown to contribute to tumor cell migration and resistance to cancer therapeutics (Ma et al., 2011; Chang et al., 2015, 2016; Bhatt et al., 2016).

While inflammation is one of the stimuli suggested to initiate such transcriptional changes, once CSCs form, evidence suggests that they can serve to further amplify inflammatory signaling. Chemoresistant CSCs were found to express proinflammatory gene signatures, mainly due to the sustained activation of NF-kB and interferon-stimulated regulatory element (ISRE)-dependent pathways. Notably, tumor-associated macrophages in this environment protect tumor cells from chemotherapeutic agents by promoting and enhancing the tumor growth properties of CSCs (Jinushi et al., 2011). A proinflammatory signature has also been exhibited by leukemia stem cells which can promote chemoresistance by means of metabolic adaptation (Ye et al., 2016). Thus, in some cases, tumors respond to chemotherapy by altering the immunological profile of the microenvironment in part due to the direct action of CSCs, thus further enabling tumor growth (Jinushi et al., 2011; Jinushi, 2014).

In more rare cases recruited circulating stem cells, subjected to chronic inflammation in a tissue, have been proposed to act as cancer-initiating cells themselves. Houghton et al. (2004), used a mouse model to demonstrate an extremely important connection between chronic inflammation, hematopoietic stem-cell recruitment and cancer development at the site of inflammation (Houghton et al., 2004). In this study, infection by *Helicobacter pylori* in the mouse caused the recruitment and subsequent engraftment of bone marrow derived stem cells (BMDC) into the stem cell compartment of the gastric mucosa. Within their new, inflamed niche, these engrafted stem cells accumulated mutations, and eventually gave rise to gastric tumors. This study showcased a direct connection between chronic inflammation, stem cell recruitment and increased cancer development (Houghton et al., 2004).

CONCLUSION

Inflammatory signaling promotes cellular responses with critical ramifications during infection, tissue generation/regeneration, cancer and other diseases. We summarize here work which demonstrates that stem cells can respond to and participate in inflammatory cascades in a direct manner. They express receptors which detect PAMPs and DAMPs, the initial triggers of the inflammatory response. They are able to mobilize and proliferate in response to inflammation in addition to producing cytokines which further amplify the response. Accumulating evidence suggests that in cases where a pathogen is involved, the changes in stemness mediated by inflammation also have a profound influence on the lifecycle of the pathogen. This is an area which merits further research. Understanding the crosstalk between stem cells and inflammation is an important piece of the puzzle which refines our understanding of the evolutionary roles of inflammation. Furthermore, it provides indispensable tools in our quest to harness knowledge into useful therapeutics.

AUTHOR CONTRIBUTIONS

SM, CA, and TP performed literature searches, and co-wrote the review. These three authors contributed equally. KS conceived the mini review topic, performed literature searches, and co-wrote the review.

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HYPOTHESIS AND THEORY
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Senescence-Inflammatory Regulation of Reparative Cellular Reprogramming in Aging and Cancer

Javier A. Menendez[1, 2, 3]* *and Tomás Alarcón*[4, 5, 6, 7]

[1] *Metabolism and Cancer Group, Program Against Cancer Therapeutic Resistance, Catalan Institute of Oncology, Girona, Spain,* [2] *Molecular Oncology Group, Girona Biomedical Research Institute (IDIBGI), Girona, Spain,* [3] *METABOSTEM, Barcelona, Spain,* [4] *Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain,* [5] *Computational and Mathematical Biology Research Group, Centre de Recerca Matemàtica, Barcelona, Spain,* [6] *Departament de Matemàtiques, Universitat Autònoma de Barcelona, Barcelona, Spain,* [7] *Barcelona Graduate School of Mathematics, Barcelona, Spain*

The inability of adult tissues to transitorily generate cells with functional stem cell-like properties is a major obstacle to tissue self-repair. Nuclear reprogramming-like phenomena that induce a transient acquisition of epigenetic plasticity and phenotype malleability may constitute a reparative route through which human tissues respond to injury, stress, and disease. However, tissue rejuvenation should involve not only the transient epigenetic reprogramming of differentiated cells, but also the committed re-acquisition of the original or alternative committed cell fate. Chronic or unrestrained epigenetic plasticity would drive aging phenotypes by impairing the repair or the replacement of damaged cells; such uncontrolled phenomena of *in vivo* reprogramming might also generate cancer-like cellular states. We herein propose that the ability of senescence-associated inflammatory signaling to regulate *in vivo* reprogramming cycles of tissue repair outlines a threshold model of aging and cancer. The degree of senescence/inflammation-associated deviation from the homeostatic state may delineate a type of thresholding algorithm distinguishing beneficial from deleterious effects of *in vivo* reprogramming. First, transient activation of NF-κB-related innate immunity and senescence-associated inflammatory components (e.g., IL-6) might facilitate reparative cellular reprogramming in response to acute inflammatory events. Second, para-inflammation switches might promote long-lasting but reversible refractoriness to reparative cellular reprogramming. Third, chronic senescence-associated inflammatory signaling might lock cells in highly plastic epigenetic states disabled for reparative differentiation. The consideration of a cellular reprogramming-centered view of epigenetic plasticity as a fundamental element of a tissue's capacity to undergo successful repair, aging degeneration or malignant transformation should provide challenging stochastic insights into the current deterministic genetic paradigm for most chronic diseases, thereby increasing the spectrum of therapeutic approaches for physiological aging and cancer.

Keywords: reprogramming, aging, cancer, senescence, inflammation

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Jelena Krstić,
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Reviewed by:
Katerina Strati,
University of Cyprus, Cyprus
Han Li,
Institut Pasteur, France
Eumorphia Remboutsika,
BSRC "Alexander Fleming," Greece

***Correspondence:**
Javier A. Menendez
jmenendez@iconcologia.net;
jmenendez@idibgi.org

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The epistemic interest of induced pluripotent stem cells (iPSCs) to model aging and aging-related diseases largely relies on the appreciation of nuclear reprogramming as a disease-in-a-dish technology. Expression of the Yamanaka cocktail of transcription factors (i.e., *Oct4*, *Sox2*, *Klf4*, and *c-Myc*, OSKM; Takahashi and Yamanaka, 2006; Takahashi et al., 2007) is commonly viewed as an artificial, non-naturally occurring molecular modality capable of radically modifying the cellular identity of differentiated cells *in vitro* (Liu et al., 2012; Inoue et al., 2014; Sterneckert et al., 2014; Avior et al., 2016). An overlooked dimension of OSKM-driven cellular reprogramming is the potential existence of such a phenomenon as a natural process for *in vivo* tissue rejuvenation. Activation of adult stem/progenitor cells and proliferation of remaining differentiated cells are well-established mechanisms for the replacement of lost cells following injury. A physiological version of OSKM-induced reprogramming might operate as an evolutionary conserved, *bona fide* epigenetic strategy to provide self-repair and resistance to damage and disease (Cooke et al., 2014; Jessen et al., 2015; de Keizer, 2017).

There is growing support for the hypothesis that nuclear reprogramming-like phenomena inducing the short-term acquisition of epigenetic plasticity, followed by cell differentiation and replacement of damaged cells, might be a reparative route through which tissues respond to injuries and other adversities. We herein delineate a threshold model of aging and cancer based on the intercommunication between cellular reprogramming/differentiation cycles of tissue repair and the cell-autonomous and non-cell autonomous mechanisms that initiate and propagate senescence-associated inflammatory signaling.

REPARATIVE CELLULAR REPROGRAMMING *IN VIVO*: THE EVIDENCE BASES

Nuclear Reprogramming and Activation of Innate Immunity

The mere process of viral transduction that is frequently employed to deliver OSKM factors into target cells (i.e., the viral particles themselves) can elicit the expression of genes involved in innate immunity (Lee et al., 2012; O'Neill, 2012). Furthermore, only after the efficient activation of innate immunity, considered the phylogenetically oldest mechanism of defense against microbes, can the retrovirally-delivered OSKM factors successfully accomplish cellular reprogramming (Lee et al., 2012; O'Neill, 2012). Key players of innate immunity-signaling, including toll-like receptors (TLRs) such as TLR3 and the retinoic acid-inducible gene 1 receptor (RIG-1)-like receptor (RLR), appear to be necessarily involved in the nuclear reprogramming process to pluripotency (Cooke et al., 2014). Moreover, the retrovirally-induced activation of pattern recognition receptors (PRRs) such as TLRs and RIG-1, which are specialized DNA sensors charged with cell defense *via* sensing nucleic acids generally derived from microbes (e.g., viral RNA) (Broz and Monack, 2011; Newton and Dixit, 2012; Dixit and Kagan, 2013), has been found to stimulate pro-inflammatory NFκB signaling as part of the reprogramming process. Retrovirally-induced immune activation and NFκB-mediated cellular inflammation can trigger significant downstream epigenetic modifications, including decreased H3K9 methylation (indicative of gene silencing) and increased H3K4 methylation (indicative of open chromatin) of the endogenous *Oct4* and *Sox2* gene promoters, thereby facilitating nuclear reprogramming upon delivery of the stemness transcription factors (Lee et al., 2012; O'Neill, 2012). Indeed, this activation of inflammatory signaling appears to autonomously promote epigenetic plasticity by eliciting global changes in the expression and activity of several chromatin-modifying enzymes, such as upregulation of histone acetyltransferases, downregulation of histone deacetylases, and downregulation of histone methyltransferases such as DOT1L (Lee et al., 2012; O'Neill, 2012; Cooke et al., 2014; **Figure 1**).

Inflammation and Epigenetic Plasticity

The targeted modulation of epigenetic modifiers that operate as barriers to OSKM-driven reprogramming is sufficient to more efficiently generate iPSCs with fewer exogenous stemness transcription factors (Onder et al., 2012; Luo et al., 2013; Rais et al., 2013; Soria-Valles et al., 2015a,b). It thus seems reasonable to suggest that the Yamanaka cocktail simply drives induced pluripotency because they efficiently and specifically finalize the epigenetic modification of chromatin pre-initiated as part of the retrovirus-induced PRR-driven host genetic response (Lee et al., 2012; O'Neill, 2012; Cooke et al., 2014). Considering that innate immunity functions not only for the early prevention, control, or elimination of host infection, but also to warn against infection or DNA damage, to which an adaptive immune response has to be mounted, other NFκB-related pro-inflammatory damage receptors (e.g., TLR4 binding to endotoxins such as lipopolysaccharide) may likely act as epigenetic modifiers to promote more epigenetically plastic cellular states (Erdoğan et al., 2016).

In agreement with the host defense nature of inflammation-regulated reprogramming, the Yamanaka factors have been found to cooperate with soluble and contact-dependent stromal signals to accelerate the conversion of myeloid progenitors to a stable pluripotent state (Park et al., 2012). Interestingly, such extrinsic potentiation of the reprogramming capacity of somatic progenitors has been found to involve both an epigenetically permissive genome and the molecular activation of the TLR/NFκB signaling pathway (Park et al., 2012). However, a close relationship seems to exist between an optimal level of cell-autonomous inflammation and the acquisition of cell states epigenetically poised to rapidly provide phenotypic responses to environmental stresses. A landmark study from the Lopez-Otin group revealed that chronic hyperactivation of pro-inflammatory NF-κB signaling constitutes a critical impediment to nuclear reprogramming in both normal and accelerated aging (Soria-Valles et al., 2015a,b). Accordingly, hyperactivation of the histone H3 methyltransferase DOT1L, a central component of the epigenetic program that is down-regulated during retrovirally-induced activation of innate immune/NFκB inflammatory signaling to promote epigenetic plasticity (Lee et al., 2012; O'Neill, 2012; Cooke et al., 2014), was found to conversely

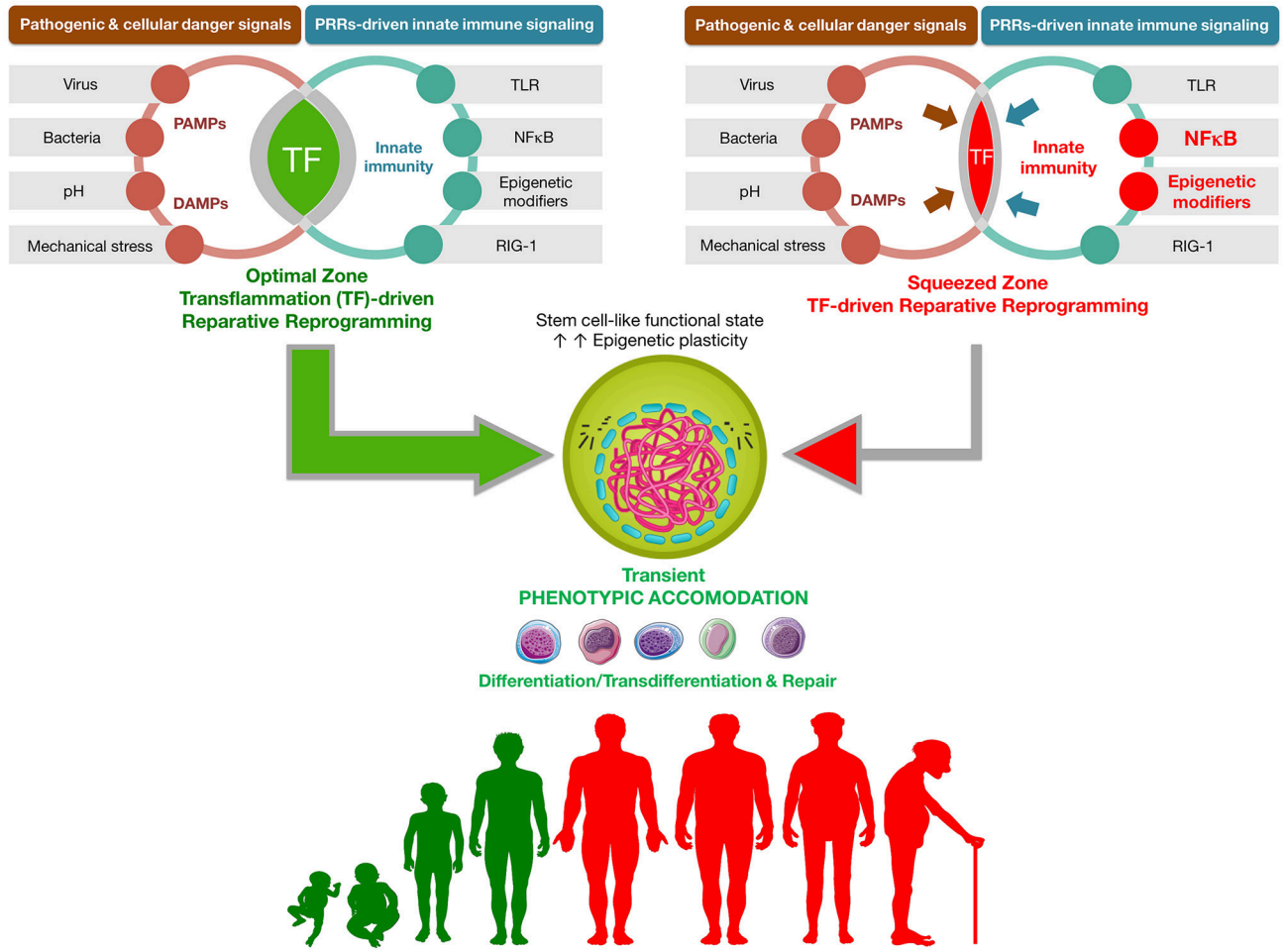

FIGURE 1 | Transflammation-driven epigenetic plasticity: a paradigmatic example of *in vivo* reparative reprogramming. Transient activation of the PAMPs-DAMPs → NFκB signaling axis may delineate an optimal zone of transflammation (TF)-driven reparative reprogramming characterized by increased epigenetic plasticity and phenotypic malleability capable of responding and adapting to injury, stress, and disease (Lee et al., 2012; O'Neill, 2012; Cooke et al., 2014). The efficiency of NFκB signaling and the level of inflammatory responses is the nodal point linking the pathogenic assault and cellular danger signals and the organization of cellular resistance and tissue repair. NFκB hyperfunction and its interaction with epigenetic modifiers would significantly squeeze the optimal zone of TF-driven reparative reprogramming, thus impairing the adequate organization of defense mechanisms. By operating as the perpetrator of inflammaging, the NFκB signaling integrates the intracellular regulation of transflammation immune responses in both aging and aging-related diseases (Salminen et al., 2008; Montgomery and Shaw, 2015).

operate as an epigenetic barrier causally involved in the loss of tissue plasticity following chronic NF-κB hyperactivation (Soria-Valles et al., 2015a,b; **Figure 1**).

Innate Immunity and Transflammation-Thresholded Cellular Reprogramming

The capacity for pathogen-associated molecular patterns (PAMPs), for example, viruses and bacteria, or damage-associated molecular patterns (DAMPs), such as mechanical stresses, pH, and oxidants, to support OSKM-driven reprogramming could have implications beyond the mere molecular insights into the cell-autonomous mechanistic barriers determining the *in-a-dish* efficiency of nuclear reprogramming. The ability of the inflammatory response to mitigate infection and clear damaged cells seems to be an evolutionary conserved process from lower organisms to mammals that might also function to promote initiation of damage repair and tissue regeneration (Karin and Clevers, 2016). When adult tissue cells are confronted with PAMPs/DAMPs, the PRR-triggered activation of downstream innate immune signaling pathways mobilizes archetypal inflammatory pathways (e.g., NF-κB, interferon regulatory factor-3) to promote an open configuration of the chromatin and, therefore, generate greater epigenetic plasticity. This phenomenon of so-called "transflammation" (Lee et al., 2012; O'Neill, 2012; Cooke et al., 2014) may act to fine-tune the rapid, but transient, adaptive adjustments to the fluidity of the cell phenotype by providing a more plastic epigenetic state for the self-repair of damaged/diseased

tissues. Beyond an optimal threshold zone of optimally and transiently activated transflammation, a successful augmentation of epigenetic and phenotypic tissue plasticity would be minimal or totally absent despite damage/disease-driven activation of cellular reprogramming-like processes (**Figure 1**). Reaching into an optimal zone of transflammation-initiated cellular reprogramming-like phenomena, followed by re-acquisition of the original or alternative cell fate, might allow tissue repair *via* replenishment or transdifferentiation of the original damaged/lost cells (Lee et al., 2012; O'Neill, 2012; Cooke et al., 2014, 2015; Sayed et al., 2015). Conversely, a chronic, cell-autonomous hyperactivation of comparable inflammation-epigenetic axes (e.g., NF-κB), rather than establishing stem-like epigenetic states, will position the damaged/stressed cell outside the optimal zone for cellular reprogramming, impeding tissue rejuvenation and generating an aging phenotype (**Figure 1**).

Permissiveness of the *In vivo* Environment for Nuclear Reprogramming-Like Phenomena

Before unequivocally suggesting that activation of reprogramming *in vivo* can be considered a host genetic program for resistance to disease and damage, it should be clarified whether the *in vivo* conditions are permissive for nuclear programming-like phenomena. By considering that cell-cell fusion is a physiological mechanism controlling not only fertilization, but also developmental processes, and that these events increase following injury and inflammation, Cosma and colleagues investigated whether stem and progenitor cells could fuse with retinal neurons and Müller glia after their transplantation into damaged retinas, and whether the *in vivo*-formed hybrids underwent nuclear reprogramming (Sanges et al., 2013). Using the eye as a model system that has low immune responses to cells and viral vectors, the authors demonstrated that, upon N-methyl-D-aspartate-induced retinal damage, mouse retinal neurons could be transiently reprogrammed back to a precursor stage. This pioneering study was the first to demonstrate that cell-fusion-mediated nuclear reprogramming of terminally differentiated cells should be viewed as a *bona fide* repair mechanism to stimulate cell and tissue regeneration in mammals *in vivo*. Soon after, a landmark study by the Serrano group established the possibility of *in vivo* nuclear reprogramming within tissues (Abad et al., 2013). These authors showed that induction of the OSKM factors in mice promoted not only the emergence of groups of dedifferentiated cells expressing the pluripotency marker *Nanog* in multiple organs, but also the generation of circulating *in vivo* iPSCs with a highly plastic, more primitive totipotent state than embryonic stem cells (ESCs) and *in vitro*-derived iPSCs (Abad et al., 2013). Permissiveness of the *in vivo* environment to reprogramming-like phenomena has been positively supported by two recent breakthrough studies from the Serrano and Izpisua-Belmonte groups; the former showing that tissue damage provides critical signals for OSKM-driven cellular reprogramming *in vivo* (Mosteiro et al., 2016), and the latter revealing how the cyclic, short-term expression of OSKM factors *in vivo* ameliorates cellular and physiological hallmarks of aging (Ocampo et al., 2016).

Nuclear Reprogramming-Induced Tumorigenesis

Using a murine system in which the expression of reprogramming factors was controlled temporally with doxycycline, the Yamanaka group demonstrated that whereas acute activation of OSKM factors leads to the formation of dysplastic lesions that spontaneously reverse upon doxycycline withdrawal, chronic induction results in the formation not only of well-differentiated teratomas but also of tumor-like undifferentiated tissues unresponsive to doxycycline withdrawal (Hobbs and Polo, 2014; Ohnishi et al., 2014a,b). The manifestation of tumorigenesis in the context of *in vivo* nuclear reprogramming might merely reflect the shared roles of transcription factors and chromatin regulators in mediating cell state transitions, which correspondingly occur during induced pluripotency and during the conversion of differentiated cells into a tumorigenic state (Suvà et al., 2013; Tung and Knoepfler, 2015). However, while gene methylation was found to be significantly perturbed in the so-called partially reprogrammed transformed cells (PRTCs), and the genomic imprints of PRTCs appeared unstable in the absence of permanent genetic aberrations, it should be noted that such epigenetic features were distinguishable from those in sporadic carcinomas. Thus, whereas DNA hypermethylation at proximal promoter regions was not evident, global DNA methylation levels were comparable with those of normal cells, indicating a lack of both site-specific DNA hypermethylation and global DNA hypomethylation that characterizes most human carcinomas (Ohnishi et al., 2014a,b). Cellular reprogramming-associated generation of undifferentiated dysplastic cells in various tissues notably resembled those of Wilms' tumors, the most common pediatric kidney cancer, as well as those of pediatric blastomas such as hepatoblastomas and pancreatoblastomas. These findings support the notion that deleterious nuclear reprogramming-associated epigenetic reorganization in certain organs and tissues at discrete developmental stages can contribute to the initiation and progression of pediatric tumors.

Pathological Versions of Nuclear Reprogramming

The occurrence of PRTCs does not in itself provide sufficient evidence that de-differentiation is involved in cancer development. The number and complexity of the molecular events required for *de novo* generation of stem cell-like cells (e.g., chromatin decondensation, loss of differentiation marks, transcriptional activation of stemness genes, suppression of competing cell lineages factors, among others) is considered to intrinsically prevent the initiation of pathological versions of nuclear reprogramming-like processes in differentiated tissues, including those of tumors (Pasque et al., 2010, 2011; Cantone and Fisher, 2013; Brooks et al., 2015). Along this line, OSKM-derived PRTCs have been viewed as mechanistically irrelevant for most common sporadic cancers that afflict the elderly,

where developmental biology is not commonly considered. Nevertheless, *in vivo* nuclear reprogramming-related PRTCs might form the basis of a new model of epigenetic tumorigenesis when looked at in depth.

Upon reactivation of OSKM factors, PRTCs fully reprogram into iPSCs, suggesting that the reorganization of epigenetic landscapes associated with chronic, unresolved nuclear reprogramming is adequate to generate epigenetically heritable cancer-like phenotypes. If pathological nuclear reprogramming reflects a cancer initiation phenomenon driven purely by epigenetic mechanisms (Goding et al., 2014; Menendez and Alarcón, 2014; Menendez et al., 2014), a testable prediction would be that those cancers in which epigenome rewiring establishes a permissive milieu for carcinogenesis but requiring additional cooperating mutations for complete malignant transformation, should behave as accelerated models of oncogenesis. In contrast to sporadic forms, familial paragangliomas associated with mutations in the succinate dehydrogenase complex and the consequent accumulation of the histone demethylase (HDM) inhibitor succinate, which establishes a hypermethylator phenotype and the epigenetic silencing of key differentiation genes (Letouzé et al., 2013; Yang and Pollard, 2013), tend to present at a younger age (Lips et al., 2006; Chetty, 2010). Patients with gliomas with gain-of-function isocitrate dehydrogenase (IDH) mutations generating the HDM oncometabolite/inhibitor 2-hydroxyglutarate (2HG), which also establishes a hypermethylator phenotype that stabilizes undifferentiated cellular states that may be targetable and expanded later by subsequent transforming mutations, are, on average, several years younger that those with wild-type IDH gliomas (Bleeker et al., 2010; Cohen et al., 2013; Popov et al., 2013; Dimitrov et al., 2015). Moreover, we have recently identified how archetypal oncometabolites such as 2HG, by endowing cells with epigenetic states refractory to differentiation, considerably enhances the global kinetic efficiency of OSKM-driven nuclear reprogramming processes to generate cancer stem cell (CSC)-like states *de novo* (Menendez and Alarcón, 2016; Menendez et al., 2016). Altogether, these observations strongly support the notion that pathological versions of nuclear reprogramming could operate as primary and causative mechanisms of cancer-associated epigenetic rewiring.

Environmental Dedifferentiation of Committed Cells into Stem Cell-Like States *In vivo*

There is accumulating robust evidence showing that non-stem cell compartments might be sources of newly generated pools of cells sharing stem-like characteristics with endogenous, adult stem cell counterparts in the same organ. The Weinberg group originally addressed this question to demonstrate that stem-like cellular states might arise *de novo* from more differentiated cell types within the human mammary gland (Chaffer et al., 2011). Moreover, a rare subpopulation of somatic cells of human breast tissue was found to be poised to actively transcribe plasticity and pluripotency markers such as *Oct4*, *Sox2*, and *Nanog*, and to acquire a plastic cell state sensitive to environmental programming (Roy et al., 2013).

Fully committed airway epithelial cells have been shown to revert to stable and functional stem cell-like states *in vivo* and, more importantly, to function as well as their endogenous adult stem cell counterparts in repairing epithelial injury (Tata et al., 2013). Upon crypt damage, $Dll1^+$ intestinal secretory progenitor cells exhibit plasticity by regaining stemness (van Es et al., 2012). Additionally, the *Wnt* target gene leucine-rich-repeat containing G-protein-coupled receptor (*Lgr5*), which marks actively dividing stem cells in *Wnt*-driven, self-renewing tissues such as small intestine and colon, stomach and hair follicles (Barker et al., 2008), can be induced to form small $Lgr5^+$ liver stem-like cells capable of generating hepatocytes and bile ducts *in vivo* (Huch et al., 2013). Upon damage, committed cells within tissues that have a low rate of spontaneous proliferation have the capacity to generate $Lgr5^+$ stem cell-like states *de novo*, which are commonly observed in actively self-renewing tissues (Barker et al., 2008; Huch et al., 2013).

The demonstration that aberrant termination of OSKM-induced reprogramming *in vivo* results in tumor development also revealed the unexpected activation of *Lgr5* (Ohnishi et al., 2014a). Since *Lgr5* expression is not present in iPSCs, and is neither transiently expressed during reprogramming of fibroblasts nor in *in vitro* generated partially reprogrammed cells, its presence in OSKM-driven tumorigenesis raised important concerns regarding the relevance of this model as a proof-of-concept for epigenetics-driven cancer development *in vivo* (Hobbs and Polo, 2014; Ohnishi et al., 2014b). However, from a reparative/regenerative perspective, it is tempting to suggest that aberrantly terminated cellular reprogramming *in vivo* recapitulates the natural functioning of a host genetic program for resistance (e.g., regeneration of $Lgr5^+$ stem-like cells from $Lgr5^-$ cell populations) that is activated upon damage (Mosteiro et al., 2016).

AGING AND CANCER: TWO SIDES OF REPARATIVE CELLULAR REPROGRAMMING

In close analogy to classical descriptions of nuclear reprogramming as a key regenerative mechanism in plants, invertebrates, teleost fishes, and amphibians (Brockes and Kumar, 2002; Jopling et al., 2011; Sugimoto et al., 2011), we are beginning to appreciate that the capacity of adult differentiated cells to generate transiently active stem-like cellular states challenges the commonly held belief that tissue-specific adult stem cells are the sole contributors to self-cell therapy (Desai and Krasnow, 2013). By functionally substituting tissue-specific stem cells, transiently reprogrammed mature committed cells might have a general role in adult tissue repair by operating in a host program for resistance to damage and other tissue adversities. *In vivo* reprogramming phenomena and consequent epigenetic plasticity, however, might also instigate tumor cell-like states by participating in the generation and maintenance of the versatility—aberrant differentiation and transdifferentiation

capacities—of the CSC-like cellular states (Friedmann-Morvinski and Verma, 2014).

Cells with non-plastic chromatin will be less likely to undergo malignant transformation, but they will also be less able to respond to danger signals and, consequently, they will be more prone to degeneration. In this regard, the cyclic and transient expression of reprogramming factors *in vivo* has recently been shown to increase lifespan in a murine model of premature aging by remodeling the chromatin landscape (Ocampo et al., 2016). Conversely, cells with more plastic chromatin will be more adaptable in the face of cell intrinsic or microenvironmental changes, but they might also provide "molecular power" on a tissue's susceptibility to undergo aging-associated degeneration or cancer-associated malignant transformation. Accordingly, chronic injury and aging have been shown to render tissues highly permissive to *in vivo* reprogramming (Mosteiro et al., 2016). The goal now is to define the key players that are involved in regulating cellular plasticity, both during physiological *in vivo* reprogramming, leading to tissue rejuvenation, and during pathological conditions, where increased plasticity-related tissue dedifferentiation associates with cancer (Marión et al., 2017).

Epigenetic Plasticity and the Archetypal Pro-Inflammatory Cytokine IL-6

We are beginning to appreciate the existence of a common mechanism for epigenetic plasticity regulated by inflammatory signaling. However, while it is widely accepted that chronic inflammation may drive pathological changes in cell phenotypes, whether an inflammatory signal that is short-term and physiological can provide a molecular scenario capable of driving self-cell therapy for resistance to damage and disease remains a matter of discussion. The archetypal pro-inflammatory cytokine IL-6, which dictates the transition from acute to chronic inflammation, might illustrate how inflammation could have both a beneficial and a harmful role in aging and cancer (Scheller et al., 2011; Rincon, 2012; Hunter and Jones, 2015; Mauer et al., 2015).

Somatic cells can detect PAMPs or DAMPs *via* PRRs such as TLRs, the activation of which triggers the generation and release of chemokines and cytokines (e.g., IL-6) that contribute to inflammatory response (Kapetanovic et al., 2015; Toubai et al., 2016). Such an acute activation of inflammatory response provokes global changes in epigenetic modifiers, favoring an open chromatin configuration and increasing epigenetic plasticity. This temporary reprogramming would replenish damaged, diseased, and lost cells in tissues challenged with danger signals. The notion that inflammatory signaling might innately operate to boost the production of stem cell-like cellular states has been supported by the discovery that IL-6 plays an early yet critical role during generation of induced pluripotency. IL-6 is involved in reprogramming to pluripotency during embryogenesis (Zolti et al., 1991; Austgulen et al., 1995). Using non-dividing heterokaryons (murine ESCs fused to human fibroblasts) in which reprogramming toward pluripotency is efficient and rapid, the (undetectable) level of IL-6 in ESCs dramatically increases 50-fold upon during nuclear reprogramming (Brady et al., 2013). Moreover, exogenous addition of IL-6 can functionally replace the oncogenic *c-Myc* component of the Yamanaka cocktail during the generation of iPSCs (Brady et al., 2013).

At sites of transient inflammation, acute resolution of inflammatory response mediated by IL-6 (and downstream activation of NF-κB) could be accompanied by beneficial tissue regeneration (Cressman et al., 1996; Taub et al., 1999; Lasry and Ben-Neriah, 2015; Chiche et al., 2017), or reparative transdifferentiation (e.g., conversion of fibroblasts into endothelial cells to increase microvascular density in response to myocardial infarction-induced ischemic injury; Cooke et al., 2015; Sayed et al., 2015). At sites of chronic inflammation, if the culmination of transient reprogramming to stem-like epigenetic states is not accompanied by a committed re-acquisition of the original or alternative (but beneficial) differentiated cell fate, unrestrained nuclear reprogramming-driven tissue plasticity might impair the repair or replacement of damaged cells and at the same time generate cancer-like cellular states.

Chronic inflammation-related reduced regenerative capacity might be accompanied by permanent changes in target tissue cells involving either their locking in unresolved stem-like states or their transdifferentiation. Thus, while the IL-6-enriched microenvironment of the ≈20% of tumors that are associated with inflammation (e.g., chronic ulcerative colitis-associated colon cancer; Coussens and Werb, 2002; Grivennikov et al., 2010; Ben-Neriah and Karin, 2011; Balkwill and Mantovani, 2012) seems to have a dominant role in facilitating tumorigenesis with expansion and maintenance of CSC-like cells, chronic esophagitis caused by gastroesophageal reflux disease can promote transdifferentiation of stratified squamous esophageal epithelium into small intestine-like columnar epithelium (i.e., Barrett's esophagus), which might later be the site of malignant transformation (Kuilman et al., 2008; Vega et al., 2014; Kapoor et al., 2015; Wang and Souza, 2016). Furthermore, tumors that do not arise because of chronic inflammation appear to later develop an IL-6-rich microenvironment, which supports tumor progression and metastasis. Thus, an IL-6-driven inflammatory feedback loop is a core epigenetic regulator of the dynamic equilibrium that converts non-stem cancer cells into CSC-like cells, and generates tumor heterogeneity in genetically distinct cancer cells (Iliopoulos et al., 2011; Korkaya et al., 2011, 2012; Krishnamurthy et al., 2014). Indeed, IL-6/NF-κB-related signaling loops, which are recognized to lead to expansion of CSC-like populations, are reminiscent to those activated during chronic inflammation and wound healing, and provide a mechanistic basis for the known link between inflammation and the promotion of aggressive cancer phenotypes.

Senescence-Associated Inflammatory Signaling (SAIS): From Organism-Wide to Local Stress Response and Para-Inflammation

The proposal that activation of *in vivo* cellular reprogramming is a host genetic program for resistance to damage and disease that could promote either "complete healing" of injured/diseased

tissue or "incomplete healing" of old or cancerous tissues must be compatible with the accepted hypothesis that the degenerative and hyperplastic pathologies of aging, the most deadly of which is cancer, are linked by a common biological phenomenon: cellular senescence.

Cellular senescence is a persistent damage response in cells experiencing unresolved or irreparable stress for a sustained period of time. It has been causally linked to a wide variety of processes including wound healing, aging, tumor prevention, and tumor progression (Rodier and Campisi, 2011; Campisi, 2013; Tchkonia et al., 2013; Muñoz-Espín and Serrano, 2014; Lasry and Ben-Neriah, 2015). The multi-faceted capabilities of senescent cells involving both beneficial (tissue repair and tumor suppression) and deleterious (aging and tumor promotion) effects on organismal health can be viewed as consequences of senescence-associated inflammatory signaling (SAIS). Despite their loss of proliferative potential, senescent cells are metabolically and transcriptionally active and express a vast number of secreted proteins. With progressing age, an organism-wide increase of the senescence-associated secretory phenotype (SASP) ensues, which comprises a range of different proteins that are well-known players in aging and age-related diseases, including matrix-remodeling metalloproteases such as MMP3, growth factors such as HGF and TGFβ, inflammatory chemokines such as CCL2 and CLL11, and prominent pro-inflammatory cytokines such as IL1α/β, IL-6, and IL-8 (Coppé et al., 2008; Kuilman and Peeper, 2009; Orjalo et al., 2009; Rodier et al., 2009; Gross et al., 2012; Acosta et al., 2013). While the SASP of a cell varies according to tissue type and stressor, its ability to maintain not only the senescent cell itself but also to propagate the stress response and impact the microenvironment through communication with neighboring cells, can lead to organism-wide phenotypes *via* systemic inflammation that is largely dependent on the core inflammatory cytokines IL-6 and IL-8.

The senescence inflammatory response (SIR) is a second type of inflammation activated in senescent cells (Pribluda et al., 2013; Aran et al., 2016). Unlike the SASP, SIR is characterized by a small number of secreted factors and it is not accompanied by recruitment of immune cells into the senescent tissue. Intriguingly, SIR has a weak association with the NF-κB signaling pathway, but mostly comprises innate immunity proteins including members of the TLR activation pathway, which has an important role in tissue homeostasis by regulating inflammatory and tissue repair responses to injury (Rakoff-Nahoum and Medzhitov, 2009). Most likely, SIR has a largely epithelial-autonomous role and seems to contribute to tissue protective senescence and to counteract tumor progression by cooperating with *bona fide* tumor suppressor genes such as *p53* (Pribluda et al., 2013; Aran et al., 2016). Inflammatory processes such as transflammation and SIR might therefore be viewed as intermediate, tissue-level stress responses and para-inflammatory states, respectively, occurring between homeostasis and overt inflammation (Medzhitov, 2008; Chovatiya and Medzhitov, 2014). Accordingly, whereas transflammation is triggered by extrinsic assaults such as pathogens or tissue injuries, SIR seems to be prompted by tissue-intrinsic assaults such as DNA damage.

SAIS: Connecting the Two Sides of Reparative Cellular Reprogramming

SAIS can be integrated with the continuum of the inflammatory spectrum that ranges from homeostatic states to (transflammation-like) stress responses (NF-κB-dependent and SIR-like), para-inflammation states, and finally overt (SASP-like) inflammation. Local stress and para-inflammation responses to extrinsic and intrinsic insults might arise first to respond to tissue stress-related danger signals (PAMPs and DAMPs) or to chronic tissue malfunction (DNA damage, senescence), later evolving into more systemic increases in the expression of major SASP factors, an organism-wide senescent phenotype that is accompanied by either immune clearance of senescent cells or attraction of inflammatory cells. Such multi-faceted, temporal organization of inflammatory phenotypes functionally converge into so-called "inflammaging," defined as the low but chronic levels of inflammation associated with many protracted pathological conditions (e.g., atherosclerosis, diabetes mellitus, autoimmune diseases, and neurodegeneration), which are thought to drive age-related decline in function (Franceschi and Campisi, 2014). The causal role of SAIS as a pivotal driver of the age-related decline in tissue homeostasis is evidenced by: (a) increased expression of genes linked to immune responses and inflammation in aging tissues; (b) chronic activation of NF-κB; (c) organism-wide elevated levels of major SASP factors such as IL-6 in a number of models of physiological and accelerated aging (Baker et al., 2008; Gregg et al., 2012; Wiley et al., 2016); and (d) a prolonged healthspan and extension in lifespan upon semigenetic clearance or pharmacological/directed elimination of senescent cells with so-called senolytic drugs (Baker et al., 2008, 2011, 2016; Zhu et al., 2015; Chang et al., 2016; Yosef et al., 2016).

The temporal continuum of SAIS closely relates to the well-recognized antagonistic pleiotropy of senescence (Campisi, 2005). Senescence can be beneficial early in life or under transient conditions of injury. For instance, senescence promotes correct patterning of the embryo (Muñoz-Espín et al., 2013; Storer et al., 2013) and, after development, SAIS can be beneficial by aiding in wound healing and limiting fibrosis following acute damage (Krizhanovsky et al., 2008; Demaria et al., 2014). However, it is later in life when SAIS, after crossing arbitrary thresholds at both the cell-autonomous and non-cell autonomous levels, is thought to be responsible for many aging-related disorders. On the assumption that terminally differentiated cells can transiently regain core stem cell-like functional properties, one could hypothesize that the beneficial or deleterious paths ensuing upon cellular reprogramming-like cycles of tissue maintenance might be dictated by cell-autonomous and non-cell autonomous SAIS capable of making tissue cells not only transiently exceed "reprogramming barriers," but also to re-acquire the original or alternative differentiated cell fate, in a difficult or easier manner. The degree of deviation from the homeostatic state might establish biological constraints delineating a multiple thresholding algorithm that isolates zones of beneficial vs. deleterious SAIS-regulated reparative cellular reprogramming (**Figure 2**).

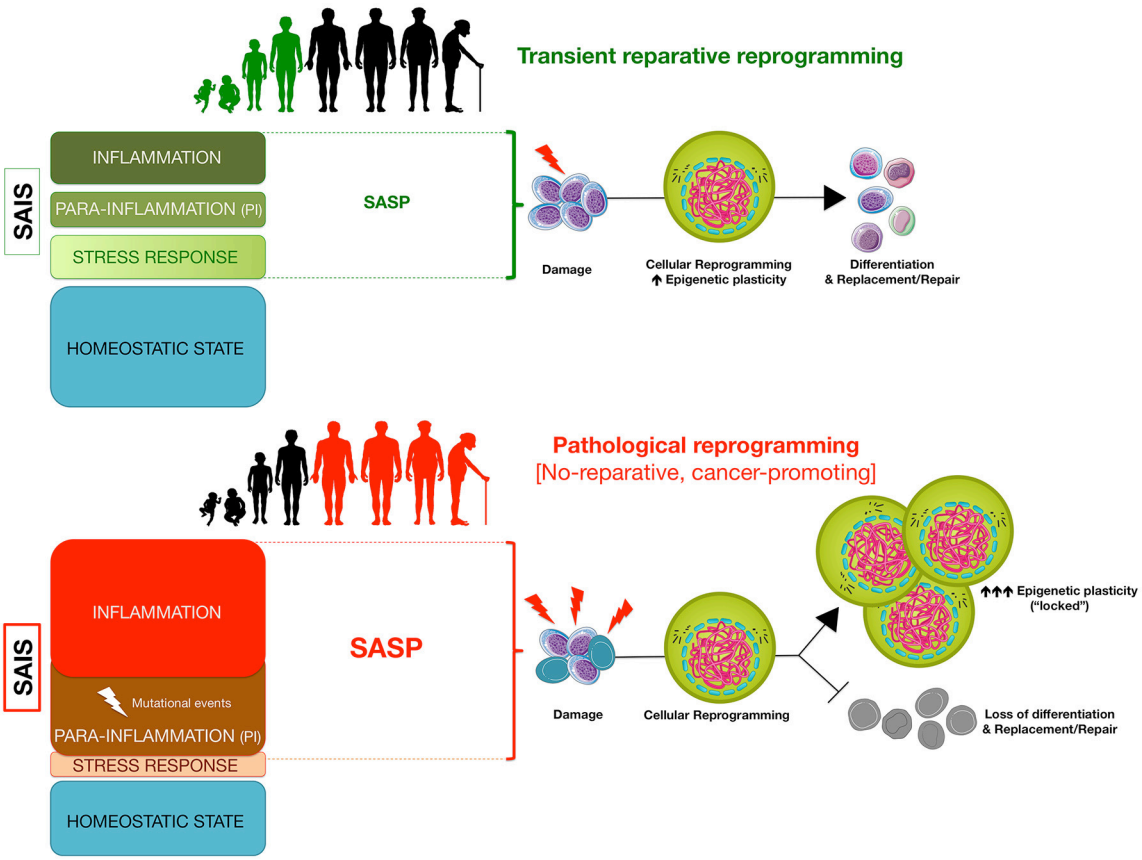

FIGURE 2 | Senescence-associated inflammatory signaling (SAIS)-regulated *in vivo* reprogramming: a threshold model of epigenetic plasticity in aging and cancer. The degree of senescence/inflammation deviation from the homeostatic state delineates a thresholding algorithm distinguishing beneficial vs. deleterious effects of *in vivo* reprogramming. First, transient activation of innate immunity and/or SASP components (e.g., IL-6) might facilitate reparative cellular reprogramming in response to acute inflammatory events. Second, NFκB-dependent and NFκB-independent (e.g., SIR) para-inflammation switches might promote a long-lasting but reversible refractoriness to reparative cellular reprogramming. Third, chronic SASP might lock cells into highly plastic epigenetic states disabled for reparative differentiation capacities.

Transflammation: Innate Immunity-Facilitated Reparative Cellular Reprogramming

From a cell-autonomous perspective, transflammation involving endogenous activation of innate immunity to PAMPs or DAMPs might be sufficient to modify the expression or activity of epigenetic modifiers to generate phenotypic fluidity for cellular responses to pathogenesis or injury (Cooke et al., 2014). If acutely resolved, such a temporal stress response-inflammatory process might allow temporary cell reprogramming and re-acquisition of the original or alternative cell fate in response to specific environmental cues, leading to beneficial tissue rejuvenation or transdifferentiation, respectively. Transflammation-driven reparative cellular reprogramming, which is expected to mostly involve increases in epigenetic plasticity to allow functional malleability without the loss in cellular identity, might operate as a *bona fide* protective response to challenge and eliminate pathogens and also to biophysical tissue damage.

The above scenario, although lacking rigorous experimental validation, is strongly supported by the recent uncoupling of rejuvenation from dedifferentiation associated with OSKM-driven reprogramming of somatic cells. First, brief exposure to OSKM factors, allowing cells to transiently transition through a plastic intermediate state without the complete loss of cellular identity, allows indirect lineage conversion of human fibroblasts to angioblast-like cells with reparative potential in ischemic pathologies (Kurian et al., 2013). Second, at the organism level, the cyclic and short-term expression of OSKM factors can transiently revert premature aging phenotypes including DNA damage responses and senescence-associated features without involving the complete loss of cellular identity (Ocampo et al., 2016).

Para-Inflammation Switches: Loss of Cellular Resilience without Tissue Repair and Rejuvenation

Since unrestricted changes in cell identity may also predispose to loss of tissue homeostasis, it is reasonable to suggest that the optimal zone for innate immunity-facilitated cellular reprogramming to operate as a reparative mechanism will be small. Whereas the lower threshold is expected to be greater than the baseline inflammatory value arising from

disturbed/perturbed physiological homeostasis, a short arbitrary "inflammatory distance" to the upper threshold should ensure that protective inflammatory repair processes might become pathogenic by altering the epigenetic states of damaged/diseased tissue cells. The narrow nature of the optimal zone for transflammation-driven reparative cellular reprogramming should have two major consequences. First, exaggerated or uncontrolled responses to PAMPs and DAMPs resulting in acute systemic hyperinflammation or repeated, overshooting of repair will overcome the upper threshold of the optimal zone of innate immunity-facilitated reparative epigenetic plasticity, thus impeding restoration of tissue homeostasis but eliciting collateral tissue damage (e.g., fibrosis). Secondly, the inability to generate new pools of stem-like cells, for instance, due to chronic baseline inflammatory scenarios exceeding the upper threshold, might hamper a crucial source of self-repair.

An unresolved scenario of continuous cell stress/tissue injury accompanied by inappropriate resolution of endogenous (e.g., metabolic and genomic damage) or exogenous (e.g., pathogens, biophysical stresses) assaults will associate with the activation of senescence-associated para-inflammatory states. The baseline SAIS level of such states will exceed the upper inflammation threshold and will be characterized by a progressive loss of cellular resilience that, however, will not be accompanied by rejuvenation-like phenomena. These para-inflammatory states, which can originate in a NF-κB-independent cell-autonomous manner (e.g., SIR) or in a more systemic NF-κB-dependent manner (Soria-Valles et al., 2016), would operate as senescence-inflammatory friend-or-foe switches that, while originally contributing to tissue protective senescence and counteracting tumor progression, can also drive reprogramming-refractory aging phenotypes and cancer-prone epithelial tissue (**Figure 2**).

Some testable predictions of the switching nature of SAIS arising from para-inflammatory states include: (a) the initial ability of para-inflammation SAIS to suppress the potential of stressed cells acquiring a malignant state will be lost in response to certain environmental and genetic clues (e.g., loss of tumor suppressor genes); and (b) the reduction in SAIS might force such para-inflammatory aging and malignant states to return to an optimal zone of transient reprogramming for rejuvenation while depleting cancer aggressiveness. Accordingly, aberrant NFκB activation is known to impair somatic cell reprogramming and to drive the aging phenotype while also promoting the expansion of CSCs *via* cell- and non-cell autonomous mechanisms (Colotta et al., 2009; Shostak and Chariot, 2011; Yamamoto et al., 2013; Terlizzi et al., 2014; Soria-Valles et al., 2016). NFκB inhibition, which is a potential therapeutic strategy to eliminate CSCs, has been shown to delay DNA damage-induced senescence and aging in mice and to significantly increase the reprogramming efficiency of fibroblasts from patients with progeria syndrome as well as from normal aged individuals (Tilstra et al., 2012; Soria-Valles et al., 2015a,b). These findings lend weight to the notion that targeting NFκB-related SAIS might modify the thresholds for reparative cellular reprogramming. Moreover, whereas para-inflammation-associated SIR is widely prevalent in cancers harboring mutations in *p53* (Aran et al., 2016), the capacity for non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin to exert protective effects against several cancers (e.g., colorectal, pancreatic, lung, and breast; Rothwell et al., 2011; Fraser et al., 2014; Streicher et al., 2014) might be related to their ability to suppress key drivers of SIR in poor prognosis, para-inflammated tumors (Aran et al., 2016). Interestingly, NSAIDs can enhance cellular reprogramming even in the absence of *Sox2* and *c-Myc* (Yang et al., 2011), thus providing further evidence for the thresholding capacity of inflammation-epigenetic axes to determine the optimal zones of cellular reprogramming-driven phenotypic plasticity.

Chronic SASP and Loss of Tissue Homeostasis: The "Stem-Lock" Zone

From a non-cell autonomous perspective, if the loss of differentiation features following reprogramming is not accompanied by re-acquisition of the original or alternative differentiated cell fate, the resulting tissue plasticity might impair the repair or replacement of damaged cells. The ability of SASP-associated pro-inflammatory cytokines to regulate stemness and nuclear reprogramming raises the notion that a SASP-impaired local environment could interfere with tissue rejuvenation by imposing the so-called "stem-lock" state (de Keizer, 2017). Chronic inflammatory conditions *via* exposure to IL-1, which normally functions as a key "emergency" signal and a master regulator of SASP by inducing downstream effectors such as IL-6, has been shown to impair tissue homeostasis and to induce an aged appearance of the hematopoietic system by restricting stem cell differentiation (Pietras et al., 2016). Moreover, biological conditions linked to chronic senescence, such as tissue injury or aging, favor *in vivo* OSKM-driven reprogramming *via* enhanced production of IL-6 as shown by the appearance of *Nanog*-positive cells in the vicinity of senescence areas (Mosteiro et al., 2016).

While counterintuitive, the ability of SASP factors including IL-6 to transiently create a permissive environment for *in vivo* reprogramming capable of inducing cellular plasticity and tissue regeneration (Ritschka et al., 2017), a prolonged promotion of such progenerative response might reduce tissue rejuvenation and promote aging by self-enhancing futile cycles of SASP/IL-6-driven reparative cellular reprogramming. Compared with young tissues containing few senescent cells where transient SAIS might cause temporary reprogramming and differentiation/proliferation to replenish cells, the prolonged accumulation of senescent cells in tissues that are old or under high levels of stress (e.g., following medical procedures such as chemotherapy) might be accompanied by a defective clearance of damaged, senescent cells, which can promote further SASP accumulation. A situation of chronic SASP secretion might not only counter the continued regenerative stimuli by promoting cell-intrinsic senescence arrest in single damaged cells but also paradoxically impose a permanent, locked gain of stem cell-like cellular states with blocked differentiation capabilities in surrounding cells (**Figure 2**). Such a scenario of prolonged survival of senescent cells and enhanced phenotypic plasticity of neighboring cells would drive a loss of tissue homeostasis by impeding the reparative replenishment of damaged cells. As mentioned earlier, core SASP factors such as IL-6 can mimic the

effects of *in vivo* reprogramming (Mosteiro et al., 2016), thereby favoring the emergence of CSC-like cellular states in neighboring cancer cells (Cahu et al., 2012; Chang et al., 2015). Thus, chronic SASP-driven loss of tissue homeostasis might go hand-in-hand with an accelerated generation of trade-off forms of undifferentiated types of cells with CSC-like states. Accordingly, the protracted presence of senescent cells that can promote local and systemic SASP in stressed normal tissue has recently been shown to cause and exacerbate short- and long-term effects of genotoxic stresses ranging from weakness and fatigue in skeletal muscle to CSC-related cancer recurrence (Demaria et al., 2017).

SENESCENCE-INFLAMMATORY REGULATION OF REPARATIVE CELLULAR REPROGRAMMING IN AGING AND CANCER: A THRESHOLD MODEL OF EPIGENETIC PLASTICITY

Nuclear reprogramming-like phenomena inducing transient epigenetic plasticity followed by cell differentiation and replacement of damaged/diseased cells may constitute a previously unrecognized route through which human tissue responds to injury, stress, and disease. This may lead to either acutely resolved tissue repair (e.g., transient gain of epigenetic plasticity upon acute transflammation events) or, alternatively, to undesirable, chronically unresolved tissue damage (e.g., lasting gain of epigenetic plasticity upon chronic SASP responses). Aging and cancer might thus be viewed as the consequence of an unrestricted/unresolved stimulation of futile, non-reparative *in vivo* reprogramming-driven epigenetic plasticity in response to chronic, but reversible, senescence-inflammatory signaling.

We propose that the regulation of cellular reprogramming/differentiation cycles of tissue repair by the cell-autonomous/non-cell autonomous mechanisms that initiate and propagate SAIS might suffice to outline a threshold model of epigenetic plasticity in aging and cancer. A better understanding of the biological constraints that determine how the map of SAIS-regulated reparative *in vivo* reprogramming is dynamically thresholded may provide therapeutic approaches for aging and cancer (**Figure 3**). A first open question is whether "epigenetic bursts" of innate immunity-facilitated reparative epigenetic plasticity operate as physiological mimickers of the transient amelioration of tissue functions without inducing complete dedifferentiation, as apparently occurs upon short-term induction of OSKM factors in animal models (Mahmoudi and Brunet, 2016; Ocampo et al., 2016). In such a scenario, the discovery and validation of small molecules, more likely

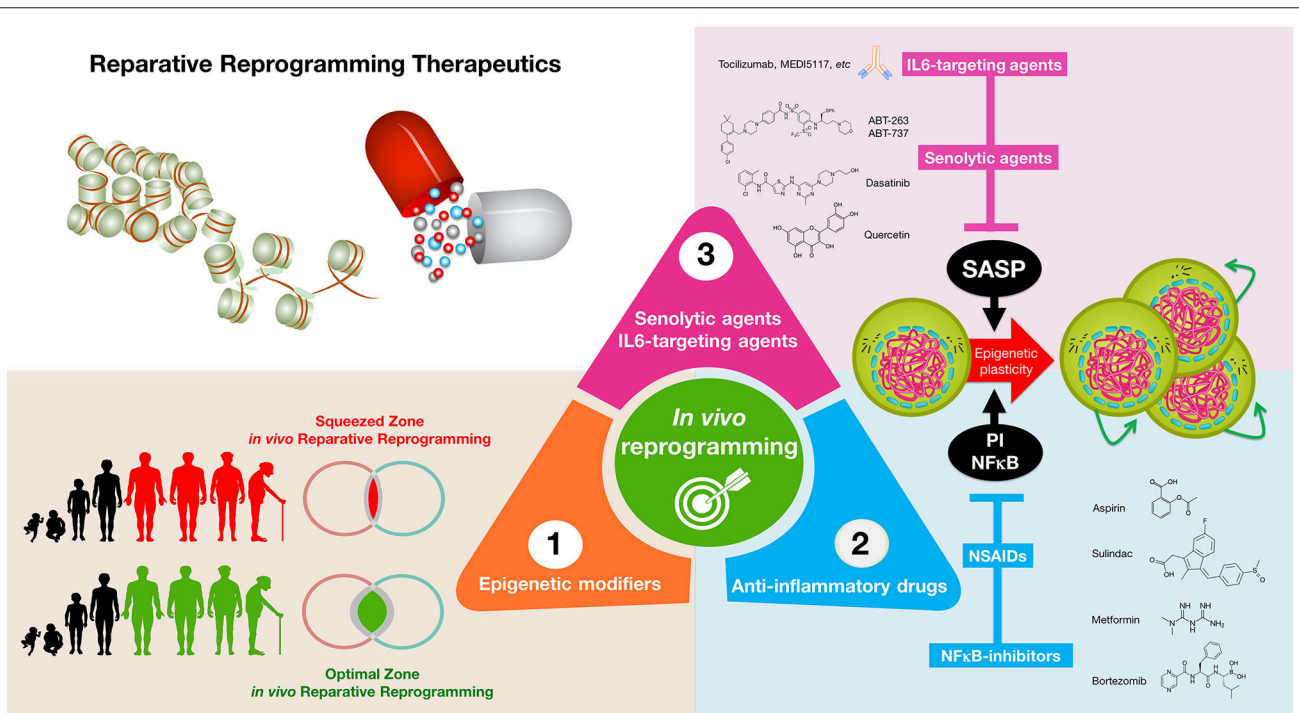

FIGURE 3 | Reparative reprogramming therapeutics: enhancing the body's self-cell therapy for resistance to damage and disease. A cellular reprogramming-centered view of epigenetic plasticity as a fundamental dimension of a tissue's capacity to undergo successful repair may provide new therapeutic approaches for aging and cancer. (1) *Epigenetic modifiers:* small molecules capable of mimicking the transient amelioration of tissue functions occurring upon short-term induction of OSKM-induced nuclear reprogramming (Mahmoudi and Brunet, 2016; Ocampo et al., 2016) might increase epigenetic plasticity and to enhance regeneration in aging tissues; (2) *anti-inflammatory drugs:* NFκB-targeting drugs and commonly employed NSAIDs might help reduce some aging- and cancer-promoting inflammatory feedback loops to reestablish the functioning of reparative reprogramming; (3) *IL-6-targeting and senolytic agents:* IL-6 blockade and senescent cell ablation might help unlock the chronic epigenetic plasticity of SASP-damaged tissues to successfully achieve tissue rejuvenation if accompanied by reparative differentiation phenomena.

epigenetic modifiers, capable of widening or re-establishing the optimal zone of physiological *in vivo* reprogramming would be expected to increase epigenetic plasticity and to enhance regeneration in aging tissues. A second open question is whether commonly employed NSAIDs (e.g., aspirin, sulindac derivatives; Chan and Detering, 2013; Gurpinar et al., 2014) and NFκB-targeting drugs (e.g., bortezomib, metformin; Hirsch et al., 2013; Zhou et al., 2015) can reestablish stress response-inflammatory thresholds compatible with reparative reprogramming while eliminating aging- and cancer-promoting inflammatory feedback loops. Finally, a third open question concerns the clarification of how senescent cells operate as *bona fide* sources of *in vivo* reprogramming. The discovery of the first generation of senolytic drugs (Kirkland and Tchkonia, 2015; Zhu et al., 2015, 2016; Chang et al., 2016) along with therapeutics targeting core SASP components such as IL-6 (Krishnamurthy et al., 2014; Kim et al., 2015; Heo et al., 2016; Zhong et al., 2016) might be viewed as an obvious therapeutic avenue to stimulate *in vivo* reprogramming-driven tissue rejuvenation. However, it should be acknowledged that an ideal anti-aging therapy would need not only to "unlock" the chronic epigenetic plasticity of SASP-damaged tissues, but also to stimulate differentiation of stem cell-like states to successfully achieve tissue rejuvenation (de Keizer, 2017). Nonetheless, it would be interesting to evaluate whether, beyond IL-6 blockade, senescent cell ablation might also ameliorate the efficacy of cancer treatment modalities by impeding the replenishment of treatment-refractory CSCs that might *de novo* arise by cellular reprogramming-like phenomena of non-CSC tumor counterparts.

The consideration of a cellular reprogramming-centered view of epigenetic plasticity as a fundamental element of a tissue's capacity to undergo successful repair, aging degeneration or malignant transformation should provide stochastic insights into the current deterministic genetic paradigm for aging-related diseases, thereby increasing the spectrum of therapeutic approaches for physiological aging and cancer.

AUTHOR CONTRIBUTIONS

JM and TA conceived the idea for this project and wrote the manuscript.

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Stimulating Fracture Healing in Ischemic Environments: Does Oxygen Direct Stem Cell Fate during Fracture Healing?

*Katherine R. Miclau[1,2,3], Sloane A. Brazina[1,2], Chelsea S. Bahney[1,2], Kurt D. Hankenson[4,5], Thomas K. Hunt[6], Ralph S. Marcucio[1,2] and Theodore Miclau[1,2]**

[1] Department of Orthopaedic Surgery, University of California, San Francisco, CA, USA, [2] Zuckerberg San Francisco General Hospital, Orthopaedic Trauma Institute, San Francisco, CA, USA, [3] Harvard College, Cambridge, MA, USA, [4] Department of Small Animal Clinical Science and Department of Physiology, Michigan State University, East Lansing, MI, USA, [5] Department of Orthopaedic Surgery, University of Pennsylvania, Philadelphia, PA, USA, [6] Department of Surgery, University of California, San Francisco, CA, USA

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***Correspondence:**
Theodore Miclau
miclaut@orthosurg.ucsf.edu

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Bone fractures represent an enormous societal and economic burden as one of the most prevalent causes of disability worldwide. Each year, nearly 15 million people are affected by fractures in the United States alone. Data indicate that the blood supply is critical for fracture healing; as data indicate that concomitant bone and vascular injury are major risk factors for non-union. However, the various role(s) that the vasculature plays remains speculative. Fracture stabilization dictates stem cell fate choices during repair. In stabilized fractures stem cells differentiate directly into osteoblasts and heal the injury by intramembranous ossification. In contrast, in non-stable fractures stem cells differentiate into chondrocytes and the bone heals through endochondral ossification, where a cartilage template transforms into bone as the chondrocytes transform into osteoblasts. One suggested role of the vasculature has been to participate in the stem cell fate decisions due to delivery of oxygen. In stable fractures, the blood vessels are thought to remain intact and promote osteogenesis, while in non-stable fractures, continual disruption of the vasculature creates hypoxia that favors formation of cartilage, which is avascular. However, recent data suggests that non-stable fractures are more vascularized than stable fractures, that oxygen does not appear associated with differentiation of stem cells into chondrocytes and osteoblasts, that cartilage is not hypoxic, and that oxygen, not sustained hypoxia, is required for angiogenesis. These unexpected results, which contrast other published studies, are indicative of the need to better understand the complex, spatio-temporal regulation of vascularization and oxygenation in fracture healing. This work has also revealed that oxygen, along with the promotion of angiogenesis, may be novel adjuvants that can stimulate healing in select patient populations.

Keywords: fractures, bone, repair, ischemia, oxygen, stem cell, differentiation, stimulation

INTRODUCTION

Ischemia, the restriction of blood supply to tissues, leads to hypoxic and nutrient-deficient environments and results in decreased cellular metabolism and proper tissue functioning, including reduced fracture repair (Lu et al., 2007; Miedel et al., 2013). Although many of the mechanisms regarding ischemia's inhibitory effect on wound healing remain unknown, preclinical and clinical experiments have been conducted to analyze the role of vasculature in fracture healing. The literature overwhelmingly supports the critical role of blood supply in the complex process of skeletal regeneration.

Musculoskeletal disorders—any injury or disorder that affects the muscles, bones, and joints —are the second leading cause of disability worldwide with the fourth greatest impact on overall health (Woolf and Pfleger, 2003). Within the United States alone, musculoskeletal disorders currently affect half of all adults and 75% of people over 65. With each passing year, these conditions only become more of a burden as the population ages (Weinstein et al., 2014). In particular, as the lifespan of the average American increases with improvements in healthcare, nutrition, and living conditions, rates of osteoporosis and osteoarthritis in the elderly are increasing rapidly, leading to a dramatic rise in the number of geriatric fractures. Each year, fractures affect 15 of every 1,000 people worldwide and occur at rates of 15 million fractures per year in the US. Of these 15 million fractures, an estimated 10–15% will not heal properly (Einhorn, 1995).

Fracture treatment is associated with an enormous societal burden secondary to direct and indirect recovery costs. In 2010, trauma surpassed cardiovascular disease as the leading healthcare cost burden in the US, accruing $21 billion a year in trauma-related visits to medical facilities and accounting for 6.6% of the total cost of hospital care in the US (Allison Russo et al., 2004). Costs of these direct treatments represent only 20% of the economic burden of trauma-related injuries. The remaining 80% is due to the indirect costs of productivity loss, as approximately half of all individuals suffering fractures do not return to work within the first 6 months of recovery (Kanakaris and Giannoudis, 2007).

Impaired healing exacerbates the economic burden of fracture-related conditions, particularly in instances of non-union. In the US alone, nearly 100,000 non-union cases—fractures characterized by failure to heal within 9 months post-injury, and lack of progress toward union, as demonstrated by radiogram, within 3 months—are treated each year, with each patient accruing an estimated $11,333 in direct and indirect costs (Dickson et al., 1995). In 1994, a report demonstrated that ∼$14.6 million is spent annually to treat delayed union—failure to achieve union by 6 months post-injury—and non-union fractures (Kanakaris and Giannoudis, 2007). This colossal economic burden imposed on the United States health care system motivates our need to better understand the various factors responsible for impaired fracture healing and to develop more effective treatments.

Evidence suggests that compromised vascularity is a leading cause of non-union conditions, making the treatment of ischemic fractures a viable target to reduce US healthcare spending. Approximately 46% of fracture patients with accompanying vascular injuries experience impaired bone healing, which is significantly higher than the average 10% non-union rate (Dickson et al., 1995). In cases with vascular-related comorbidities such as diabetic angiopathy and trauma-related extensive soft tissue damage, blood flow is often compromised at the site of the fracture, thereby impeding the comprehensive vascular response necessary for proper bone regeneration and highlighting ischemia as a primary risk factor.

CLINICAL RELEVANCE

Although the effect of ischemia on fracture healing and the mechanism of bone regeneration under hypoxic conditions has been studied in murine models, this work has yet to be translated to *in vivo* human studies. Few clinical studies have addressed the role of ischemia in fracture repair. Dickson et al. retrospectively evaluated the prognosis of healing as a function of arterial injury in tibial fractures and found that open tibia fracture patients presenting with arterial occlusion have a significantly higher rate of delayed union and non-union (Dickson et al., 1994). Avany et al. demonstrated a 50% incidence of arterial occlusion among tibial non-union patients (Arany et al., 1980), and Dietz et al. reported that tibial non-unions in chronically ischemic limbs healed after the arterial supply was restored to normal (Deitz et al., 1989). These three clinical studies illustrate a clear correlation between a lack of blood supply to injured tissue and impaired fracture healing as a result. Impaired healing of diabetic foot ulcers serves as another example of the importance of vascularization and oxygenation in wound healing. Diabetic foot ulcers occur in 15% of all diabetic patients and are accompanied by prolonged hypoxia and inadequate angiogenesis. Decreased wound vascularization associated with diabetic foot ulcers is associated with impaired mobilization and homing of endothelial progenitor cells (EPCs), and a decrease in VEGF levels (reviewed in: Guo and Dipietro, 2010). Studies demonstrate that the mobilization of EPCs to diabetic foot ulcers in patients is reversible through treatment with moderate hyperoxia, which suggests that ischemia induced by prolonged hypoxia contributes to compromised healing. (Liu and Velazquez, 2008)

Clinical analyses of healing rates for patients with impaired vascularization supports the importance of adequate blood supply during fracture healing. Within a population free of chronic disease, the risk of non-union ranges from 10 to 15% (Einhorn, 1995). This figure is increased to nearly 50% when fractures are accompanied by impaired vasculature (Dickson et al., 1994, 1995). Several non-traumatic risk factors are associated with vasculature disease that may also contribute to an increased incidence of delayed union and non-union (Buza and Einhorn, 2016). Diabetics experience approximately two to three times longer fracture healing than non-diabetic patients (Mehta et al., 2010), and diabetes is associated with decreased angiogenesis (Abaci et al., 1999; Galiano et al., 2004). A higher proportion of fractures in smokers result in non-union or delayed union compared to non-smokers (Castillo et al., 2005); one study has shown that tibial healing times were 62% longer

in smokers compared to non-smokers (Schmitz et al., 1999). Cigarette smoking has long been associated with cardiovascular disease (Benowitz and Burbank, 2016) and defective angiogenesis (Ejaz and Lim, 2005), but nicotine has also been shown to have angiogenic activities (Heeschen et al., 2001). No definitive studies examining the effect of smoking or nicotine on angiogenesis after fracture have been performed to date. Elderly patients heal more slowly than young adult patients (Green et al., 2005), and research has shown that angiogenesis is delayed during fracture healing in older animals (Lu et al., 2008a). As evident by these findings, many patient populations are at risk for compromised fracture healing.

ROLE OF VASCULATURE DURING FRACTURE HEALING

While clinical studies clearly demonstrate the necessity of the vasculature for efficient healing, the specific role(s) that the vasculature plays is unknown. Long bone fractures are almost always accompanied by vascular disruption in the surrounding soft tissues, creating a hematoma around the fracture site. Interestingly, the blood vessels that couple angiogenesis and osteogenesis appear to be unique (Kusumbe et al., 2014). The vasculature serves at least two important functions during fracture repair: delivery of nutrients to the damaged tissue, and transport of cells to the healing fracture site. Blood vessels provide oxygen and nutrients to the site of injury that are necessary for cell survival. Additionally, the blood supply is also a route for inflammatory cells and other cell types that are recruited from systemic sources to the fracture site. Vasculature may also provide important signals that help regulate the process of bone fracture repair (Bahney et al., 2015; Hu et al., 2017).

EFFECT OF OXYGEN LEVELS ON STEM CELL DIFFERENTIATION DURING FRACTURE HEALING

Mechanical stability is associated with the mode of healing at the fracture site (Carter and Giori, 1991; Claes and Heigele, 1999). Rigidly stabilized fractures heal through intramembranous ossification (Willenegger et al., 1971; Le et al., 2001), a process in which stem cells located in the periosteum and endosteum differentiate directly into osteoblasts that form bone directly (Thompson et al., 2002; Colnot, 2008). In contrast, fractures that are not rigidly stabilized heal primarily through endochondral ossification with a small amount of direct bone formation in the periosteum and endosteum (Probst and Spiegel, 1997; Hankemeier et al., 2001). During endochondral ossification stem cells differentiate into chondrocytes, which then transform into osteoblasts to form the new bone. Clinically, most fractures in humans heal through a combination of intramembranous and endochondral ossification (Urist and Johnson, 1943; Hak et al., 2014).

The differences in vascularity between bone and cartilage may provide some insight into these two different mechanisms of fracture healing: bone is a highly vascularized tissue, while cartilage is completely devoid of blood vessels. Therefore, one possibility is that in a stabilized environment the blood supply is more intact than in a non-stable environment, and this favors direct bone formation. In contrast, in non-stable environments disruption to the blood supply may favor chondrogenesis due to localized hypoxia (Probst and Spiegel, 1997; Malda et al., 2003), which leads to a large cartilage callus that eventually transforms to bone as the vasculature invades. This idea is supported by observations that chondrogenesis of articular chondrocytes proceeds *in vitro* much more efficiently in cultures that have reduced oxygen levels (Murphy and Polak, 2004), suggesting that oxygen levels may play a role in differentiation of chondrocytes and osteoblasts.

While the data on the effects of oxygen on skeletogenic stem cells is still in flux (selected work is shown in **Table 1**), that concerning vascular stem cells is not. Differentiation of vascular stem cells after injury occurs at the site of injury and in proportion to the concentration of oxygen. However, other conditions must be satisfied: lactate must be elevated and pH must be depressed. These conditions are met largely by inflammatory cells that have been stimulated by the injury, the initial hypoxia, and the elevated aerobic production of lactate (Trabold et al., 2003; Vander Heiden et al., 2009). It is likely that once the process is initiated, the effect of oxygen may be to stimulate lactate production, or at least not lower it until inflammation begins to resolve (Trabold et al., 2003; Vander Heiden et al., 2009). Continued movement and re-injury is therefore expected to enhance the angiogenic response to unstable fractures. Interestingly, the hyperplastic effect of carefully controlled traction may or may not fit this assumption.

To address the role of the vasculature and oxygenation during fracture healing, we performed a series of experiments to examine the relationship among mechanical stability, vascularization, oxygenation, and stem cell differentiation into chondrocytes and osteoblasts during fracture healing in mice. While tissue oxygen levels during fracture healing have been measured by other investigators in the past (e.g., Brighton and Krebs, 1972 and **Table 1**), the methods relied on placement of microprobes to measure oxygen levels (Brighton et al., 1971). This approach provides excellent detection methods at a single time point and location, but does not allow repeated measures at the same location. In our work, we used Electron Paramagnetic Spectroscopy (EPR) to measure oxygen levels (Khan et al., 2007). In this method, electron paramagnetic particles are implanted into the fracture site and the amount of oxygen interacting with the surface of the particle is measured via EPR. Repeated measurements can be taken on the same animal from the same location, and using histology, the position of the oxygen measurement within the fracture site can be determined.

Using this approach, we determined that oxygen levels did not differ between non-stabilized and stabilized fractures. There was an initial reduction in oxygen to hypoxic levels in both fracture groups, which rebounded within 24 h, and no differences in oxygen levels were apparent between groups by day 3. Further, we observed an increased vascular supply in the lower limb of animals with non-stabilized fractures compared to uninjured tibiae and stabilized fractures at early time points (Lu et al.,

TABLE 1 | Select works illustrating effects of oxygen on skeletal cells.

Measuring Oxygen at the Fracture Site	References
C.T. Brighton, R.B. Heppenstall, and D.A. Labosky, An oxygen microelectrode suitable for cartilage and cancellous bone. Clin Orthop Relat Res 80 (1971) 161-6.	Brighton et al., 1971
C.T. Brighton, and A.G. Krebs, Oxygen tension of nonunion of fractured femurs in the rabbit. Surg Gynecol Obstet 135 (1972) 379-85.	Brighton and Krebs, 1972
C. Lu, M. Rollins, H. Hou, H.M. Swartz, H. Hopf, T. Miclau, and R.S. Marcucio, Tibial fracture decreases oxygen levels at the site of injury. Iowa Orthop J 28 (2008) 14-21.	Lu et al., 2008b
Lu, N. Saless, X. Wang, A. Sinha, S. Decker, G. Kazakia, H. Hou, B. Williams, H.M. Swartz, T.K. Hunt, T. Miclau, and R.S. Marcucio, The role of oxygen during fracture healing. Bone 52 (2013) 220-9.	Lu et al., 2013
EFFECT OF OXYGEN ON FRACTURE HEALING	
R.B. Heppenstall, C.W. Goodwin, and C.T. Brighton, Fracture healing in the presence of chronic hypoxia. J Bone Joint Surg Am 58 (1976) 1153-6.	Heppenstall et al., 1976
Lu, N. Saless, X. Wang, A. Sinha, S. Decker, G. Kazakia, H. Hou, B. Williams, H.M. Swartz, T.K. Hunt, T. Miclau, and R.S. Marcucio, The role of oxygen during fracture healing. Bone 52 (2013) 220-9.	Lu et al., 2013
EFFECT OF OXYGEN ON STEM CELLS	
W.L. Grayson, F. Zhao, B. Bunnell, and T. Ma, Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. Biochem Biophys Res Commun 358 (2007) 948-53.	Grayson et al., 2007
P. Malladi, Y. Xu, M. Chiou, A.J. Giaccia, and M.T. Longaker, Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. Am J Physiol Cell Physiol 290 (2006) C1139-46.	Malladi et al., 2006
L.F. Raheja, D.C. Genetos, A. Wong, and C.E. Yellowley, Hypoxic regulation of mesenchymal stem cell migration: the role of RhoA and HIF-1alpha. Cell Biol Int 35 (2011) 981-9.	Raheja et al., 2011
A. Wong, E. Ghassemi, and C.E. Yellowley, Nestin expression in mesenchymal stromal cells: regulation by hypoxia and osteogenesis. BMC Vet Res 10 (2014) 173.	Wong et al., 2014
C.C. Tsai, T.L. Yew, D.C. Yang, W.H. Huang, and S.C. Hung, Benefits of hypoxic culture on bone marrow multipotent stromal cells. Am J Blood Res 2 (2012) 148-59.	Tsai et al., 2012
EFFECT OF OXYGEN ON OSTEOBLASTS	
D. Wu, J. Malda, R. Crawford, and Y. Xiao, Effects of hyperbaric oxygen on proliferation and differentiation of osteoblasts from human alveolar bone. Connect Tissue Res 48 (2007) 206-13.	Wu et al., 2007
S.M. Warren, D.S. Steinbrech, B.J. Mehrara, P.B. Saadeh, J.A. Greenwald, J.A. Spector, P.J. Bouletreau, and M.T. Longaker, Hypoxia regulates osteoblast gene expression. J Surg Res 99 (2001) 147-55.	Warren et al., 2001
D.S. Steinbrech, B.J. Mehrara, P.B. Saadeh, J.A. Greenwald, J.A. Spector, G.K. Gittes, and M.T. Longaker, Hypoxia increases insulinlike growth factor gene expression in rat osteoblasts. Ann Plast Surg 44 (2000) 529-34; discussion 534-5.	Steinbrech et al., 2000
D.S. Steinbrech, B.J. Mehrara, P.B. Saadeh, G. Chin, M.E. Dudziak, R.P. Gerrets, G.K. Gittes, and M.T. Longaker, Hypoxia regulates VEGF expression and cellular proliferation by osteoblasts *in vitro*. Plast Reconstr Surg 104 (1999) 738-47.	Steinbrech et al., 1999
O.C. Tuncay, D. Ho, and M.K. Barker, Oxygen tension regulates osteoblast function. Am J Orthod Dentofacial Orthop 105 (1994) 457-63	Tuncay et al., 1994
EFFECT OF OXYGEN ON CHONDROCYTES	
E.G. Meyer, C.T. Buckley, S.D. Thorpe, and D.J. Kelly, Low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression. J Biomech 43 (2010) 2516-23.	Meyer et al., 2010
P. Malladi, Y. Xu, M. Chiou, A.J. Giaccia, and M.T. Longaker, Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. Am J Physiol Cell Physiol 290 (2006) C1139-46.	Malladi et al., 2006
M. Hirao, N. Tamai, N. Tsumaki, H. Yoshikawa, and A. Myoui, Oxygen tension regulates chondrocyte differentiation and function during endochondral ossification. J Biol Chem 281 (2006) 31079-92.	Hirao et al., 2006
Y. Xu, P. Malladi, M. Chiou, E. Bekerman, A.J. Giaccia, and M.T. Longaker, *In vitro* expansion of adipose-derived adult stromal cells in hypoxia enhances early chondrogenesis. Tissue Eng 13 (2007) 2981-93.	Xu et al., 2007

2011), which confirms results of earlier studies in large animals (Claes et al., 2002). These observations are in contrast to the prediction that mechanical instability would lead to disruptions to the vasculature supply and reduced oxygen levels, and others have observed decreased vascularity in non-stabilized fractures when observed several weeks post-injury (Lienau et al., 2005).

We also examined localized oxygen levels in order to determine the relationship between oxygen levels at early time points and tissue formation later. Using EPR, we measured tissue oxygen levels prior to and during stem cell differentiation, and then by histology determined the location of the electron paramagnetic particles to relate oxygen levels to tissue formation. Again, we observed an initial decrease in oxygen levels that rebounded quickly; however, we did not observe consistent relationships between levels of oxygen and formation of cartilage or bone. These results suggest that oxygen levels do not direct differentiation of stem cells into chondrocytes and osteoblasts that cartilage comprising the fracture callus is well perfused, which agrees with work showing that the living growth plate in mice is also well perfused (Farnum et al., 2006).

In addition to the descriptive work outlined above, we manipulated oxygen levels experimentally by creating either an ischemic, or altered oxygen environment, and assessing healing. To create an ischemic environment, we removed the femoral artery of mice and observed that healing was significantly affected. We observed delayed healing and decreased bone and cartilage formation in non-stabilized fractures, and delayed bone formation in stabilized fractures. However, we did not observe a shift in the differentiation of stem cells into chondrocytes and osteoblasts (Lu et al., 2007). Since ischemia does not address the role of oxygen in isolation, we altered systemic oxygen levels by placing animals in hypoxic and hyperoxic chambers during the healing period. Chronic hypoxia delayed healing. This has been observed previously (Heppenstall et al., 1976), and is also predicted using computer modeling approaches (OReilly et al., 2016). We did not observe a change in differentiation of osteoblasts and chondrocytes in response to altered oxygen levels. Stabilized fractures healed via intramembranous ossification, and non-stabilized fractures healed primarily by endochondral ossification, independent of the level of inspired oxygen. From these experiments, we concluded that oxygen levels were important for fracture healing generally. Indeed, others have shown that transient hypoxia may actually stimulate fracture repair (Muinos-Lopez et al., 2016).

However, in our work, oxygen was not associated with directing differentiation of osteoblasts and chondrocytes during bone healing.

Other studies have led to different conclusions. Mice that lack thrombospondin-2 (TSP2), an anti-angiogenic matricellular protein, have increased vascularity and an increase in the proportion of bone to cartilage during fracture healing. Similarly, delivery of TSP2 decreases vascularity and decreases the ratio of bone to cartilage during fracture healing (Taylor et al., 2009; Burke et al., 2013). *In silico* modeling studies have also suggested that mechanical stress combined with oxygen levels act together to regulate osteoblast and chondrocyte differentiation (Carlier et al., 2015; OReilly et al., 2016). The reasons for these discrepancies are unknown, but warrant further study. Local oxygen gradients may be more important than the absolute level of tissue oxygenation during fracture healing, as was observed for vascular repair (Knighton et al., 1981). However, assessing oxygen gradients *in vivo* is difficult.

OXYGEN CAN STIMULATE BONE HEALING

Given the importance of the blood supply for bone healing, and the increased risk that vascular injury imposes on fracture healing, developing therapies to overcome ischemic insult may provide novel therapies to stimulate repair. One approach is to treat the ischemia directly by increasing the amount of inspired oxygen in order to increase the local tissue oxygenation levels (Lu et al., 2008b). When animals with fracture and ischemia are housed in hyperoxic chambers, healing was improved compared to normoxic controls (Lu et al., 2013). Furthermore, inspiration of 100% oxygen stimulated healing in animals with a combined lung injury and bone fracture (Kemmler et al., 2015), and hyperbaric oxygen stimulates repair of critical sized defects treated with autologous bone grafts (Grassmann et al., 2015). These observations suggest that increasing inspired oxygen in patients may aid fracture healing, but more work is required to understand safety concerns regarding the potential effect of increased reactive oxygen species (e.g., Gokturk et al., 1995).

Interestingly, hyperoxic animals had significantly more vasculature than animals housed in hypoxic conditions. While this agrees with previous work on the role of hyperoxia and angiogenesis (Hopf et al., 2005; Grassmann et al., 2015), in general, this is contrary to the notion that hypoxia stimulates angiogenesis by stabilizing Hypoxia inducible factor 1α (HIF1α), which increases expression of Vascular Endothelial Growth Factor (VEGF; reviewed in: Fong, 2009). Stabilization of HIF1α leads to increased expression of VEGF during fracture healing (Shen et al., 2009), but this is not necessarily due to hypoxia. Lactate inhibits activity of the prolyl hydroxylases that mark HIF1α for degradation by the proteasome, and lactate is produced by aerobic respiration in wound beds (Ghani et al., 2004; Hunt et al., 2007), including the fracture site (Lu et al., 2013). This aerobically-derived lactate is able to induce angiogenesis by induction of VEGF expression in well-oxygenated environments (Hunt et al., 2007). Hence, while hypoxia is able to induce VEGF expression and angiogenesis (Fong, 2009), the same degree of sustained hypoxia can inhibit angiogenesis (Hopf et al., 2005) due in part to failure of the basement membrane to form around new blood vessel sprouts (Hunt et al., 2007; **Figure 1**).

Modulating angiogenesis using biochemical signals is also a potentially valuable avenue to pursue (Carano and Filvaroff, 2003). Thrombospondins (TSP1 and TSP2) are matricellular proteins that inhibit angiogenesis. Genetic removal of TSP2 leads to increased angiogenesis after injury and rapid tissue healing (Kyriakides et al., 1999; Krady et al., 2008). During healing of ischemic fractures, removal of *TSP2* led to better healing that was accompanied by increased vascularity (Miedel et al., 2013). Similar outcomes have been observed after blocking

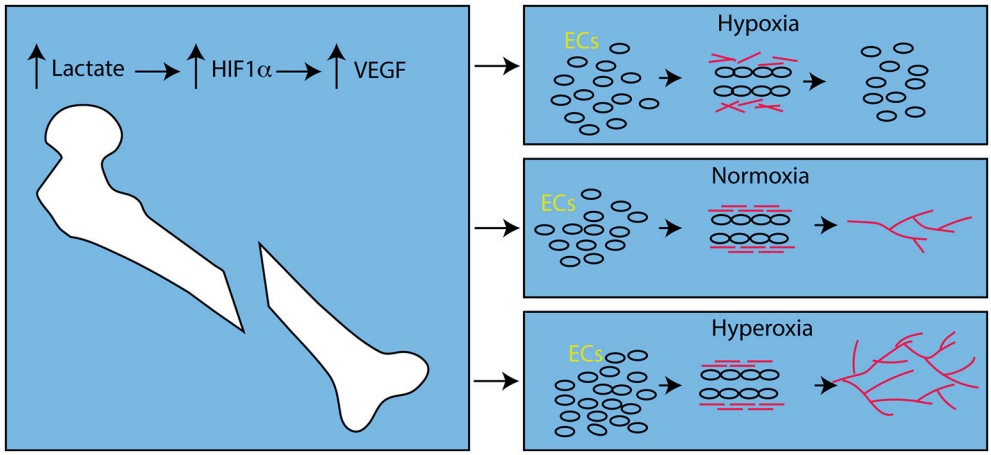

FIGURE 1 | Effect of Oxygen Levels on Angiogenesis after Fracture. After bone fracture, lactate, produced by aerobic metabolism, stabilizes HIF1α and leads to up-regulation of VEGF. In hypoxic conditions endothelial cells (ECs, round circles) respond to VEGF, proliferate and form tubes. However, collagen (red bars) does not assemble and the vascular sprouts disintegrate. In the presence of oxygen, the collagen forms cross-links and the basement membrane stabilizes the newly formed angiogenic sprouts and angiogenesis proceeds (red lines). In hyperoxic conditions this process is amplified and angiogenesis is more robust.

Tsp1 activity (Isenberg et al., 2008) and blocking the TSP receptor, CD47, suggesting that this signaling pathway may be a good target for stimulating repair in patients with concomitant ischemia.

CONCLUSION

Ischemia impairs fracture healing, which contributes to a significant number of complications in fracture patients, and accrues a large societal cost burden. Very few clinical studies evaluating the consequences and underlying mechanisms of prolonged hypoxia and a lack of vascularization on fracture repair have been conducted, but recent preclinical studies have isolated the effects of ischemia on stem cell differentiation and fracture healing. In contrast to opinion, oxygen levels do not appear associated with stem cell differentiation into chondrocytes and osteoblasts, and oxygen is required for robust angiogenesis during fracture healing. However, work by others shows that low oxygen is an important driver of chondrogenesis during fracture healing (Meyer et al., 2010) and may direct chondrogenesis during development (Hirao et al., 2006). These contradictory outcomes suggest that the role of oxygen in stem cell differentiation requires further study. Results from this new work may lead to novel therapies to stimulate fracture healing in patients with ischemia based on oxygen delivery (Yang et al., 2013; Koga et al., 2014) and angiogenic agents. However, further research is needed to determine the effective time-course of oxygen treatment, as well as the specific cellular processes that are affected by oxygen levels.

AUTHOR CONTRIBUTIONS

All authors participated in the discussion and intellectual contribution that was the foundation of the article. KM, SB, and CB wrote the draft manuscript and all of the authors contributed to the revision and production of the final version. Each of the authors approved the final version to be published and agree to be accountable for all aspects of the work.

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Co-Inflammatory Roles of TGFβ1 in the Presence of TNFα Drive a Pro-inflammatory Fate in Mesenchymal Stem Cells

*Shalom Lerrer[1], Yulia Liubomirski[1], Alexander Bott[2], Khalid Abnaof[2], Nino Oren[1], Afsheen Yousaf[2], Cindy Körner[2], Tsipi Meshel[1], Stefan Wiemann[2] and Adit Ben-Baruch[1]**

[1] Faculty of Life Sciences, Department of Cell Research and Immunology, Tel Aviv University, Tel Aviv, Israel,
[2] Division of Molecular Genome Analysis, German Cancer Research Center (DKFZ), Heidelberg, Germany

High plasticity is a hallmark of mesenchymal stem cells (MSCs), and as such, their differentiation and activities may be shaped by factors of their microenvironment. Bones, tumors, and cardiomyopathy are examples of niches and conditions that contain MSCs and are enriched with tumor necrosis factor α (TNFα) and transforming growth factor β1 (TGFβ1). These two cytokines are generally considered as having opposing roles in regulating immunity and inflammation (pro- and anti-inflammatory, respectively). Here, we performed global gene expression analysis of human bone marrow-derived MSCs and identified overlap in half of the transcriptional programs that were modified by TNFα and TGFβ1. The two cytokines elevated the mRNA expression of soluble factors, including mRNAs of pro-inflammatory mediators. Accordingly, the typical pro-inflammatory factor TNFα prominently induced the protein expression levels of the pro-inflammatory mediators CCL2, CXCL8 (IL-8), and cyclooxygenase-2 (Cox-2) in MSCs, through the NF-κB/p65 pathway. In parallel, TGFβ1 did not elevate CXCL8 protein levels and induced the protein expression of CCL2 at much lower levels than TNFα; yet, TGFβ1 readily induced Cox-2 and acted predominantly *via* the Smad3 pathway. Interestingly, combined stimulation of MSCs by TNFα + TGFβ1 led to a cooperative induction of all three inflammatory mediators, indicating that TGFβ1 functioned as a co-inflammatory cytokine in the presence of TNFα. The cooperative activities of TNFα + TGFβ1 that have led to CCL2 and CXCL8 induction were almost exclusively dependent on p65 activation and were not regulated by Smad3 or by the upstream regulator TGFβ-activated kinase 1 (TAK1). In contrast, the TNFα + TGFβ1-induced cooperative elevation in Cox-2 was mostly dependent on Smad3 (demonstrating cooperativity with activated NF-κB) and was partly regulated by TAK1. Studies with MSCs activated by TNFα + TGFβ1 revealed that they release factors that can affect other cells in their microenvironment and induce breast tumor cell elongation, migration, and scattering out of spheroid tumor masses. Thus, our findings demonstrate a TNFα + TGFβ1-driven pro-inflammatory fate in MSCs, identify specific molecular mechanisms involved, and propose that TNFα + TGFβ1-stimulated MSCs influence the tumor niche. These observations suggest key roles for the microenvironment in regulating MSC functions, which in turn may affect different health-related conditions.

Keywords: bone marrow-derived mesenchymal stem cells, NF-κB, pro-inflammatory mediators, Smad3, TGFβ1, TNFα

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Institute for Medical Research, Serbia

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Nemanja Jovicic,
University of Kragujevac, Serbia
Henning Walczak,
Imperial College London, UK

***Correspondence:**
Adit Ben-Baruch
aditbb@tauex.tau.ac.il

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INTRODUCTION

Mesenchymal stem cells (MSCs) are characterized by high plasticity and have critical roles in regulating physiological and pathological processes, in health and disease (1–3). MSC differentiation into different lineages and their versatile activities reflect, among others, their response to microenvironment cues residing at specific niches. Among the signals regulating the migration patterns taken by MSCs and their functional diversification are cytokines, which typically are regarded as key regulators of acquired immunity or inflammation (4, 5). Bone marrow (BM)-derived MSCs share the bone niche with hematopoietic cells and with their products and often encounter immune/inflammatory modulators in remote organs following their migration to these sites (4, 5). The interactions of MSCs with immune and inflammatory cells and with the factors they release may have a strong impact on the way the MSCs then affect their surrounding microenvironment.

While the understanding of MSC regulation by their intimate microenvironments has been improved recently (5–8), much is yet to be revealed. Here, we aimed to unravel the regulation of MSC phenotypes and functions by cytokines that are typically present at MSC niches (4, 5). Particularly, we determined the fate of BM-derived MSCs upon exposure to tumor necrosis factor α (TNFα) and transforming growth factor β1 (TGFβ1) that have been associated with opposing roles in immune and inflammatory activities; these two cytokines are coexpressed in specific niches also harboring MSCs (4, 5, 9, 10).

TNFα is a strong pro-inflammatory cytokine that has key roles in promoting leukocyte recruitment to injured/infected sites through induction of expression of adhesion molecules and of inflammatory chemokines (9, 11–13). The nature of TGFβ1 is more complex: in the presence of IL-1β + IL-6/IL-21/IL-23, TGFβ1 can promote Th17-mediated pro-inflammatory responses; yet, TGFβ1 is mostly identified as a very potent anti-inflammatory and immunosuppressive cytokine, opposing the activities of TNFα, inducing the generation of T regulatory cells, and mediating the anti-inflammatory activities exerted by such cells (10–15).

Despite their general opposing roles in immune regulation, TNFα and TGFβ1 coexist and act simultaneously in specific niches, where their joint activities may also influence the fate of MSCs and their respective functions. One such example is the bone, where macrophage-derived TNFα and TGFβ1 induce the migration of MSCs and their differentiation to osteoblasts, thus promoting bone formation (16, 17). Recent findings suggest that reduced bone-forming activities in MSCs are connected to excessive inflammatory conditions that are ensued with increased age, possibly reflecting changes in the microenvironment and its cytokine contents, which may include TNFα and TGFβ1 (6, 18, 19).

The tumor microenvironment (TME) provides another example for potential coregulation of MSC activities by TNFα and TGFβ1 (20–23). While in pathogen-induced immunity, TGFβ1-mediated suppression may follow TNFα-driven inflammatory processes and shut them off, in malignancy the two processes coexist and eventually they both promote disease progression (20–26). Recent published reports indicate that the pro-tumoral activities of TNFα and TGFβ1 are manifested through their impact on the cancer cells and on cells of the TME, such as MSCs that populate the tumors (27–32). In response to TNFα, MSCs gain a pro-inflammatory phenotype that drives forward the metastatic cascade (20, 21, 28, 33, 34). In parallel, TGFβ1-stimulated MSCs release factors that act directly on tumor cells and promote their invasive properties (31, 32). Moreover, a recent report demonstrated that the tumor-enhancing activities of TNFα-primed adipose tissue-derived MSCs are mediated by TGFβ1, suggesting close interactive relationships between these two seemingly opposing cytokines (27).

These studies have led us to hypothesize that in microenvironments containing both TNFα and TGFβ1, the two cytokines regulate MSC functions through separate/shared mechanisms and that as a result of such molecular effects, the MSCs then affect cells at their intimate surroundings. The results of our current study indeed support this hypothesis. We demonstrate that in the presence of TNFα, TGFβ1 expressed pro-inflammatory activities and that jointly the two cytokines have increased the pro-inflammatory phenotype of BM-derived MSCs more than each cytokine alone. This was evidenced by increased protein levels of CCL2, CXCL8 (IL-8), and cyclooxygenase-2 (Cox-2), which are well identified as strong pro-inflammatory factors (35–39). This cooperativity between TNFα + TGFβ1 reflected channeling of their signals to different molecular paths: activation of NF-κB regulated the induction of CCL2 and CXCL8 while activation of Smad3 played a major role in inducing Cox-2 elevation. Our findings also identified divergent roles for the pathway of TGFβ-activated kinase 1 (TAK1) in regulating TNFα + TGFβ1-induced CCL2/CXCL8, compared to Cox-2-induced expression, in the MSCs. Of note, as a consequence of the joint activities of TNFα + TGFβ1 stimulation, the MSCs released factors that have led to elevated migratory and scattering processes in breast tumor cells.

Taken together, the findings of the current study demonstrate the functional relevance of the microenvironment in shaping the functions of MSCs and provide a proof of concept to the notion that TNFα + TGFβ1-stimulated MSCs affect their surroundings. These findings can contribute to an improved understanding of the way MSCs are regulated by the microenvironment and the way they impact their intimate milieu, demonstrating potential relevance of such events to physiological and pathological conditions in which MSCs are key tissue determinants.

MATERIALS AND METHODS

Origin and Growth of MSCs

Human BM-derived MSCs were purchased from Lonza (Cat# PT-2501; Lonza, Walkersville, MD, USA). The cells were validated as MSCs by Lonza, by marker criteria (positive for CD44, CD29, CD105, and CD166; negative for CD45, CD14, and CD34) and differentiation to adipogenic, chondrogenic,

and osteogenic lineages. MSCs of six different donors were used in the study. The cells were thawed in MSC growth medium (MSCGM; Cat# PT-3001; Lonza) and then were subcultured every 5–7 days, for up to 10 passages, in MSCGM or enriched Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Beit Ha'emek, Israel), including 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin, and 4 mM L-glutamine (all from Biological Industries).

MSC Stimulation

Following overnight incubation in "experimental medium" (DMEM containing the above-mentioned supplements without FBS, or with 0.5% FBS, as appropriate for experimental conditions; see figure legends), MSCs were stimulated with TNFα (50 ng/ml; Cat# 300-01; PeproTech, Rocky Hill, NJ, USA) and/or TGFβ1 (10 ng/ml; Cat# 100-21; PeproTech). TNFα concentration was selected based on our previous studies (40–43), as well as other cell systems (e.g., Ref. 44–46). TGFβ1 concentration was selected based on a literature search (47–49) and preliminary titration analysis (data not shown). In all procedures, control non-stimulated cells were treated with the diluents of the cytokines (= vehicle control). In array experiments, MSCs were stimulated with TNFα or TGFβ1 for 1, 3, 7, 14, and 24 h. In signaling experiments, the stimulation time was 10 min, and in functional assays it was 24 h.

Illumina Beadchip Array Analyses

Processing and Normalization

Following MSC stimulation by the cytokines (as described above), total-RNA of frozen cell pellets was isolated using miRNeasy Mini kit (Cat# 217004; Qiagen, Hilden, Germany) according to manufacturer's protocols. The quality of total RNA was checked by gel analysis using the total RNA Nano chip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Genome-Wide Gene Expression Profiling

This step was performed using HumanHT-12 v4 BeadChips (Illumina, San Diego, CA, USA) in the Genomics and Proteomics Core Facility at the German Cancer Research Center (DKFZ), Heidelberg, Germany. Hybridization was performed at 58°C, in GEX-HCB buffer (Illumina Inc.) at a concentration of 100 ng cRNA/µl, unsealed in a wet chamber for 20 h. Spike-in controls for low-, medium-, and highly abundant RNAs were added, as well as mismatch control and biotinylation control oligonucleotides. Raw probe intensities were extracted, background-corrected, normalized, and summarized to expression levels using the variance stabilization normalization method (50). The complete dataset was deposited at ArrayExpress (51, 52) (accession numbers E-MTAB-5421 and E-MTAB-5420).

Gene Ontology (GO) Analysis

All differentially expressed genes were scanned at each time point for enrichment in the gene sets of the GO project terms (53). Enrichment analysis was performed for the different time points individually by taking the negative value of the logarithm of uncorrected *p*-values [−log (*p*-value)] as ranking scores for the transcript. Gene sets of GO terms were then tested for their association with these ranking scores *via* a univariate logistic regression-based method as described in the studies of Sartor et al. (54) and Montaner and Dopazo (55). Resulting *p*-values of GO terms were then adjusted according to Benjamini–Yekutieli's method for false discovery rate (FDR) control under dependency (56). Significant GO terms are reported at a cutoff value of FDR ≤ 0.001 in **Figure 1** and at a cutoff value of FDR ≤ 0.01 in Table S1 in Supplementary Material.

Differential Gene Expression Analyses

These analyses were performed *via* the "Limma" method (57, 58) that uses linear models and empirical Bayes. At 1, 3, 7, 14, and 24 h after stimulation (TNFα or TGFβ1), sample sets of each stimulation were compared to their counterpart vehicle-treated control cells (0 and 24 h). Statistical dependencies of samples within time points and replicates were considered *via* a factorial design matrix in "Limma". Corrections for multiple testing were performed using Benjamini–Hochberg's method (59), and significant differentially expressed genes were reported at a cutoff value of FDR ≤ 0.005 and absolute $\log_2$ fold change ≥ 1.5 (= fold change ≥ 2.8).

Quantitative Real-time Polymerase Chain Reaction (qPCR)

Following global profiling, the upregulated expression of mRNAs was validated by qPCR analysis, at the 3–14-h range, following MSC stimulation. Two procedures were used: (1) quantification of PTGS2, CX3CL1, EPSTI1, ANGPTL4, PTHLH, and PLAU expression levels: total RNA was isolated using the EZ-RNA kit (Cat# 20-400; Biological Industries). RNA samples were used for generation of first-strand complementary DNA synthesis using the M-MLV reverse transcriptase (Cat# AM2044; Ambion, Austin, TX, USA). Quantification of cDNA targets by qPCR was performed on Rotor Gene 6000 (Corbett Life Science, Concorde, NSW, Australia). Transcripts were detected using Absolute Blue qPCR SYBR Green ROX mix (Cat# AB-4163/A; Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. The sequences of the primers are listed in Table S2A in Supplementary Material. In each reaction, two pairs of specific primers were used, which had been designed to span different exons. Data were normalized to the housekeeping gene RPS9. Dissociation curves for each primer set indicated a single product after the 40 cycles used for analysis (except for CX3CL1: 50 cycles), and no-template controls were negative. Quantification was performed by standard curves, within the linear range of quantification. (2) Quantification of CCL2, CXCL8, NGF, IL6, LIF, HBEGF, CSF2, MMP1, MMP3, VEGFC, FGF1, and IL12A expression levels: mRNAs were isolated using miRNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. cDNA synthesis was performed with Revert Aid H Minus first Strand cDNA Synthesis Kit (Thermo Fisher Scientific), and qPCR amplifications of specific

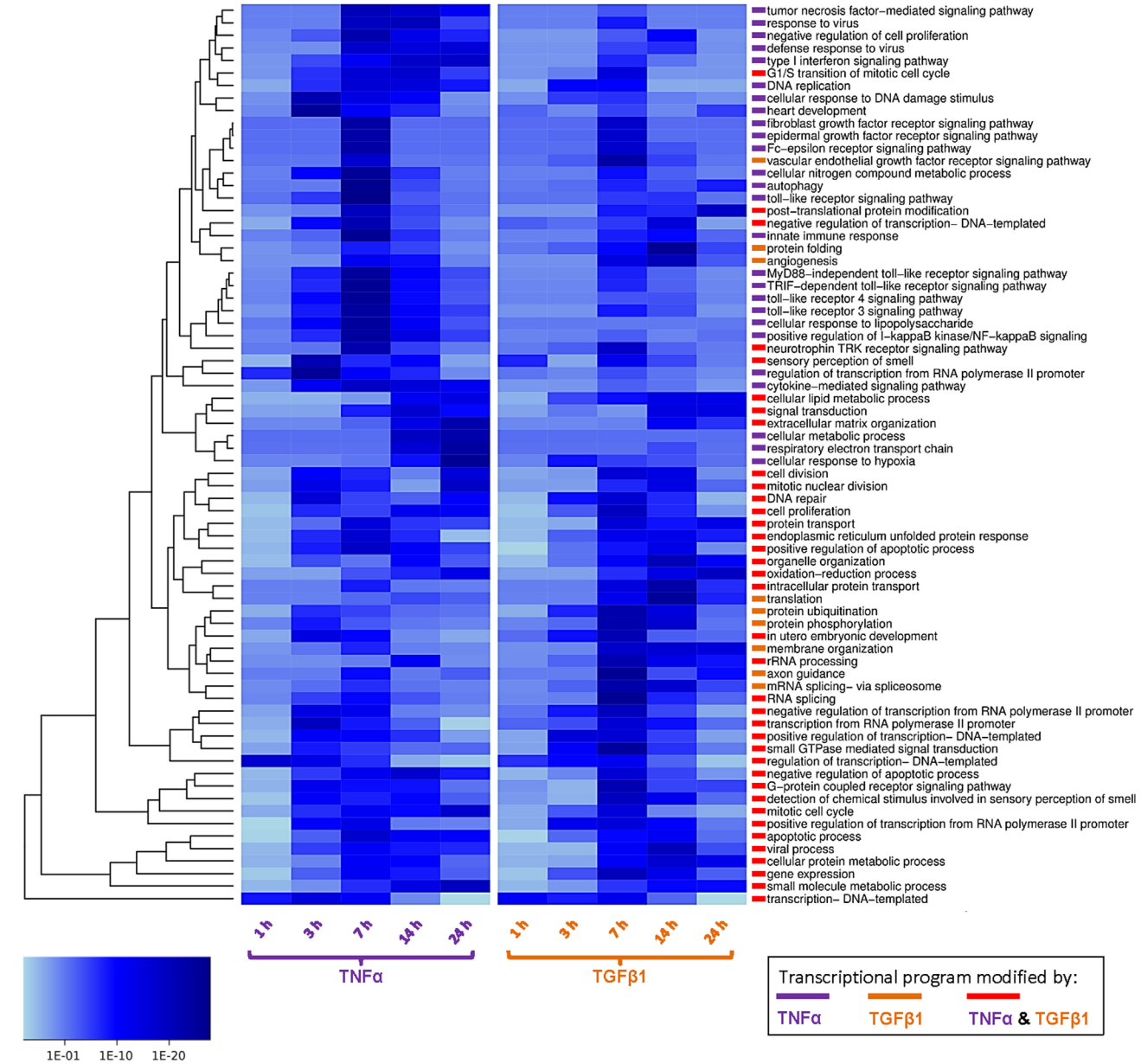

FIGURE 1 | **Continued**

FIGURE 1 | Continued
TNFα and TGFβ1 modify private and shared transcriptional programs in MSCs. Human BM-derived MSCs of Donor #1 were stimulated by TNFα (50 ng/ml) or TGFβ1 (10 ng/ml) or treated by a vehicle control, as illustrated in the experimental design of Figure S1A in Supplementary Material (cytokine concentrations were selected as described in Section "Materials and Methods"; experiment performed in FBS-free medium). RNA was subjected to Illumina Beadchip array analyses, and the complete dataset was deposited at ArrayExpress [(51, 52); accession numbers E-MTAB-5421 and E-MTAB-5420]. **(A)** The figure presents the transcriptional programs modified in MSCs at different time points following their stimulation by TNFα or TGFβ1. Data are presented by *p*-value scaling ($p \leq 0.001$ after Benjamini–Yekutieli correction for multiple testing). All the transcriptional programs that are demonstrated include ≥10 genes. **(B)** Venn diagram showing the number of transcriptional programs significantly affected only by TNFα, only by TGFβ1, or by both cytokines.

genes were performed in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Probes from Universal Probe Library (UPL; Roche Diagnostics GmbH, Mannheim, Germany) were used to increase primer specificity. Analysis was performed by using $2^{-\Delta\Delta CT}$. The sequences of the primers and the UPL probes used are listed in Table S2B in Supplementary Material. Data were normalized to the housekeeping genes GAPDH and HPRT.

Western Blotting

Following MSC stimulation by the cytokines (as described above), the cells were lysed in RIPA lysis buffer and conventional Western blot (WB) procedures were performed, using antibodies (Abs) directed against the following proteins: phosphorylated (P)-p65 [Cat# 3033; Cell Signaling Technology (CST), Danvers, MA, USA]; total (T)-p65 (Cat# 4764 or Cat# 8242; CST); IκBα (Cat# 4814; CST); P-Smad3 (Cat# 9520; CST); T-Smad3 (Cat# 9523; CST); T-TAK1 (Cat# 4505; CST); Cox-2 (Cat# PA1725; Boster Immunoleader, Pleasanton, CA, USA); Abs directed against GAPDH (Cat# ab9485; Abcam, Cambridge, UK); or β-tubulin (Cat# ab6046; Abcam) served for loading controls. Then, membranes were reacted with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG, as appropriate (Cat# 111-035-003; Cat# 115-035-071, respectively; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), subjected to enhanced chemiluminescence (Cat# 20-500; Biological Industries), and visualized using Kodak Medical X-RAY processor (Carestream Health, Rochester, NY, USA).

ELISA Assays

Following MSC stimulation by the cytokines (as described above), cell conditioned media (CM) were collected and cleared by centrifugation. Extracellular expression levels of CCL2 and CXCL8 in CM were determined by ELISA, using standard curves at the linear range of absorbance with recombinant human (rh) CCL2 and CXCL8 (Cat# 300-04 and #200-08M, respectively; PeproTech). The following Abs were used (all from PeproTech): for CCL2: coating mouse monoclonal Abs (Cat# 500-M71); detecting biotinylated rabbit polyclonal Abs (Cat# 500-P34Bt). For CXCL8: coating rabbit polyclonal Abs (Cat# 500-P28); detecting biotinylated rabbit polyclonal Abs (Cat# 500-P28Bt). After the addition of HRP (Cat# 016-030-084; Jackson ImmunoResearch Laboratories), the substrate TMB/E solution (Cat# ES001; Millipore, Temecula, CA, USA) was added, the reaction was stopped by addition of 0.18 M H_2SO_4, and absorbance was measured at 450 nm.

Analysis of METABRIC and TCGA Patient Datasets

The correlation between expression levels of CCL2, CXCL8 and PTGS2 (Cox-2) in patients expressing high/low levels of TNFα + TGFβ1 was performed using gene expression data from the RNA-Seq-based TCGA dataset, including data from 1,215 breast cancer patients (60). Here, patients were divided into quartiles based on the expression levels of TNFα and of TGFβ1. Patients were individually assigned to low expression (i.e., lower quartile) or high expression (upper quartile) of each cytokine. Expression of the target genes in patients exhibiting high expression of both cytokines, low expression of both cytokines, or high expression of one and low expression of the other was illustrated in box plots. Patients belonging to the second and third quartiles for one of the cytokines were not considered in this analysis. Patient numbers in the different groups were as follows: TNFα-high + TGFβ1-high = 85 patients; TNFα-high + TGFβ1-low = 33 patients; TNFα-low + TGFβ1-high = 48 patients; and TNFα-low + TGFβ1-low = 105 patients. Statistical analysis was performed with two-tailed Mann–Whitney test.

The TCGA dataset was used also to determine the associations between the expression levels of CCL2, CXCL8 and PTGS2 (Cox-2) in the patient cohort. A similar analysis was performed with the METABRIC dataset (61), including data from 1,992 breast cancer patients. The following probes were used: CCL2 ILMN_1720048; CXCL8 ILMN_2184373; and PTGS2 (Cox-2) ILMN_2054297. In both datasets, $\log_2$-transformed expression values were outlined as scatter plots. Correlation coefficients and *p*-values were analyzed using Spearman correlation.

Transfection of siRNAs in MSCs

Transient siRNA transfections were performed using the Lipofectamine RNAiMAX transfection reagent (Cat# 56531; Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions, with the following siRNAs (all from Dharmacon, Lafayette, CO, USA): p65 siRNA pool (Cat# L-003533-00); Smad3 siRNA pool (Cat# L-020067-00); TAK1 siRNA pool (Cat# L-003790-00); and non-targeting control siRNA pool (Cat# D-001810-10). After 16 h, the medium was replaced with experimental medium (described above) for additional 24–48 h, and the cells were then stimulated by the cytokines as described above.

Breast Tumor Cell Cultures

The human breast tumor cell lines MDA-MB-231 (HTB-26™) and MCF-7 (HTB-22™) were obtained from ATCC (Manassas, VA, USA) and grown in enriched DMEM. To generate mCherry-expressing MDA-MB-231 and MCF-7 cells, two rounds of retroviral infections were performed as previously described (42), with minor technical adaptations. Seventy-two hours following the second infection, infected cells were selected with 1 μg/ml (MDA-MB-231) or 4 μg/ml (MCF-7) of puromycin (Cat# P-1033; A.G. Scientific, San Diego, CA, USA) for 7 days.

Stimulation of Breast Tumor Cells with MSC-Derived CM: Morphology and Migration Assays

mCherry-expressing breast tumor cells were plated in enriched DMEM medium for 24 h, then the medium was replaced by the following: (1) control medium; (2) medium containing TNFα + TGFβ1 at the same concentrations used for MSC stimulation (as above); (3) CM derived from vehicle-stimulated MSCs; and (4) CM derived from TNFα + TGFβ1-stimulated MSCs. The media of Groups 1 and 2 were kept in the same conditions as MSC-derived CM of Groups 3 and 4. All media were filtered through a 0.45 μm membrane prior to addition to the tumor cells. Following addition of media from Groups 1–4 to MDA-MB-231 cells (for 48–72 h) and to MCF-7 cells (for 48 h), morphology was determined by fluorescent microscopy. Transwell migration of MCF-7 cells was performed in inserts with 8 μm pore size (Cat# 3422; Corning, Cambridge, MA, USA), in which the upper compartment of the inserts was precoated with fibronectin (20 μg/ml, diluted in serum-free DMEM; Cat# 03-090-1; Biological Industries) for 1 h at 37°C. The inserts were placed in new wells, containing DMEM supplemented with 10% FBS in the lower compartment. A total of 1×10^5 viable MCF-7 cells (pretreated for 48 h with the different MSC-derived CM or the respective control media, as described above) were added to the upper compartment of the inserts in serum-free DMEM. Following 21–22 h of incubation, the cells on the upper surface of the insert were removed, and the filters were fixed in ice-cold methanol and stained with Hemacolor (Cat# 1.11661; Merck, Darmstadt, Germany). Migrating cells were photographed at ×40 magnification and counted. Data are presented as number of cells in five fields that cover most of the insert.

Stimulation of Breast Tumor Cells with MSC-Derived CM: Tumor Spheroid Assays

Six-well plates were incubated overnight on a rocker with 1.2% poly(2-hydroxyethyl methacrylate) (Cat# P3932; Sigma) diluted in ethanol. mCherry-expressing MCF-7 cells were plated in the coated wells, in DMEM/F12 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin (all from Biological Industries), 0.4% BSA (Cat# 0332-TAM; Amresco, Solon, OH, USA), B-27 serum-free supplement (Cat# 17504044; Gibco, Life technologies, Grand island, NY, USA), 20 ng/ml rh-basic FGF (Cat# 100-18B; PeproTech), 20 ng/ml rh-EGF (Cat# 236-EG; R&D systems, Minneapolis, MN, USA), and 5 μg/ml insulin (Cat# I9278; Sigma). After 72 h, tumor spheroids were collected, centrifuged (1,200 rpm for 7 min, + 4°C) and resuspended in the different MSC-derived CM or the respective control media (as described above). Tumor spheroids were photographed daily using fluorescent microscopy.

Data Presentation and Statistical Analyses

The statistical analyses of mRNA arrays and METABRIC/TCGA analyses were described in their respective sections. Other *in vitro* experiments were performed in $n \geq 3$ independent experimental repeats, with MSCs from ≥ 2 different donors, as indicated in respective figure legends. Data of TNFα + TGFβ1-induced functional assays with siRNAs are presented in two biological replicates, one in the main body of the manuscript and one in Supplementary Material. The results of ELISA and migration assays were compared by two-tailed unpaired Student's *t*-test. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

TNFα and TGFβ1 Induce Different, yet Partly Overlapping Transcriptome Signatures in MSCs

To determine the impact of TNFα and TGFβ1 on BM-derived MSCs, we performed genome-wide expression analysis, in which we identified mRNAs that were upregulated or downregulated in response to each of the two cytokines. To this end, MSCs of Donor #1 were exposed to TNFα (50 ng/ml) or TGFβ1 (10 ng/ml) for 1, 3, 7, 14, and 24 h or to vehicle control (0 and 24 h), in two biological replicates (Figure S1A in Supplementary Material). Following Illumina Beadchip analysis, unsupervised clustering of total-mRNA expression proved high reproducibility between replicates (Figure S1B in Supplementary Material). The analyses demonstrated that: (1) all four vehicle samples (two of time "0 h" and two of time "24 h") were clustered together indicating that no changes had occurred in unperturbed conditions; (2) the 1-h samples of each of the cytokines clustered outside of the vehicle samples, indicating that differential regulation of genes is evident already at this early time point; and (3) each cytokine induced, in a kinetics-dependent manner, a private transcriptional program.

Then, GO enrichment analysis was performed, identifying transcriptional programs that were modified at the different time points by TNFα or TGFβ1. In this analysis, we focused on biological processes that include 10 or more genes ($n \geq 10$), with significance of $p \leq 0.001$ (compared to vehicle-stimulated cells; **Figure 1A**). TNFα stimulation modified the expression of 63 programs and TGFβ1 of 46 programs (**Figures 1A,B**). Altogether, we identified 72 programs that were modified by the two cytokines, with 37 programs overlapping between TNFα and TGFβ1 (51%; **Figures 1A,B**). Some of the processes that were modified by both cytokines reflected the potential impact of TNFα- and TGFβ1-stimulated MSCs on their microenvironment, including pathways such as oxidation–reduction processes, angiogenesis, and extracellular matrix organization (Table S1 in Supplementary Material).

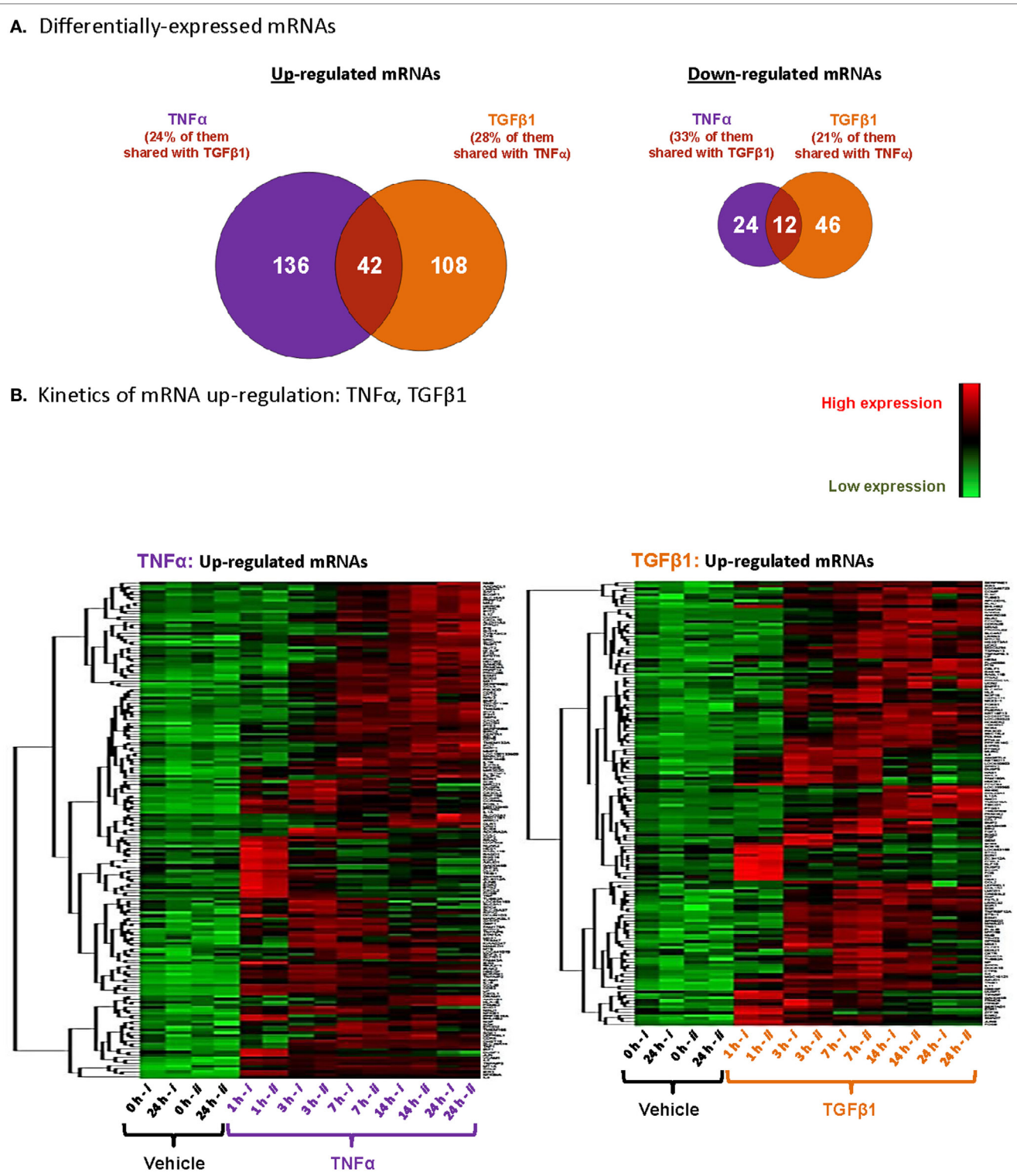

FIGURE 2 | TNFα and TGFβ1 induce private and shared modifications of mRNA expression in MSCs, in a time-dependent manner. Based on the array analyses described in **Figure 1** with MSCs of Donor #1, total numbers of upregulated and downregulated mRNAs were determined. **(A)** Venn diagram, demonstrating the patterns of gene regulation in MSCs stimulated by TNFα or TGFβ1. The figure includes mRNAs modified by $\log_2$ fold change ≥ 1.5 (= fold change ≥ 2.8) with $p \leq 0.005$ after Benjamini–Hochberg correction for multiple testing, compared to vehicle-treated cells. A specific gene was considered as up- or downregulated if it has passed these cutoffs at one of the time points included in the analyses. **(B)** Visualization of upregulated mRNAs after unsupervised clustering, using the same criteria as in panel **(A)**. Each column represents a single replicate of each specific treatment (i, ii), and each row demonstrates a single upregulated mRNA.

Analysis of TNFα- or TGFβ1-deregulated mRNAs (cutoff: $\log_2$ fold change ≥ 1.5 = fold change ≥ 2.8; $p \leq 0.005$) had identified a total of 178 mRNAs that were upregulated and 36 mRNAs that were downregulated following TNFα stimulation (in at least one of the time points; **Figure 2A**). After TGFβ1 stimulation, 150 mRNAs were upregulated and 58 mRNAs were downregulated (**Figure 2A**). Interestingly, a substantial proportion of the upregulated mRNAs—24% of those affected by TNFα and 28% of those affected by TGFβ1—were shared between the two cytokines. Shared downregulated mRNAs were also identified: 33% of TNFα-regulated genes and 21% of TGFβ1-regulated genes.

Furthermore, kinetics analysis demonstrated that major time-dependent alterations in transcriptional programs were induced after 3–14 h of stimulation by TNFα or TGFβ1 (**Figure 2B**). We selected 18 upregulated mRNAs to follow up on (**Figure 3**), focusing on secreted factors and pro-inflammatory mediators that can potentially impact the microenvironment of MSCs. Of these 18 mRNAs, six were induced only by TNFα, five were induced only by TGFβ1, and seven were induced by both cytokines. Increased mRNA expression of these 18 genes was validated by qPCR in MSCs from two additional donors—Donors #2 and #3 (**Figure 3**)—thus confirming the original array findings obtained in MSCs of Donor #1 (described above).

To add clinical relevance to target selection, we analyzed the TCGA and METABRIC datasets of breast cancer patients, which included data from 1,215 patients and 1,992 patients, respectively. First, the TCGA dataset demonstrated that the expression of three pro-inflammatory genes, which are well established as tumor-promoting factors in breast cancer (37–39, 62–64)—CCL2, CXCL8 and PTGS2 that codes for Cox-2—was significantly elevated in breast tumors that expressed high levels of both TNFα and TGFβ1 together [**Figure 4A**; similar analyses could not be performed with the METABRIC dataset because TNFα and TGFβ1 (mostly the latter) were not properly detected in the original array that generated the dataset]. Moreover, in both the TCGA and the METABRIC datasets, the expression levels of CCL2, CXCL8 and PTGS2 were highly coregulated with each other in patient breast tumors (TCGA: **Figure 4B**; METABRIC: Figure S2 in Supplementary Material). Together, these findings suggest that tumors containing high levels of TNFα and TGFβ1 are enriched with the inflammatory and tumor-promoting mediators CCL2, CXCL8 and Cox-2, and that all three inflammatory mediators are coregulated in human breast tumors. Thus, our findings propose that since TNFα and TGFβ1 often coreside at the breast TME (20–23), their joint expression in tumors may induce the expression of CCL2, CXCL8 and Cox-2 in intratumoral MSCs.

The above observations, demonstrating that TNFα and TGFβ1 stimulation induced elevated CCL2, CXCL8 and Cox-2 expression in MSCs, and connecting the expression of these pro-malignancy mediators with high expression of TNFα and TGFβ1

			TNFα-induced fold change in:			TGFβ1-induced fold change in:		
	Gene	Time point	mRNA-array Donor #1	qPCR Donor #2	qPCR Donor #3	mRNA-array Donor #1	qPCR Donor #2	qPCR Donor #3
Induced in mRNA-array by both TNFα and TGFβ1	CCL2	7 h	18.1	56.7	23.1	7.0	3.2	2.3
	CXCL8	7 h	194.1	1363.5	1998.2	6.6	1.1	2.2
	PTGS2 (Cox-2)	3 h	5.2	4.1	25.8	7.2	5.6	32.0
	NGF	7 h	2.7	3.8	6.1	3.0	2.6	1.7
	IL6	7 h	8.9	9.2	8.8	10.0	3.8	2.9
	LIF	7 h	7.3	8.4	6.5	12.3	7.3	4.4
	HBEGF	7 h	8.0	1.6	2.2	24.9	9.3	9.0
Induced in mRNA-array only by TNFα	CX3CL1	3 h	10.6	163.7	127.9	0.94	0.41	0.63
	CSF2 (GM-CSF)	7 h	4.6	68.4	382.7	0.98	0.57	0.80
	MMP1	14 h	3.8	143.4	31.3	0.95	1.6	0.73
	MMP3	14 h	6.3	38.7	23.1	1.1	1.2	1.1
	VEGFC*	14 h	2.6	2.9	3.1	0.47	0.94	0.96
	EPSTI1	14 h	3.9	5.1	3.2	0.37	0.33	0.89
Induced in mRNA-array only by TGFβ1	ANGPTL4	3 h	3.9 (NS)	1.4	3.2	7.6	3.2	4.6
	FGF1 (aFGF)	7 h	1.2	0.45	0.58	3.9	2.2	4.3
	PTHLH (PTHrP)	14 h	1.3	0.99	0.64	4.3	10.4	26.9
	PLAU (uPA)	14 h	0.50	0.16	0.13	7.3	3.6	8.8
	IL12A	14 h	1.6	2.7	2.1	2.5	5.7	7.1

FIGURE 3 | qPCR validations of TNFα- or TGFβ1-upregulated mRNAs. Following MSC stimulation by TNFα or TGFβ1 and the global gene analysis described in previous figures, upregulation of 18 selected mRNAs was validated. All 18 mRNAs were modified in the original array analysis by $\log_2$ fold change ≥ 1.5 (= fold change ≥ 2.8; * = fold change 2.6) with $p \leq 0.005$ (after Benjamini–Hochberg correction for multiple testing, compared to vehicle-treated cells). The qPCR validations were performed with MSCs of two different donors (Donor #2 and Donor #3). The 18 selected mRNAs were validated by qPCR at time points in which they have undergone significant changes in the array analysis.

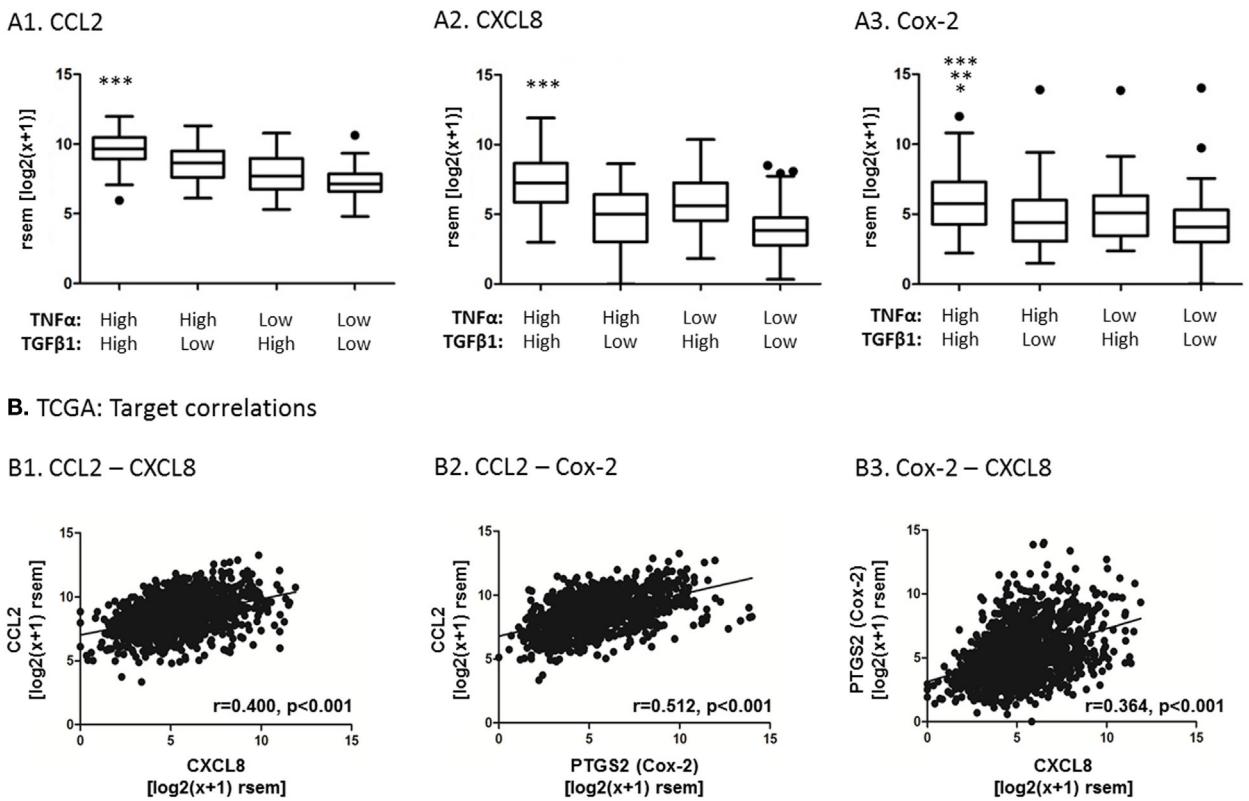

FIGURE 4 | **Analyses of human breast tumors demonstrate coordinated expression of CCL2, CXCL8 and Cox-2, and higher levels of their expression in tumors enriched with both TNFα and TGFβ1**. Patient data analysis was performed with the TCGA dataset of human breast cancer, including 1,215 patient samples. **(A)** Associations between CCL2 (A1), CXCL8 (A2), Cox-2 (A3) expression and expression levels of TNFα and TGFβ1 in the tumors, performed by quartile analysis, as described in Section "Materials and Methods". The black dots above the plots are outliers that are outside of the whiskers. Statistical analysis was performed by two-tailed Mann–Whitney test. In panel A1, $^{***}p < 0.001$ for CCL2 expression levels in the TNFα-high + TGFβ1-high patient group, compared to all other patient groups. In panel A2, $^{***}p < 0.001$ for CXCL8 expression levels in the TNFα-high + TGFβ1-high patient group, compared to all other patient groups. In panel A3, $^{***}p < 0.001$ for Cox-2 expression levels in the TNFα-high + TGFβ1-high patient group, compared to the TNFα-low + TGFβ1-low group, $^{**}p < 0.01$ compared to the TNFα-high + TGFβ1-low group and $^{*}p < 0.05$ compared to the TNFα-low + TGFβ1-high group. **(B)** Associations between CCL2, CXCL8 and Cox-2 expression levels in the patient cohort. The correlation coefficient (*r*) and statistical significance between two mRNAs in each graph were determined by Spearman correlation analysis. rsem, RNA-Seq by Expectation Maximization. Similar analysis performed with the METABRIC dataset of patient samples is demonstrated in Figure S2 in Supplementary Material.

in patient samples, have led us to determine the impact of TNFα and TGFβ1 on the protein expression levels of CCL2, CXCL8 and Cox-2 in the MSCs. In line with its strong pro-inflammatory nature, TNFα has potently induced the expression of CCL2 and CXCL8 at the protein level [as we had demonstrated before (41)] and of Cox-2 as well, in the MSCs (**Figure 5**). TGFβ1 upregulated the mRNA expression of CCL2 in the MSCs but to lower extent than TNFα (**Figure 3**) and has promoted CCL2 expression to only a small extent at the protein level (**Figure 5A1**; Figure S3 in Supplementary Material), as could be expected from a cytokine which is not a typical pro-inflammatory mediator. The relatively minor induction of CXCL8 mRNA by TGFβ1—compared to TNFα—did not come into effect at the protein level (**Figure 5A2**). Yet, TGFβ1 potently induced the protein expression of Cox-2, even to stronger extent than TNFα (**Figure 5A3**), revealing a potential pro-inflammatory activity for this cytokine.

As TNFα and TGFβ1 are both expressed in MSC-containing niches (as alluded in Section "Introduction"), we next asked what will be the impact of joint stimulation by both TNFα and TGFβ1 together on the MSCs. The findings shown in **Figures 5A1–A3** demonstrate cooperativity between the two cytokines, leading to greater effect than their individual impacts. Most importantly, such cooperative effects were evident not only for Cox-2 that was strongly induced by TGFβ1 but also for CCL2 and CXCL8, on which TGFβ1 had a weak or no effect when it was administered alone. These findings indicate that in the presence of TNFα, TGFβ1 acts as a "co-inflammatory" factor that promotes the activities of the classical pro-inflammatory cytokine TNFα. Together, the joint activities of TNFα and TGFβ1 induced a pro-inflammatory phenotype in BM-derived MSCs, demonstrating that factors of the microenvironment can have a strong impact on the fate of MSCs and on the secreted factors they produce.

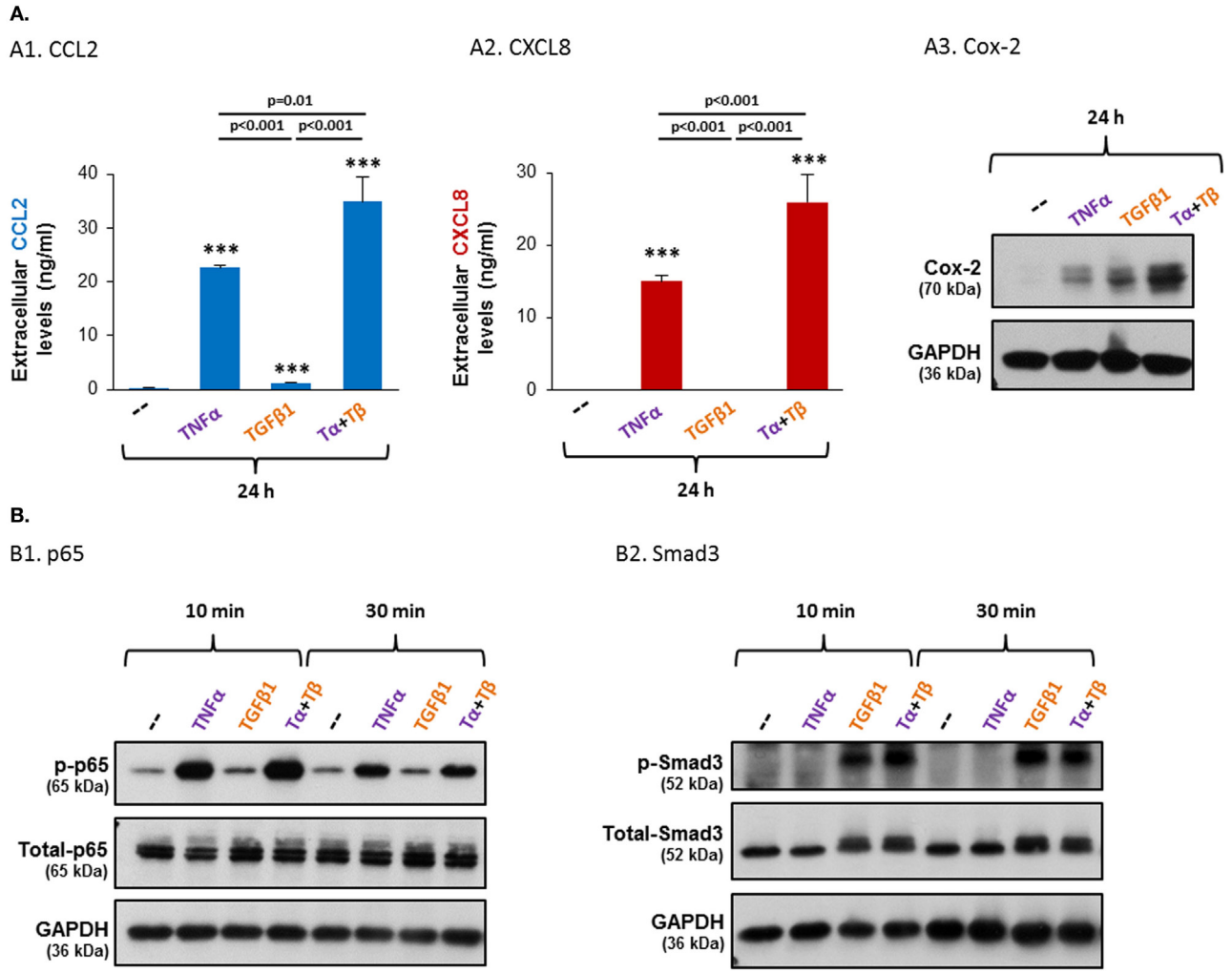

FIGURE 5 | TNFα and TGFβ1 cooperate in inducing the protein expression levels of CCL2, CXCL8 and Cox-2, and induce the activation of the NF-κB and Smad3 transcription factors, respectively, in MSCs. Human BM-derived MSCs were stimulated with vehicle ("--"), TNFα (50 ng/ml), TGFβ1 (10 ng/ml), or TNFα + TGFβ1 (same concentrations). Tα + Tβ = TNFα + TGFβ1. **(A)** Protein expression levels of CCL2, CXCL8 and Cox-2. Following 24 h of stimulation (FBS-free medium for CCL2/CXCL8 and 0.5% FBS-containing medium for Cox-2) the extracellular expression of CCL2 [(A1); see also Figure S3 in Supplementary Material] and CXCL8 (A2) was determined by ELISA in cell supernatants, in the linear range of absorbance. ***$p \leq 0.001$ compared to vehicle-treated cells. (A3) Cox-2 expression levels were determined by WB; GAPDH was used as a loading control. **(B)** Following 10 and 30 min of stimulation (FBS-free medium), the phosphorylation levels of p65 (B1) and Smad3 (B2) were determined by WB; GAPDH was used as a loading control. In all panels, the findings are representatives of $n \geq 3$ independent experiments, performed with MSCs of two to three different donors, which have shown similar results.

The Cooperative Induction of CCL2/CXCL8 by Joint TNFα + TGFβ1 Stimulation Is Differently Regulated by NF-κB and Smad3 than the Cooperative Induction of Cox-2

To identify the molecular mechanisms regulating the joint activities of TNFα + TGFβ1 on MSCs, we analyzed the canonical transcription factors activated by the two cytokines: (1) TNFα activates the NF-κB/p65 pathway (65, 66), which was found in our published findings to regulate TNFα-induced elevation of CCL2 and CXCL8 expression in BM-MSCs (41). The other canonical pathway activated by TNFα, of AP-1, was demonstrated in our past study to be irrelevant in this context (41). (2) The canonical Smad3 pathway that is activated by TGFβ1 (67–69) was investigated in parallel to NF-κB.

Activation analyses indicated that TNFα + TGFβ1 stimulation of MSCs induced prominent phosphorylation of p65 and Smad3 (**Figure 5B**). p65 was activated by TNFα + TGFβ1 to the same extent as by TNFα alone, and the activation level of Smad3 following TNFα + TGFβ1 stimulation was similar to its activation by TGFβ1 alone. These findings suggested that in the combined TNFα + TGFβ1 stimulation each cytokine activated its respective canonical pathway—TNFα activated p65 and TGFβ1 activated Smad3—and that the functional cooperativity between TNFα + TGFβ1 was due to cooperative

activities of p65 and Smad3. Yet, our findings using p65 siRNA and Smad3 siRNA revealed a more complex mode of regulation of TNFα + TGFβ1 activities in the MSCs, as demonstrated further below.

To determine the roles of NF-κB and Smad3 in TNFα- or TGFβ1-induced increases in CCL2/CXCL8 and Cox-2 we used siRNAs to p65 (NF-κB) and Smad3 (efficacies of p65 and Smad3 downregulation are demonstrated in Figures S4A,B in Supplementary Material, respectively). Under these conditions, the induction of CCL2, CXCL8 and Cox-2 by TNFα alone was markedly dependent on p65 activities (**Figure 6A**). In parallel, when TGFβ1 acted alone, it promoted the expression of CCL2 and of Cox-2 in a Smad3-dependent mechanism (**Figure 6B**; CXCL8 was not investigated because it was not affected by TGFβ1 at the protein level, as demonstrated in **Figure 5A2**).

To test pathway-specificity of TNFα + TGFβ1 effects, we knocked-down both p65 and Smad3 together and then stimulated the MSCs with TNFα + TGFβ1 (efficacies of p65 and

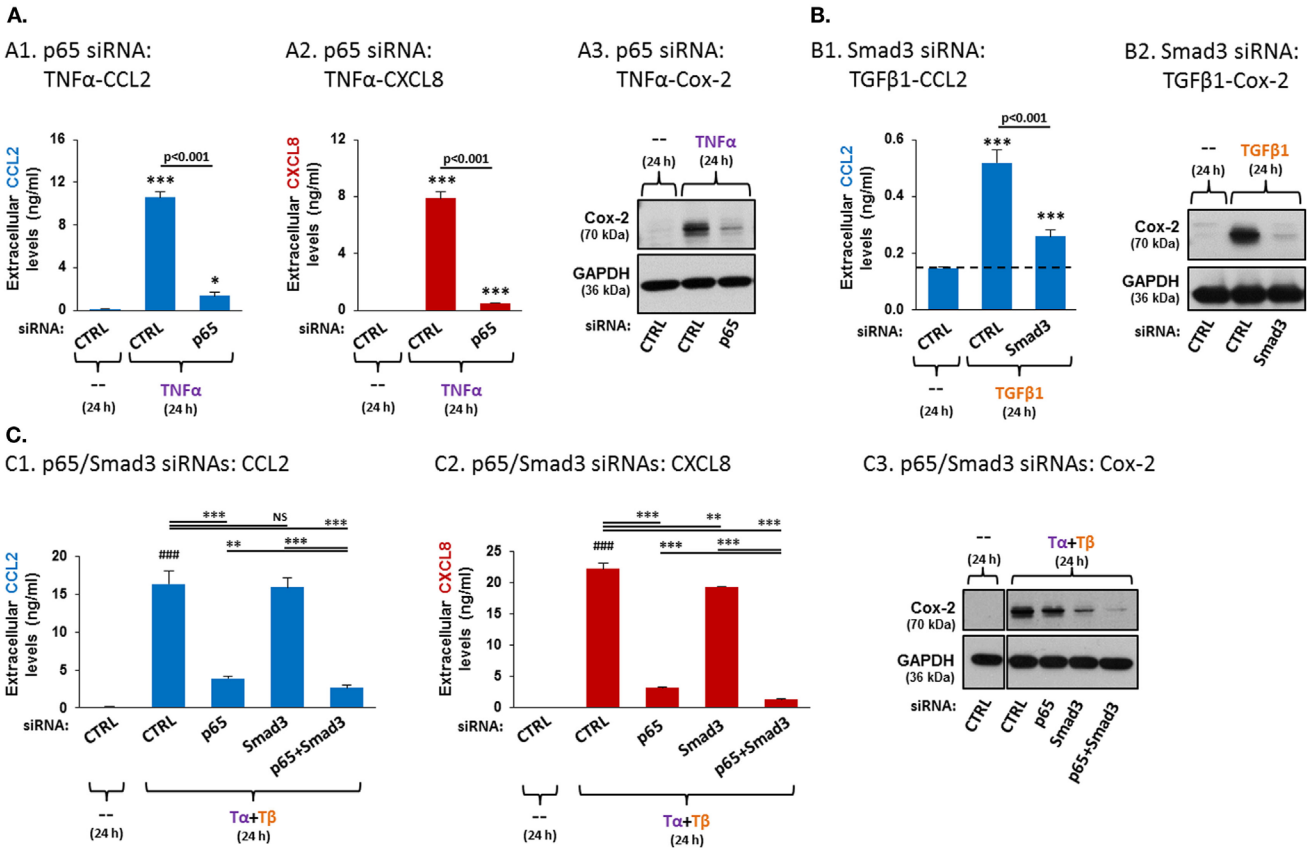

FIGURE 6 | Following TNFα + TGFβ1 stimulation of MSCs, p65 is the major regulator of CCL2 and CXCL8 expression, while Smad3 is the predominant regulator of Cox-2 expression, cooperating with NF-κB activation. Human BM-derived MSCs were transiently transfected with control siRNA, with siRNA to p65 or with siRNA to Smad3 and were stimulated as described below. siRNA concentrations were selected based on preliminary titration analysis (data not shown). The expression of CCL2, CXCL8 and Cox-2 was determined as described in **Figure 5**. **(A)** p65 siRNA effects on TNFα-mediated induction of CCL2 (A1), CXCL8 (A2) and Cox-2 (A3). Following transfection with control siRNA ("CTRL", 30 nM) or siRNA to p65 (30 nM; efficacy of p65 downregulation is demonstrated in Figure S4A in Supplementary Material), the cells were stimulated with vehicle ("--") or TNFα (50 ng/ml) for 24 h (0.5% FBS-containing medium). Comment: findings on p65 siRNA effects on TNFα-induced CCL2 and CXCL8 were demonstrated in our published report (41), but in different conditions than in the current study. $^{***}p \leq 0.001$, $^{*}p < 0.05$ compared to vehicle-treated, control siRNA-transfected cells. **(B)** Smad3 siRNA effects on TGFβ1-mediated induction of CCL2 (B1) and Cox-2 (B2). Following transfection with control siRNA ("CTRL", 30 nM) or siRNA to Smad3 (30 nM; efficacy of Smad3 down-regulation is demonstrated in Figure S4B in Supplementary Material), the cells were stimulated with vehicle ("--") or TGFβ1 (10 ng/ml) for 24 h (0.5% FBS-containing medium). $^{***}p \leq 0.001$ compared to vehicle-treated, control siRNA-transfected cells. **(C)** The effects of p65 siRNA and Smad3 siRNA on TNFα + TGFβ1-mediated induction of CCL2 (C1), CXCL8 (C2) and Cox-2 (C3). MSCs were transfected with control siRNA ("CTRL", 60 nM) or with siRNAs to p65 (30 nM p65 siRNA + 30 nM control siRNA), Smad3 (30 nM Smad3 siRNA + 30 nM control siRNA) or siRNAs to both p65 + Smad3 (30 nM each; efficacies of p65 and Smad3 downregulations are demonstrated in Figures S4C1,C2 in Supplementary Material). Then, the cells were stimulated with vehicle ("--") or TNFα (50 ng/ml) + TGFβ1 (10 ng/ml) for 24 h (0.5% FBS-containing medium). Tα + Tβ = TNFα + TGFβ1. $^{***}p \leq 0.001$, $^{**}p \leq 0.01$. NS, not significant. $^{\#\#\#}p \leq 0.001$ compared to vehicle-treated, control siRNA-transfected cells. In all panels, the findings are representatives of $n = 3$ independent experiments, performed with MSCs of two different donors, which have shown similar results. In panel C and in Figures S4C1,C2 in Supplementary Material, all the results were obtained in parallel with MSCs of one donor; similar findings, obtained in MSCs from another donor, are demonstrated in Figure S5 in Supplementary Material.

Smad3 downregulation are demonstrated in Figures S4C1,C2 in Supplementary Material). The findings of **Figure 6C** demonstrate that p65 knockdown induced a prominent reduction in the release of CCL2 and CXCL8 from TNFα + TGFβ1-stimulated MSCs, while Smad3 knockdown had no or a very minimal effect and did not add much to p65 downregulation in reducing CCL2 and CXCL8 expression (**Figures 6C1,C2**; Figure S5 in Supplementary Material demonstrates similar findings in MSCs of another donor—see explanation in Section "Data Presentation and Statistical Analyses"). In contrast, p65 knockdown had only a small effect on the cooperative induction of Cox-2 by TNFα + TGFβ1, but Smad3 downregulation led to substantial reduction in TNFα + TGFβ1-induced Cox-2 expression (**Figure 6C3**; another donor—Figure S5 in Supplementary Material). The strong elevation in Cox-2 expression following TNFα + TGFβ1 stimulation was almost totally abrogated by joint siRNA-induced downregulation of p65 and Smad3 expression (**Figure 6C3**; another donor—Figure S5 in Supplementary Material). Overall, our findings demonstrate that the elevated expression of CCL2 and CXCL8 in response to the cooperative activities of TNFα + TGFβ1 was mediated primarily by NF-κB activation, whereas the cooperative induction of Cox-2 by TNFα + TGFβ1 stimulation was mostly dependent on Smad3.

The Cooperative Induction of CCL2/CXCL8 by Joint TNFα + TGFβ1 Stimulation Is Differently Regulated by TAK1 than the Cooperative Induction of Cox-2

In parallel to the canonical pathways activated by TNFα and TGFβ1, the two cytokines share the ability to activate the pathway of the MAP3K named TAK1 (69–72). Previous studies have indicated that TAK1 activation leads to activation of NF-κB, but does not induce direct activation of Smad3. Activated TAK1 undergoes posttranslational modifications including K63-linked activating ubiquitination as well as phosphorylation at serine/threonine residues (70, 73–77), which are difficult to detect at endogenous levels with existing experimental tools (data not shown). These activation-associated posttranslational modifications of TAK1 can be reflected by a smeared gel mobility shift (75–77). Accordingly, we found that stimulation of the MSCs by TNFα, alone or in the presence of TGFβ1, had induced a smeared migration shift in TAK1 (**Figure 7A**); no such migration shift was detected following TGFβ1 stimulation, suggesting that TGFβ1 did not activate TAK1 in the MSCs.

To determine the roles of TAK1 in the ability of TNFα + TGFβ1 to cooperatively induce the pro-inflammatory factors, siRNA to TAK1 was used, demonstrating high efficiency in downregulating TAK1 expression [Figure S6 in Supplementary Material; the common inhibitor of TAK1, 5Z-7-oxozeaenol, was not used due to recent reports raising concerns on its specificity (78, 79)]. Published reports indicate that downstream of TAK1 activation, IKK is activated and leads to phosphorylation of IκBα; upon phosphorylation, this negative regulator of NF-κB is destined for degradation, thus enabling the activation of p65 (80). Therefore, in this part of the study, we first asked what is the influence of TAK1 siRNA on IκBα expression levels following costimulation of the MSCs by TNFα + TGFβ1. Our findings indicate that following MSC stimulation by TNFα + TGFβ1, IκBα was diminished in the cells (**Figure 7B1**; another donor—Figure S7 in Supplementary Material). This finding agrees with our previous results showing that this combined stimulation induces NF-κB activation (**Figure 5B1**). Yet, siRNA to TAK1 did not affect IκBα degradation following TNFα + TGFβ1 stimulation, suggesting that TAK1 is only minimally involved in the regulation of the NF-κB pathway in TNFα + TGFβ1-stimulated MSCs, if at all (**Figure 7B1**; another donor—Figure S7 in Supplementary Material). Accordingly, when we determined the effects of siRNA to TAK1 on the activation of p65 or of p38, which is another typical downstream target of TAK1 (73, 81, 82), we found out that TAK1 downregulation by siRNA led to only minor reduction in the activation of p65 (**Figure 7B2**; another donor—Figure S7A3 in Supplementary Material) or of p38 (Figure S6A2 in Supplementary Material; another donor—Figure S7A4 in Supplementary Material). As expected, TAK1 did not regulate Smad3 activation in MSCs (**Figure 7B2**; another donor—Figure S7A4 in Supplementary Material).

In view of the strong involvement of p65 in the cooperative induction of CCL2 and CXCL8 by TNFα + TGFβ1 stimulation (**Figures 6C1,C2**) and of the minimal reduction in p65 activation following TAK1 downregulation (**Figure 7B2**; Figure S7A3 in Supplementary Material), it was not surprising that TAK1 knockdown (efficacies—Figure S6B1 in Supplementary Material) did not impact the expression of CCL2 and CXCL8 (**Figures 7C1,C2**; another donor—Figure S7B in Supplementary Material). However, TAK1 downregulation did lead to reduced production of Cox-2 following TNFα + TGFβ1 stimulation of the MSCs (**Figure 7C3**; another donor—Figure S7C in Supplementary Material). These latter findings agree with the relatively lower involvement of p65 in TNFα + TGFβ1-induced Cox-2 expression, compared to TNFα + TGFβ1-induced CCL2 and CXCL8 expression in the MSCs (**Figure 6C**). Thus, our findings reveal divergent roles for TAK1 in regulating the expression of different TNFα + TGFβ1-induced pro-inflammatory mediators in MSCs.

TNFα + TGFβ1-Stimulated MSCs Release Factors That Promote Elongation, Migration, and Scattering of Breast Tumor Cells

TNFα and TGFβ1 are both expressed in many tumors (20–26) and, as our findings so far indicate, act in cooperativity to promote the pro-inflammatory phenotype of MSCs, which also prevail in breast tumors (20–23). Moreover, signaling in MSCs *via* TNFα and TGFβ1 has been strongly associated with tumor progression (20–23). To follow on the above-mentioned observations, we were interested in identifying the combined impact of TNFα and TGFβ1 on the generation by MSCs of factors that may contribute to increased tumor cell motility. To this end, we determined the effects of factors that were secreted by MSCs—following their activation by TNFα + TGFβ1 together—on characteristics of

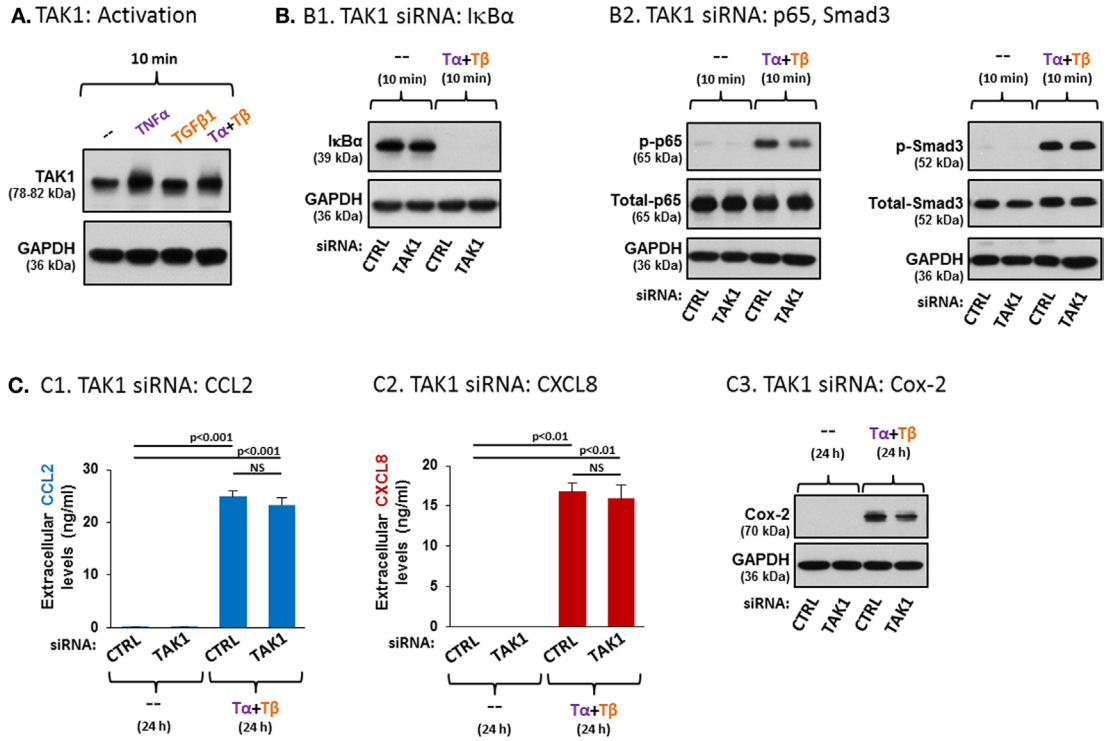

FIGURE 7 | TAK1 plays divergent roles in regulating TNFα + TGFβ1 cooperatively-induced CCL2/CXCL8 and Cox-2 expression in MSCs. (A) Human BM-derived MSCs were stimulated with vehicle ("--"), TNFα (50 ng/ml), TGFβ1 (10 ng/ml), or TNFα + TGFβ1 (same concentrations) for 10 min (FBS-free medium). Tα + Tβ = TNFα + TGFβ1. TAK1 expression was determined by WB; GAPDH was used as a loading control. The findings are representatives of $n = 3$ independent experiments, performed with MSCs of three different donors, which have shown similar results. **(B,C)** The effects of TAK1 downregulation on MSC signaling and functions. siRNA concentrations were determined by preliminary titration analysis (data not shown). **(B)** Signaling: human BM-derived MSCs were transiently transfected with control siRNA ("CTRL", 50 nM) or with siRNA to TAK1 (50 nM; efficacy of TAK1 downregulation is demonstrated in Figure S6A1 in Supplementary Material). Following siRNA transfection, the cells were stimulated with vehicle ("--") or with TNFα (50 ng/ml) + TGFβ1 (10 ng/ml) for 10 min (0.5% FBS-containing medium). Tα + Tβ = TNFα + TGFβ1. (B1) The expression levels of IκBα were determined by WB; GADPH was used as loading control. (B2) p65 and Smad3 activation levels were determined as described in **Figure 5**. p38 activation levels are demonstrated in Figure S6A2 in Supplementary Material. **(C)** Function: human BM-derived MSCs were transiently transfected with control siRNA ("CTRL", 50 nM) or with siRNA to TAK1 (50 nM; efficacies of TAK1 downregulation are demonstrated in Figures S6B1,B2 in Supplementary Material). Following siRNA transfection, the cells were stimulated with vehicle ("--") or with TNFα (50 ng/ml) + TGFβ1 (10 ng/ml) for 24 h (0.5% FBS-containing medium). Tα + Tβ = TNFα + TGFβ1. The impact of TAK1 siRNA on the expression of CCL2 (C1), CXCL8 (C2) and Cox-2 (C3) was determined as described in **Figure 5**. NS, not significant. In all panels, the findings are representatives of $n = 3$ independent experiments, performed with MSCs of two different donors, which have shown similar results. Similar findings, obtained in MSCs from another donor, are demonstrated in Figure S7 in Supplementary Material (each panel with its corresponding validation of downregulation efficacy).

breast tumor cells that are connected to increased motility and spreading. To specifically isolate the effects delivered by factors released by the stimulated MSCs from signals that may be induced in the tumor cells by the cytokines themselves [as shown in Ref. (24, 83–90)], a control group consisting of TNFα + TGFβ1 stimulation (without factors of the MSCs) was included in the analyses.

In this part of the study, two human breast tumor cells were addressed: the highly motile human MDA-MB-231 triple negative cells and the relatively less invasive MCF-7 luminal-A human breast tumor cells (91, 92). Following stimulation of the MSCs for 24 h by TNFα + TGFβ1 or by their vehicles, the CM of cytokine-stimulated or of vehicle-exposed cells (Groups 4 and 3, respectively) were transferred to mCherry-expressing breast tumor cells. Control tumor cells were grown in parallel with medium alone (Group 1) or with TNFα + TGFβ1 only (Group 2).

In view of the high basal motility of MDA-MB-231 cells, we chose to determine the impact of the different CM on the generation of elongated morphology of these cells. This has been connected to elevated tumor cell motility (93, 94). The results of **Figure 8A** indicate that CM derived from TNFα + TGFβ1-stimulated MSCs (Group 4) have induced an elongated morphology in the tumor cells. The results indicated that the influence of the CM derived from TNFα + TGFβ1-stimulated MSCs (Group 4) was much stronger than the effects induced on the tumor cells by the CM obtained from vehicle-treated MSCs (Group 3) and was also more evident than the impact of the cytokines themselves (Group 2; photos demonstrating enlarged cell magnifications of MDA-MB-231 in another experiment are demonstrated in Figure

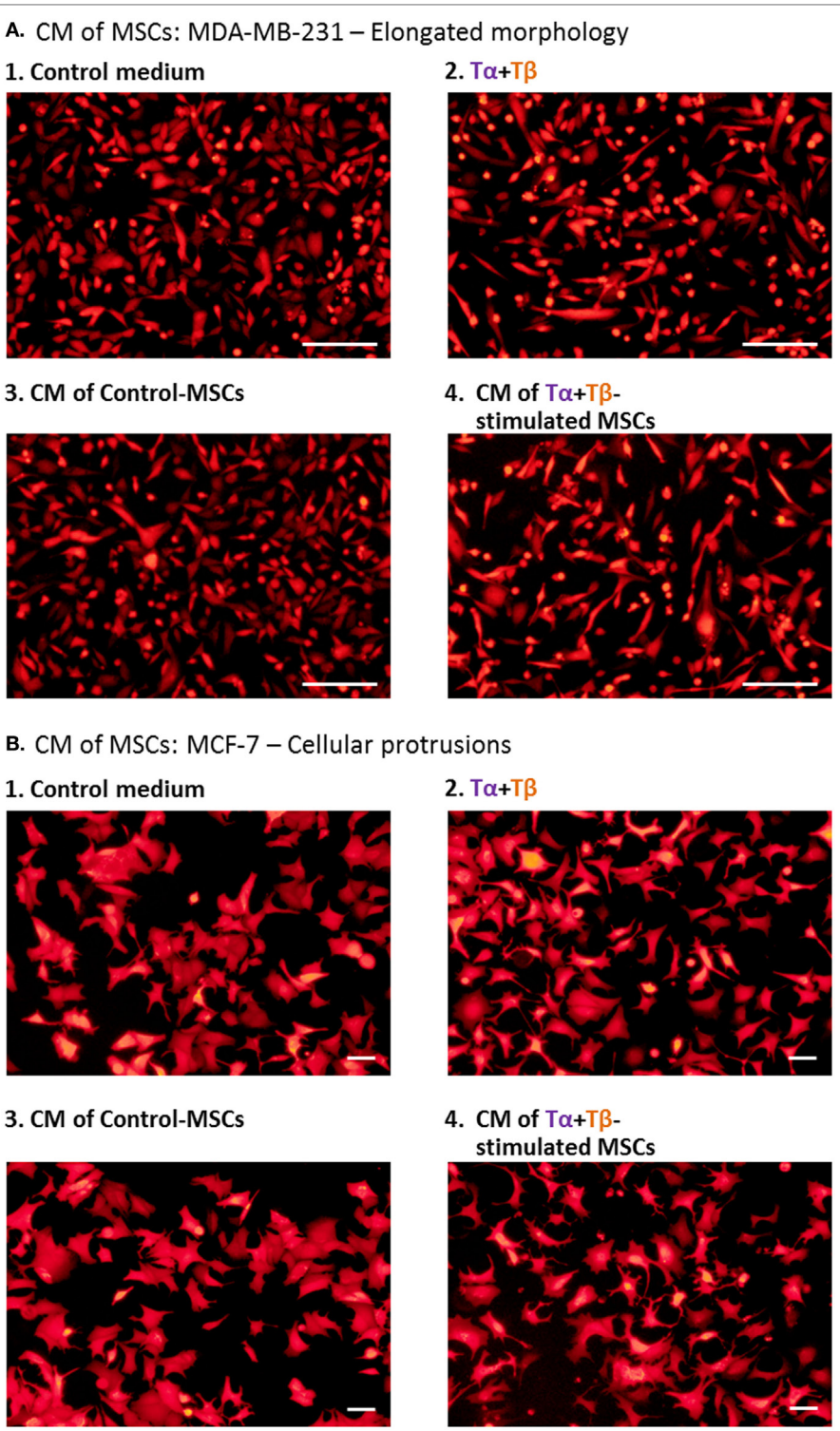

FIGURE 8 | **Factors released by TNFα + TGFβ1-stimulated MSCs induce cellular elongation and formation of cellular protrusions in human breast cancer cells**. Human BM-derived MSCs were stimulated with vehicle ("Control-MSCs") or TNFα (50 ng/ml) + TGFβ1 (10 ng/ml), in 0.5% FBS-containing medium; Tα + Tβ = TNFα + TGFβ1. In parallel, samples of "Control medium" (not exposed to MSCs), with or without the stimulating cytokines, were kept in identical conditions. Twenty-four hours later, all different media were filtered (0.45 µm pores) and applied to mCherry-expressing MDA-MB-231 cells for 48–72 h, in different experiments **(A)** or to mCherry-expressing MCF-7 cells for 48 h **(B)**. Cancer cells were then washed and photographed. CM = conditioned media. Scale bar: 200 µm in MDA-MB-231 cells and 50 µm in MCF-7 cells. The pictures are representatives of $n \geq 3$ independent experiments, performed with MSCs of two to three different donors, which have shown similar results. For MDA-MB-231 cells, enlarged pictures of cells, obtained in MSCs from another experiment, are demonstrated in Figure S8 in Supplementary Material.

S8 in Supplementary Material). These findings indicate that factors released by TNFα + TGFβ1-stimulated MSCs enhanced a malignancy-related characteristic in the tumor cells.

In parallel, the relatively low basal motility phenotype of MCF-7 cells has motivated us to ask if factors released by TNFα + TGFβ1-stimulated MSCs would increase the formation of cellular protrusions and motility in MCF-7 cells. Here, we found that the CM of TNFα + TGFβ1-stimulated MSCs (Group 4) have led to formation of definite cellular protrusions that were generally more intense than in the relevant control groups (**Figure 8B**); such protrusions were strongly connected in other studies to increased EMT and scattering of these cells (86, 95). Moreover, when we determined the motility of MCF-7 cells in response to migration-inducing factors that are present in serum, we found that cancer cells cultured in the presence of TNFα + TGFβ1-derived CM (Group 4) revealed more robust migratory ability than tumor cells exposed to the other treatments, including CM derived from vehicle-treated MSCs (Group 3) or the cytokines alone (Group 2; **Figures 9A1,A2**).

To further investigate the impact of factors released by TNFα + TGFβ1-stimulated MSCs on tumor cell motility, we determined the scattering of MCF-7 cells from spheroid tumor masses, demonstrated by our published study to correlate with a more aggressive behavior of the cells (86). Using a 3D spheroid-based detachment assay, we found that MCF-7 cells treated by CM of TNFα + TGFβ1-stimulated MSCs (Group 4) had very high capabilities of scattering out of the 3D tumor spheroids (**Figure 9B**). A remarkable difference was revealed between MCF-7 cells exposed to CM derived from TNFα + TGFβ1-stimulated MSCs (Group 4) and cancer cells exposed to the two other relevant treatments: CM of vehicle-treated MSCs (Group 3) or to the two cytokines only (Group 2).

All of the above findings demonstrate the high ability of CM of TNFα + TGFβ1-stimulated MSCs (Group 4) to promote motility-related functions of breast tumor cells *in vitro*; importantly, the effects of CM derived from TNFα + TGFβ1-stimulated MSCs were more pronounced than the effects of CM derived from vehicle-treated MSCs (Group 3) or of the cytokines alone (Group 2). These findings clearly indicate that factors produced by MSCs following TNFα + TGFβ1 stimulation induced a motile phenotype in cancer cells, which can potentially contribute to elevated aggressiveness.

DISCUSSION

Mesenchymal stem cells, their functions and modes of regulation, have been extensively studied during the last several years. The growing interest in these cells stems from their ability to give rise to cartilage, bone, muscle, and fat lineages (1–3); their activities in sites of hematopoiesis and inflammation (4, 5); their prominent roles in controlling malignancy (96–98); and their potential use as tools for gene delivery and tissue regeneration (7, 8). Stemming out from different tissue origins, the high plasticity of MSCs and their abundance at many organs emphasize the need to identify how their functions are regulated by factors of their intimate microenvironments.

Accordingly, in this study we were interested in deciphering the combined effects of TNFα + TGFβ1, both being coexpressed in different niches and conditions (16, 17, 20–23), on MSCs. Although TNFα and TGFβ1 very often have opposing roles in immune/inflammatory regulation (9–13), we found out that they join forces and act in cooperativity to promote the pro-inflammatory phenotype of MSCs. In the presence of TNFα, TGFβ1 turned into a co-inflammatory cytokine whose functions promoted the activities mediated by the classic pro-inflammatory cytokine TNFα. These findings add to recent reports on the ability of TGFβ to promote immune responses by reversing the suppressive activity of MSCs on T cell proliferation (99).

The findings obtained in our study reveal that the functional cooperativity between TNFα + TGFβ1 leads to activation of NF-κB and Smad3, yet the involvement of the two transcription factors in regulating the pro-inflammatory phenotype of the MSCs is complex. When acting alone, TNFα-induced expression of CCL2, CXCL8 and Cox-2 depended on NF-κB activation, agreeing with published reports on direct binding of p65 to the promoter/enhancer regions of these genes [e.g., Ref. (100–102)]. In parallel, when the MSCs were stimulated only by TGFβ1, the induction of CCL2 and Cox-2 highly depended on Smad3 activities, reflecting the presence of Smad3-binding sites in these two genes (103, 104). Yet, the cooperative induction of CCL2, CXCL8 and Cox-2 by joint stimulation with TNFα + TGFβ1 was due to modified balance between NF-κB and Smad3.

Specifically, NF-κB activation was almost exclusively involved in TNFα + TGFβ1-induced CCL2 and CXCL8 without a significant involvement of Smad3. In contrast, the cooperative activities of TNFα + TGFβ1 leading to elevated expression of Cox-2 were mostly dependent on Smad3 activation. These findings may reflect the importance of cooperativity between different transcription factors regulating the concerted transcription of different pro-inflammatory targets in MSCs, as has been reported in other systems [e.g., Ref. (100, 105)]. Most evidently, this is the case for the TGFβ1-Smad3 pathway: the wide variety of TGFβ1 activities reflect the physical interactions of Smad3 with different transcription factors, leading to one response when interacting with one specific transcription factor while generating another response upon interaction with a different transcription factor (106, 107). Indeed, this complex mode of TGFβ1-induced Smad3 activation may stand in the basis of joint Smad3 and p65 activities in TNFα + TGFβ1-stimulated MSCs, when the Cox-2 response was induced. Cooperativity between different transcription factors may also be involved in TNFα + TGFβ1-induced elevation in CCL2 and CXCL8 expression: the phosphorylation levels of p65 upon TNFα + TGFβ1 stimulation were not higher than with TNFα alone, proposing that TGFβ1 stimulation has not changed the activation level of p65 but rather modified the cooperativity between p65 and other transcription factors that act together with it on the relevant genes.

Our findings on the roles of TAK1 reveal additional aspects of differential regulation of CCL2/CXCL8 compared to Cox-2. It is interesting to note that unlike other cell systems (81, 108), TAK1

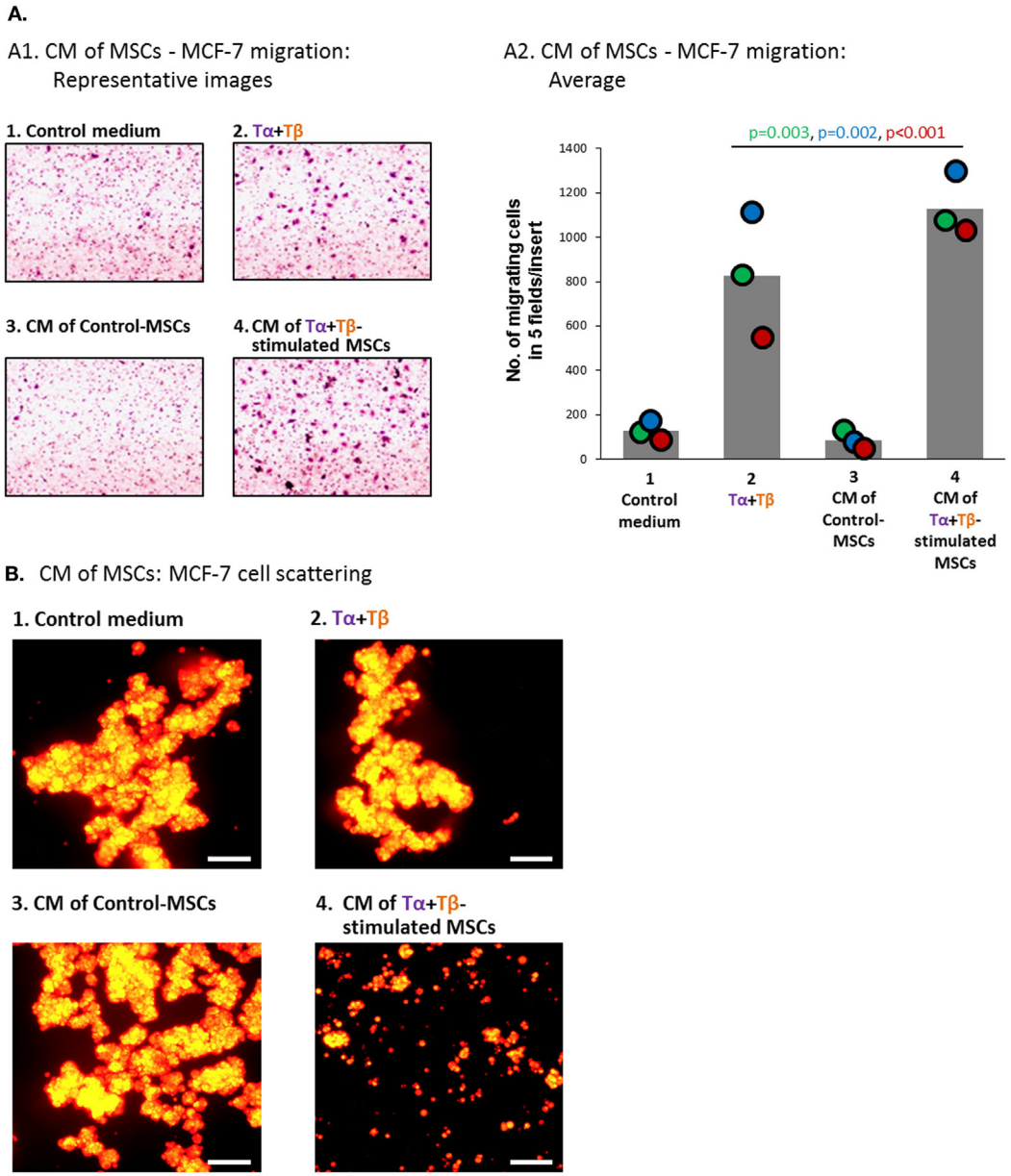

FIGURE 9 | **Factors released by TNFα + TGFβ1-stimulated MSCs induce elevated migration and scattering of MCF-7 breast cancer cells**. Human BM-derived MSCs were stimulated with vehicle ("Control-MSCs") or TNFα (50 ng/ml) + TGFβ1 (10 ng/ml) [0.5% FBS-containing medium in panel **(A)** and FBS-free medium in panel **(B)**]; Tα + Tβ = TNFα + TGFβ1. In parallel, samples of "Control medium" (not exposed to MSCs), with or without the stimulating cytokines, were kept in identical conditions. Twenty-four hours later, all different media were filtered (0.45 µm pores) and applied to mCherry-expressing MCF-7 cells for 48 h **(A)** or 96 h **(B)**. Then, functional assays were performed. CM = conditioned media. **(A)** Migration of MCF-7 cells toward 10% FBS-containing medium. (A1) Representative pictures of part of the high-resolution fields, ×40 magnification, of one of three independent experiments performed with MSCs of two different donors. (A2) Bar graph demonstrating the average number of cells migrating in each cell group, obtained in three independent experiments (total of five pictures/insert in each experiment); colored dots represent the number of migrating cells in each of these same three experiments, with corresponding color-coded *p*-values indicated. **(B)** Scattering of MCF-7 cells out of tumor spheroids. Scale bar = 200 µm. The pictures are representatives of $n > 3$ independent experiments, performed with MSCs of three different donors that have shown similar results.

was not a key regulator of NF-κB activation in the MSCs; it also did not regulate the expression of CCL2 and CXCL8 while partly controlling the expression of Cox-2. The roles of TAK1 in this system nicely reflect the fact that different targets of TNFα + TGFβ1 are regulated in a divergent manner by NF-κB activation, being almost exclusively involved in CCL2/CXCL8 induction and only partly active in Cox-2 induction upon TNFα + TGFβ1 stimulation.

Thus, in the current study we identify molecular mechanisms induced by joint activities of different factors that are coexpressed at specific niches/conditions. Cancer is a major clinical implication in which the joint activities of TNFα + TGFβ1 on MSCs are very relevant. It has been demonstrated that the two cytokines cooperate in driving epithelial-to-mesenchymal processes and the generation of cancer stem cells in breast cancer, colorectal cancer, and other malignancies (24–26, 43). Our findings propose that when the TME is enriched with both cytokines, as may often be the case in malignancy (20–26), TNFα + TGFβ1 would act not only on the tumor cells but also may induce the release of pro-inflammatory and tumor-promoting factors by MSCs. The factors released by the MSCs, which are cells with well-established tumor-promoting roles (109–113), can contribute to cancer progression by promoting two complementing processes: (1) they may enrich the TME with pro-inflammatory mediators that have been identified as major contributors to tumor progression, such as CCL2, CXCL8 and Cox-2 (37–39, 62, 63, 114); (2) In parallel, the factors released by the TNFα + TGFβ1-stimulated MSCs, pro-inflammatory and others, may act directly on the cancer cells to promote their migratory and invasive properties, as we have shown in the current study.

Therefore, on the whole, the presence of both TNFα and TGFβ1 at tumor sites, combined with the factors they induce in MSCs (as we have demonstrated), may have pro-malignancy effects that act on the TME as well as directly on the cancer cells, to promote their pro-invasive potential. Along these lines, in recent preliminary studies, we have generated mRNA expression profiles of breast tumor cells grown in the presence of CM derived from TNFα + TGFβ1-stimulated MSCs. In this ongoing study, we identified that such CM has led to elevated expression in the tumor cells of molecules that control the organization of the actin cytoskeleton and of microtubules, and promote migration/invasion, matrix degradation, and metastasis in breast cancer: Rho GTPase 1, laminin gamma 2, LIM-only protein FHL2, MMP9, tubulin β3, ICAM-1, MMP13, zyxin, WASP interacting protein, and myosin X. Thus, it is expected that in future studies we will be able to identify the molecules that drive the pro-migratory phenotype of breast tumor cells following their exposure to factors released by TNFα + TGFβ1-stimulated MSCs.

Our findings demonstrating the joint power of TNFα + TGFβ1 + the factors they induce in MSCs on cancer cells provide proof-of-concept to the fact that MSCs are strongly affected by their microenvironment, and as a result secrete soluble mediators that modify their surroundings. These observations are of high relevance to different physiological and pathological settings in which the two cytokines are coexpressed, alongside with MSCs. First and outmost, the pro-inflammatory phenotype gained by TNFα + TGFβ1-stimulated MSCs is very relevant to immune regulation, where MSCs are playing important roles. MSCs are generally considered as having one of two phenotypes: (1) "pro-inflammatory" MSC1 cells: in microenvironment low in inflammatory signals, these cells polarize to a pro-inflammatory phenotype, inducing the generation of activated T cells; and (2) "anti-inflammatory" MSC2 cells: when the microenvironment is enriched with pro-inflammatory mediators, MSC2 cells turn into the anti-inflammatory/immunosuppressive type (115, 116). Our findings raise questions on the way these two subpopulations of MSCs would act when exposed to both TNFα + TGFβ1, because in contrast to the general view seeing them as having opposing forces in immune regulation—pro-inflammatory *vs.* anti-inflammatory/immunosuppressive, respectively—we demonstrate in this study that the MSCs gained an enhanced pro-inflammatory phenotype when stimulated jointly by TNFα + TGFβ1. Obviously, this issue deserves in-depth investigation of its own; yet, it is worth mentioning some relevant findings from our current study: although the mRNA array analysis of our study indicated that many pro-inflammatory mediators are induced by the cytokines, it has demonstrated that TNFα and TGFβ1 did not modify the expression levels of immune molecules associated with immune suppression, including IDO1, IDO2, CTLA-4, and PD-L1.

Much beyond regulation of immune activities, many other health-related conditions may be affected by MSC exposure to both TNFα and TGFβ1 together. Our current understanding of fracture healing suggests that in this setting both cytokines are necessary for inducing MSC migration and/or MSC activities that are required for fracture healing (16, 17). As a result of pro-inflammatory processes that are ensued by TNFα, macrophages that are recruited to bones release chemokines such as CCL2, which promote MSC recruitment and function. In parallel, macrophages release TGFβ1 that promotes the proliferation and differentiation of MSCs, thus enhancing processes of bone repair (16, 17). However, the delicate equilibrium between these factors may be impaired in aging individuals by extensive pro-inflammatory processes, whose trigger(s) are not fully identified (6, 18, 19). Our findings raise the possibility that such increased pro-inflammatory phenotype may be gained by the MSCs due to their exposure to TNFα and TGFβ1 simultaneously, at the bone niche.

The relevance of cooperative TNFα + TGFβ1 activities on MSCs can be further extended to pathological conditions in which the two cytokines are coexpressed, alongside with the presence of MSCs, or in which MSC-based therapies are considered. These include pulmonary diseases (8, 117), cardiomyopathy (118–120), and possibly also Alzheimer's disease (121, 122). In these conditions, it is possible that the combined activities of TNFα + TGFβ1 promote—through their impact on MSCs—the pro-inflammatory nature of the microenvironment.

Moreover, such mechanisms may be particularly relevant when MSCs are considered as therapeutic tools. Gene-modified MSCs which are delivered from exogenous sources may be affected by the intimate microenvironment residing in their respective niche, which may be enriched with both TNFα and TGFβ1. In response to the two cytokines, such MSCs may turn into pro-inflammatory reservoirs, acting in unbalanced and undesired manners and affecting the functions of other cells in their vicinity. If so, it would be desired to inhibit the joint activities of TNFα and TGFβ1 by targeting their receptors or downstream mediators. The molecular complexity revealed in our study suggests that combined targeting of multiple pathways may be required. Eventually, it is the intricate nature of molecular pathways driven by different microenvironment stimuli that will dictate the therapeutic measures that are needed in each setting.

To follow up on our observations, it is important to note that not only cooperativity may be taking place between TNFα + TGFβ1 as we have demonstrated but also other modes of cross talks between them may take place as well. A cascade-type of interaction was found in adipose tissue-derived MSCs, which upon priming with TNFα released TGFβ1 that, in turn, elevated the malignancy phenotype of breast tumor cells (27). Here, it is interesting to note that in our studies TNFα did not induce an elevated expression of TGFβ1 mRNA, and TGFβ1 did not increase TNFα expression (as indicated in the mRNA array). Thus, it is possible that regulation of MSCs depends not only on the content of microenvironmental factors but also on the tissue origin of the MSCs.

Overall, our findings indicate that the microenvironment has a strong impact on the phenotype of MSCs and their functions. The content of the microenvironment and the origin of MSCs may be critical factors in driving molecular processes that eventually affect tissue cells in their vicinity. These findings have strong implications not only on MSCs that are found natively at different niches but also on therapeutic modalities that are based on MSCs delivered to different tissues from external sources.

AUTHOR CONTRIBUTIONS

SL was responsible for gathering most of the experimental data and their analysis and had instrumental contribution to study design, data interpretation, and drafting the manuscript; YL contributed to data gathering throughout the whole study and supported additional aspects of the project; AB participated in design and data gathering of mRNA arrays and the validation of mRNA expression by qPCR; KA has performed bioinformatics analyses of mRNA array data; NO participated in WB analyses; AY participated in analysis of mRNA array data; CK performed the analyses of METABRIC and TCGA datasets; TM has supported the qPCR analyses; SW participated in study conception and design; AB-B was the principal investigator responsible for study conception and design and for complete manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00479/full#supplementary-material.

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TGF-β1 Pretreatment Improves the Function of Mesenchymal Stem Cells in the Wound Bed

Deepraj Ghosh[1], *Daniel J. McGrail*[2] *and Michelle R. Dawson*[1,3]*

[1] Department of Molecular Pharmacology, Physiology, and Biotechnology, Brown University, Providence, RI, USA, [2] Department of Systems Biology, University of Texas MD Anderson Cancer Center, Houston, TX, USA, [3] School of Engineering, Brown University, Providence, RI, USA

The wound healing process initiates after injury to a tissue and involves a series of orchestrated events to minimize the invasion of foreign matters such as bacteria and efficiently regenerate the damaged tissue. A variety of cells must be recruited to the tissue during wound healing. However, this process is severely disrupted in patients suffering from chronic illness, including diabetes, leading to impaired healing or non-healing wounds. Current avenues of treatment include negative-pressure therapy, wound debridement, growth factor replacement, and cell-based therapies. Among these therapies, mesenchymal stem cells (MSCs) delivery to the wound holds a very high promise due to the innate abilities of MSCs that include immunogenicity, plasticity, and self-renewal. Bone marrow derived MSCs have been shown to promote more rapid wound healing by increased cytokine production in diabetic mice. However, the lack of understanding of the mechanical and chemical interaction of the transplanted MSCs with the factors present in the regenerative niches limits their efficacy in the wound bed. In this study, we sought to understand how the changes in MSC biochemical and biophysical properties can affect their function *in vitro* and *in vivo*. We demonstrate that pretreatment of MSCs with the mechano-stimulatory soluble factor transforming growth factor (TGF-β1), which is highly expressed in injury sites, improves wound closure in a syngeneic murine wound model. This improved wound closure correlated with increased invasion into the wound bed. *In vitro* studies demonstrated that TGF-β1 pretreatment expedited wound closure by increasing adhesion, traction force, and migration even after removal of the stimulus. Furthermore, this response was mediated by the cytoskeletal protein focal adhesion kinase. Taken together, this study suggests that defined chemical stimuli can benefit site specific adaptability of MSCs to improve their function and therapeutic usefulness.

Keywords: wound healing, mesenchymal stem cells (MSCs), transforming growth factor—β1 (TGF-β1), adhesion, migration, focal adhesion kinase (FAK)

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***Correspondence:**
Michelle R. Dawson
michelle_dawson@brown.edu

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INTRODUCTION

Skin is the outermost tissue that acts as a barrier against foreign matter incursion into the body. Injury to skin initiates the wound healing process, which includes three distinct but temporally overlapping series of events namely, inflammation, proliferation, and remodeling (Diegelmann and Evans, 2004; Guo and Dipietro, 2010; Takeo et al., 2015). During inflammation, platelets secrete

a host of soluble factors to attract neutrophils and macrophages, and these cells subsequently remove the contaminants from the wound bed and protect wound site from further infection. Once the inflammation process subsides, the healing process enters the proliferation stage which is characterized by fibroblast migration, matrix deposition and granulation tissue formation, angiogenesis, and re-epithelialization. Finally, the healing process moves to the remodeling phase which requires simultaneous deposition and degradation of extracellular matrix including collagen isoforms, to form scar tissue and rebuild the native architecture of the skin. However, the progress of the healing events are severely disrupted in elderly patients and patients suffering from chronic illness, including diabetes, ischemia, or hypertension, leading to non-healing chronic wounds (Gosain and DiPietro, 2004; Brem and Tomic-Canic, 2007; Guo and Dipietro, 2010). Chronic wounds are caught in a state of constant inflammation which disrupts the initiation of later phases and it is characterized by impaired production of cytokines and growth factors and reduced angiogenesis (Werner and Grose, 2003; Barrientos et al., 2008; Lazaro et al., 2016). Nearly 1% of the population in developed countries suffers from chronic wounds (Simka and Majewski, 2003; Gottrup, 2004). In United States alone, chronic wounds affect 6.5 million people and incur an estimated annual cost of USD 25 billion (Sen et al., 2009). Conventional treatment of the chronic wounds includes surgical debridement, biological dressing, pressure offloading, and topical growth factors. However, these methods provide very limited results in most cases (up to 50%) and new therapies are required to overcome the current challenges of chronic wound management (Greer et al., 2013; Dreifke et al., 2015; Rowan et al., 2015; Sessions et al., 2017). Recent studies have indicated that cell based therapies can provide a suitable alternative treatment method for multiple pathological conditions including, wound healing (Chen et al., 2012; Rodriguez-Menocal et al., 2015; Duscher et al., 2016).

Mesenchymal stem cells (MSCs) are multipotent cells that actively participate in tissue repair and regeneration *in vivo*. MSCs can be isolated and expanded with relative ease and their immunogenicity sets them as the ideal candidate for both autologous and allogeneic transplantation in wound healing (Bobis et al., 2006; Murphy et al., 2013). Both preclinical studies with animal models and clinical studies have demonstrated effectiveness of MSCs in accelerating chronic wound healing (Wu et al., 2007a; Jackson et al., 2012; Isakson et al., 2015; Kim et al., 2015; Kirby et al., 2015). MSCs added (topical delivery) or recruited (systemic delivery) to the wound bed are believed to participate in healing process by one of the following mechanisms: (1) releasing paracrine factors e.g., cytokines, and (2) differentiating into cell phenotypes e.g., keratinocytes, required for regeneration (Wu et al., 2007b; Chen et al., 2008; Sasaki et al., 2008; Kuo et al., 2016). Despite the recent progress in evaluating MSC based therapies for wound healing, the distinct interaction of MSCs with the mechanical and chemical cues present in wound bed is not fully understood and this currently limits the extensive use of MSCs in clinical capacity. Previous studies have reported that wound stiffness increases dramatically during the healing process and modifies the function of underlying cells, e.g., fibroblasts, in both normal and pathological conditions (Goffin et al., 2006; López et al., 2010; Liu et al., 2010a; Chao et al., 2013; Klingberg et al., 2013; Darby et al., 2014). In addition to the biochemical signals present in the wound milieu, such mechanical cues e.g., matrix elasticity, can also affect the MSC function profoundly (Engler et al., 2006; Kilian et al., 2010; Tse and Engler, 2011; MacQueen et al., 2013). Studies with polyacrylamide (PA) substrate of varying elasticity have reported stiffness dependent migration of cells in the context of wound healing (Ng et al., 2012; Raab and Discher, 2016). It is now widely accepted that substrate stiffness can influence a myriad of fundamental properties and functions of diverse cell types including MSCs (Discher et al., 2005, 2009; Eyckmans et al., 2011; Janmey and Miller, 2011; Charras and Sahai, 2014; Humphrey et al., 2014). In this study, we sought to understand the effect of inducing phenotypical changes (including mechanical properties) in MSCs on its function in wound bed and wound healing rate. We have previously characterized the mechanical and chemical response of MSCs *in vitro* to soluble factors present in regenerative niches (Ghosh et al., 2014). Transforming growth factor β1 (TGF-β1), a pleiotropic protein belonging to the TGF-β superfamily, regulates a myriad of cell functions including, proliferation, differentiation, adhesion, migration, and apoptosis (Massagué, 1998; Heldin et al., 2009; Watabe and Miyazono, 2009). TGF-β1 plays a significant role throughout the phases of wound healing (Gilbert et al., 2016). Our studies with soluble factor TGF-β1 provided enhanced mechanical response with cytoskeletal remodeling and stiffening of MSCs (Ghosh et al., 2014). TGF-β1 treated MSCs also provided molecular response to indicate adhesive strengthening, ECM remodeling and differentiation (Ghosh et al., 2014). This study sought to understand if TGF-β1 pretreatment induced modification in MSC phenotype can alter their *in vitro* and *in vivo* behavior. We hypothesized that migrating MSCs that disseminated throughout the wound bed would contribute to the formation of granulation tissue, which would constrict the wound for more rapid wound closure. Improved MSC migration could also improve the spatial and temporal activity of growth factors and cytokines since they were secreted from MSCs that disseminated throughout the wound tissue. Injection of TGF-β1 pretreated MSCs at the periphery of skin wounds resulted in increased wound closure rates compared to control MSCs. TGF-β1 pretreated MSCs also demonstrated greater distribution toward the center of the wound compared to control cells. The persistent characteristics of TGF-β1 pretreated cells can be beneficial for treatment of chronic wounds, where cell functions are arrested due to rapid degradation of soluble factors. To better understand the effects of TGF-β1 pretreatment, we performed multiple *in vitro* functional analyses of MSCs up to 24 h after removal of initial stimulus. TGF-β1 treatment resulted in dramatically elongated morphology and this phenotype was maintained even after 24 h of removal of the stimulus. Similarly, TGF-β1 pretreated cells sustained the enhanced surface expression of α_v, β_1, and β_3 integrins as determined by flow cytometry and subsequently displayed higher adhesive strength compared to control cells. To better understand the initial cell attachment process, we used 34 kPa PA substrates that closely match the stiffness of

the wound bed (Goffin et al., 2006; Discher et al., 2009). TGF-β1 pretreated cells adhered and spread more efficiently on the PA substrates and generated significantly higher traction forces. TGF-β1 pretreatment also enhanced the soluble factor-mediated migration of MSCs. Additionally, using small molecule inhibitors to disrupt certain well known pathways associated with cell functions, we found that focal adhesion kinase (FAK) signaling is key for enhanced performance of TGF-β1 pretreated cells.

MATERIALS AND METHODS

Materials

IMDM, DMEM, L-glutamine, penicillin-streptomycin, and trypsin were purchased from Mediatech and fetal bovine serum (FBS) was purchased from Atlanta Biologicals. Recombinant human TGF-β1, PDGF and IGF-I proteins and flow cytometry antibodies were purchased from Biolegend. Recombinant proteins were solubilized in phosphate buffered solution (PBS) containing 1% bovine serum albumin (BSA) and stored in −80°C as per manufacturer's recommendation. All other reagents were purchased from VWR unless otherwise specified.

MSC Isolation and Culture

Murine MSCs were isolated from the bone marrow of 6–10 weeks old adult male Balb/C mice (Charles River Laboratories, Wilmington, MA) and cultured in normal growth media (IMDM media supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin). Purified MSCs between passages 2–6 were used for all studies after thorough characterization (Supplementary Figure 1). All animal studies were approved by the Institutional Animal Care and Use Committee at Georgia Institute of Technology (PHS Assurance Number 3822-01).

Soluble Factor Pretreatment

Soluble factor dilutions were created from aliquots in serum-free DMEM or IMDM immediately before use. Based on literature review and our previous studies, TGF-β1 concentration of 5 ng/ml was used for treatment of MSCs (Ghosh et al., 2014). Initially, MSCs were pretreated with serum-free control media (CM), and 5 ng/ml TGF-β1 (diluted in serum free media) for 24 h. Afterwards, the stimulations were removed and both control and pretreated cells were moved to serum free or specific differentiation induction media to determine the effects of pretreatment on MSC functions. To avoid confusion, the removal of growth factor stimulus was assigned as t_0; whereas the time points for each experiment were indicated as $t_0 + t$ h. For example, centrifugation based adhesion assays described in this section were carried out at $t_0 + 24$ h.

Wound Preparation and Mesenchymal Stem Cell Transplantation

An *in vivo* punch biopsy wound healing model was used to determine the effects of MSC pretreatment on wound healing (McGrail et al., 2013). To account for mouse to mouse variation, all mice had one wound injected with control media pretreated MSCs and one TGF-β1 pretreated MSCs (Supplementary Figure 2). Briefly, hair was removed from the dorsal surface of anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine or isoflurane gas) 12-week old male Balb/C mice by shaving and Nair hair removal. One 5 mm full-thickness skin wound was made on each side of the dorsal midline using a punch biopsy tool (to trace the wound perimeter) and iris scissors (to remove the tissue). Concurrently, pretreated MSCs were detached and labeled with the lipophilic tracer dye DiD (Invitrogen). Mouse mesenchymal stem cells (5.0×10^5) suspended in a small volume of PBS solution (∼100 μl) were injected (30-gauge needle) intradermally at the wound periphery of anesthetized mice. All animal studies were approved by the Institutional Animal Care and Use Committee at Georgia Institute of Technology (PHS Assurance Number 3822-01).

Wound Healing Analysis

After seven days, animals were sacrificed and wound tissues were collected to image the fluorescently labeled MSCs. Wound tissues were stained with DAPI to highlight the nuclei and were then mounted on slide for imaging. The wound bed was imaged using Nikon Eclipse Ti inverted fluorescence microscope and an image of the entire wound area was created by stitching together all the individual images in Nikon Elements. The images were further analyzed by quantifying the fluorescence intensity across the distance between the centroid of the injection sites as previously discussed (McGrail et al., 2013).

Morphological Analysis

Control and pretreated MSCs were cultured for 24 h after the removal of stimulus ($t_0 + 24$ h) and were stained with crystal violet. Cells were then imaged with stereoscope microscope and Motic camera. Cell borders were traced manually and cell shape factors, defined as $4^*\pi^*\text{Area}/ (\text{Perimeter})^2$, were determined using Image J.

Centrifugal Force Based Adhesion Assay

Briefly, control and pretreated MSCs were trypsinized and labeled with Calcein AM. Then the cells were seeded in an uncoated 96-well plate in serum-free media. At 24 h after stimulus was removed ($t_0 + 24$ h), an initial fluorescence reading was recorded. Cells were detached by centrifuging inverted plates at 500 × g for 3 min before recording a final fluorescence reading. The adherent fraction was determined by normalizing the final florescence values with the initial pre-spin values (McGrail et al., 2013).

Flow Cytometry

Briefly, both treated and untreated cells were cultured for 24 h after the removal of stimulus ($t_0 + 24$ h), detached from surface, centrifuged, and suspended in 100 μl cold FACS buffer (2% FBS, 1mM EDTA in PBS) with one of the following anti-mouse antibodies (dilutions in parentheses): FITC-CD29 (1:100), PE-CD61 (1:100), PE-CD51 (1:20), AF-647-MVCAM1/CD106 (1:200). Following the incubation, MSCs were washed and fixed in 2% paraformaldehyde. Samples were run on a BD LSR-II flow cytometer to capture at least $n = 50{,}000$ events per sample.

Fabrication of Polyacrylamide (PA) Substrates

PA hydrogels were used as an elastic 2D cell culture platforms due to extensive characterization of their mechanical properties that can be easily tuned by controlling crosslinker concentration(Tse and Engler, 2010; Caliari and Burdick, 2016; Polacheck and Chen, 2016). PA substrates were synthesized based on the protocol described before (Tse and Engler, 2010). Briefly, glass coverslips were activated using 3-aminopropyltrimethoxysilane and a mixture of the acrylamide and bis-acrylamide solution (10% acrylamide to 0.3% bis-acrylamide) was polymerized on the activated glass coverslips (Young' modulus (E) ∼34 kPa). Substrates were coated with type I collagen solution (0.2 mg/ml) before cell culture.

Adhesion Assay

Control and pretreated MSCs were trypsinized and labeled with a transmembrane fluorescent viability marker, Calcein AM (Anaspec), in HBSS containing divalent ions Ca++ and Mg++. Then the cells could adhere for 3 h after stimulus was removed (t_0 + 3 h) in HBSS before taking an initial florescence reading in a DTX-800 Multimode Detector plate reader. A final reading was taken after removing non-adherent cells by washing with HBSS to determine the adherent fraction.

Traction Force Microscopy

Control and pretreated MSCs were seeded on collagen-coated PA substrates embedded with 200-nm fluorescent microbeads, for 24 h. Particle displacements were determined by comparison of the embedded bead positions before and after the cells were detached from the gels and used to determine traction forces with a Fourier-transform traction force cytometry in a custom-written MATLAB routine as previously described (Sabass et al., 2008).

Inhibitor Studies

PF573228, Ly294002, and SIS3 were used to inhibit FAK, PI3K, and SMAD3 mediated signaling, respectively. Range of concentrations for all the inhibitors were determined from literature reviews and MSCs were treated with 10 μM concentration (Jeon et al., 2008; Qureshi et al., 2008; Gharibi et al., 2012; Zhang et al., 2015, 2016). For adhesion studies cells control and pretreated cells were detached and suspended in inhibitor supplemented serum free media for 2 h before seeding on a 96-well plate. Then the cells could adhere for 3 h before taking an initial florescence reading. A final reading was taken after removing non-adherent cells by washing with HBSS to determine the adherent fraction. For both motility and scratch assay cells were treated with inhibitors for 2 h before adding the migration stimulating factor PDGF (15 ng/ml).

Motility Assay

Cells were treated with control and TGF-β1 supplemented media for 24 h in 10 cm dishes. Both control and pretreated MSCs were trypsinized and seeded on collagen coated 48-well plate at a sub-confluent density for 12 h. Wells were then washed to remove detached cells before staining with Hoechst 33,342 for 30 min. Cells were then treated with soluble factors before placing them on Nikon Eclipse Ti inverted epifluorescent microscope, which was maintained at 37°C and 5% carbon dioxide throughout the experiment using an *In vivo* Scientific environmental cell chamber. Images of the nucleus were taken using DAPI filter (excitation 358 nm and emission 461 nm) at multiple points at 12 min- interval for 6–8 h at 10x magnification. The locations of cell nuclei, segmented from fluorescent images, were tracked in MATLAB to define cell traces. The cell migration coefficients and directional velocities were determined by fitting the traces to the persistent random walk model (Dickinson and Tranquillo, 1993). Briefly, mean square displacements were calculated (MSD = $d^2(\tau)$) from the two-dimensional tracking data and was used for fitting the following equation: (where, d = displacement, t = time, P = persistence time, and μ = migration coefficient).

$$\langle d^2(\tau)\rangle = 4\mu\left\{t - P\left[1 - e^{-t/P}\right]\right\}$$

Scratch Assay

Cells were cultured as a confluent monolayer on collagen coated 48-well plate and a gap was created by removing cells with a pipette tip. Wells were then washed to remove detached cells before staining with Calcein AM. Cells were then treated with soluble factors before placing them on Nikon Eclipse Ti inverted epifluorescent microscope, which was maintained at 37°C and 5% carbon dioxide throughout the experiment using an *In vivo* Scientific environmental cell chamber. FITC green channel (495 nm excitation and 515 nm emission) images were taken at multiple points at 15-min interval for 12 h at 10x magnification.

Statistics

Each experiment was performed with 3 or more replicates, and all values expressed as the mean ± s.e.m. For comparison between two groups student *t*-test was used. One way Anova test with repeated measures was used to determine statistical significance of experiments involving more than two groups. For comparison between groups, Tukey's HSD post-test was used. Significance was reported as * (for $p < 0.05$), ** (for $p < 0.005$), and *** (for $p < 0.0005$).

RESULTS

TGF-β1 Pretreatment of MSCs Enhances Their Distribution in Wound Bed

MSCs have previously been used to increase the rate of wound closure in full thickness skin wounds; however, untreated MSCs used in these studies resulted in a modest change in the rate of wound healing and long-term engraftment of MSCs was not clearly established. We used a syngeneic mouse wound healing model to evaluate the effect of TGF-β1 pretreatment on dissemination of MSCs *in vivo*. Fluorescently labeled MSCs (control and TGF-β1 pretreated) were injected at the periphery of full thickness wounds in Balb/C mice. At day 0 the ratio of TGF-β1 to control MSCs treated wounds for all the mice were close to 1 suggesting similar areas; however, after day 5 normalized wound areas were ∼15% reduced suggesting significant wound closure rate with TGF-β1 pretreated MSCs compared to untreated cells (**Figures 1A,B**, Supplementary Figure 3). By day 7, wounds were

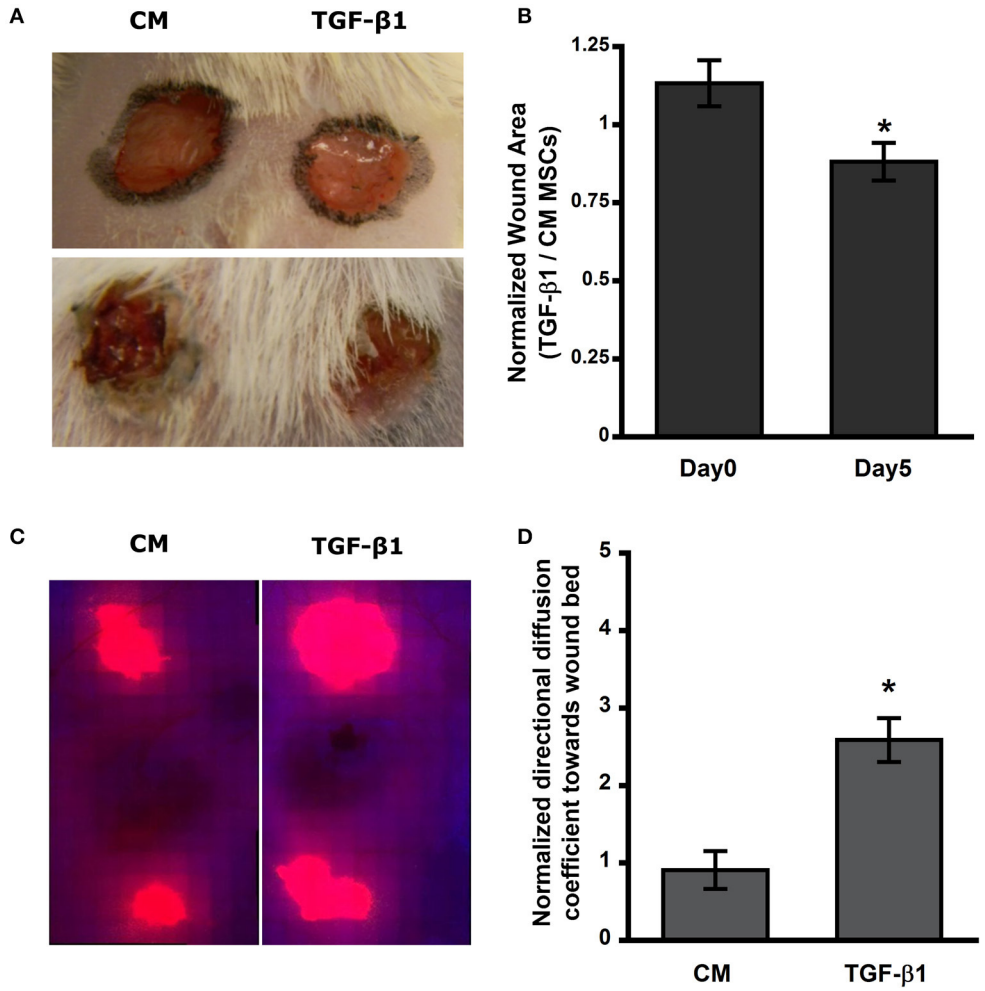

FIGURE 1 | Transforming growth factor–β1 (TGF-β1) pretreated mesenchymal stem cells (MSCs) migrate and distribute efficiently in the wound bed and improve wound closure rate in Balb/C mice. (A) Full thickness skin wound on Balb/C mice at day 0 and 5. **(B)** Open area of TGF-β1 pretreated MSCs injected wound normalized to its respective control. **(C)** Thresholded fluorescence images of skin tissue showed enhanced distribution of MSCs toward center of the wound. **(D)** Quantification of directed distribution of MSCs toward the center of the wound ($n = 3$). Values given as mean ± s.e.m.; significance is indicated as *($p < 0.05$).

mostly closed (Supplementary Figure 4). However, by analyzing the labeled MSCs in the wound bed we found that TGF-β1 pretreated cells displayed more efficient distribution toward the center of the wound (**Figures 1C,D**).

Sustained Morphological Changes at 24 h After TGF-β1 Pretreatment

We have previously demonstrated that MSCs undergo dramatic elongation in response to TGF-β1 treatment (Ghosh et al., 2014). To examine the persistent effect of TGF-β1 on cell shape, pretreated MSCs were detached and reseeded on a new surface (TCP) with serum free media in the absence of stimulus for 24 h (t_0 + 24 h). Cells were fixed and stained with crystal violet to analyze cell morphology using a cell shape factor (CSF) which varies from 0 for a line to 1 for a perfect circle. TGF-β1 pretreated cells retained the elongation effect even after 24 h (t_0 + 24 h; **Figure 2A**). Control MSCs retained their spindle shape leading to CSF close to 0.5. Still the cell shape factor of TGF-β1 pretreated cells was significantly lower (∼0.3) than its control counterpart indicating dramatic elongation ($p < 0.05$; **Figure 2B**).

TGF-β1 Pretreatment Enhances Integrin Expression and Adhesive Strength of MSCs

Next we sought to analyze the sustained effect of pretreatment on adhesive strength of MSCs using a centrifugation force based assay at 24 h ($t_0 + 24$ h). TGF-β1 pretreated cells maintained their higher adhesivity to ECM and exhibited a 4.2-fold increase in the adherent fraction compared to control (**Figure 2C**). To better elucidate the role of cell surface adhesion molecules that control cell-cell and cell-ECM interaction, we analyzed integrin subunits α_v (CD51), β_1 (CD29), β_3 (CD61), and vascular cell adhesion molecule-1 (VCAM-1, CD106) using flow cytometry at this time point (t_0 + 24 h; **Figure 2D**, Supplementary Figure 5). TGF-β1 pretreated cells displayed significantly upregulated expressions of the integrins while reducing VCAM-1 expression (**Figure 2D**).

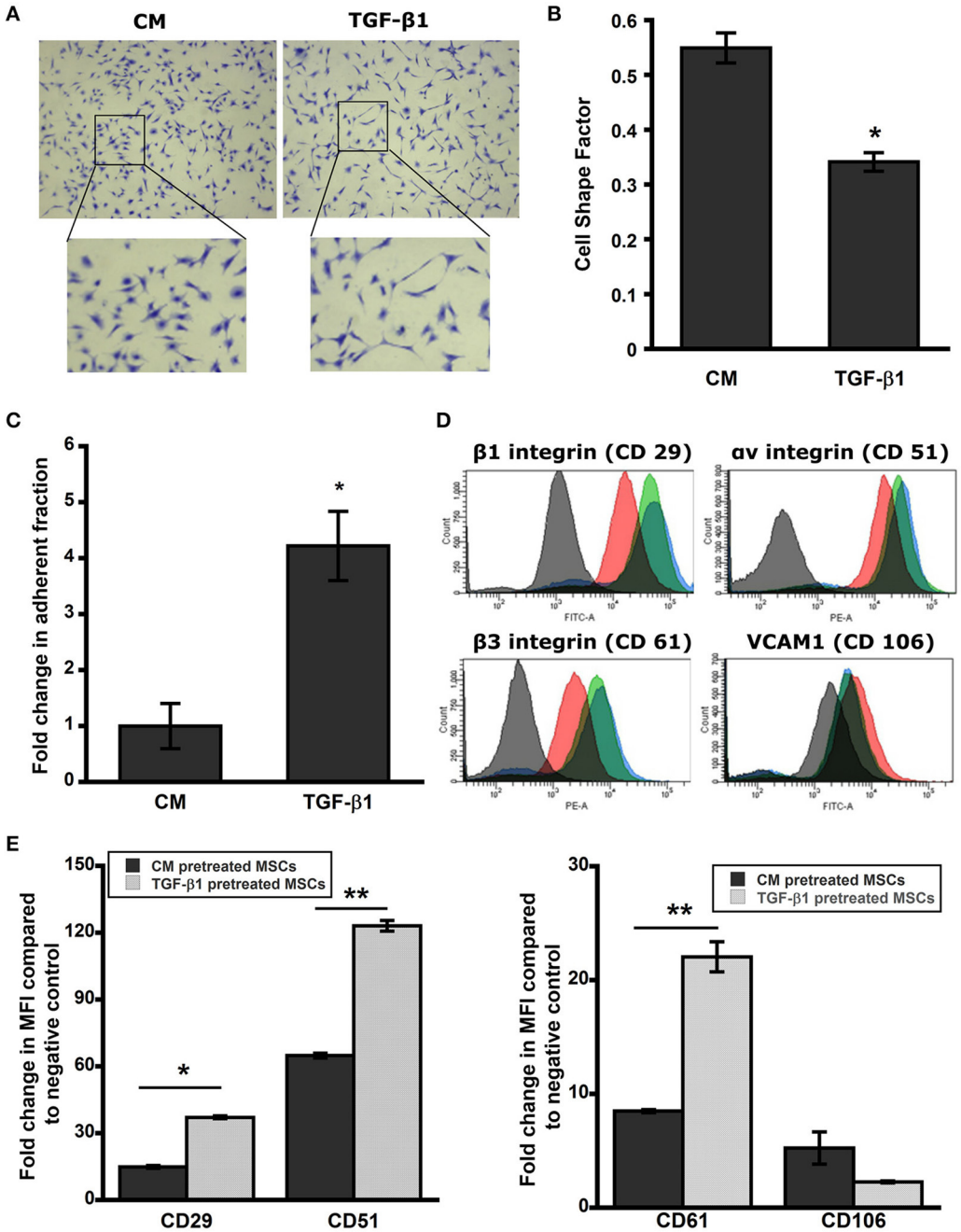

FIGURE 2 | Pretreated MSC maintain elongated phenotype. (A) Brightfield images of pretreated MSCs after 24 h stained with crystal violet. TGF-β1 pretreated MSCs sustained elongated morphology in serum free media for a period of 24 h. **(B)** Cell shape factor (CSF) was determined by analysis of bright field images with Image J. CSF was used to characterize the elongation of the cell, with a shape factor of 1 indicating a perfect circle and 0 indicating a straight line. Results are reported as average ± s.e.m. ($n = 2$). **(C)** The centrifuge-based adhesion assay was used to determine the effect of soluble factor pretreatment on the adhesion of MSCs on uncoated surface. **(D)** TGF-β1 pretreated MSCs maintain surface adhesion characteristics. Histograms from flow cytometry were analyzed using FACS-DIVA for mean fluorescence intensity (MFI). Expressions of surface integrins αv (CD51), β1 (CD 29), and β3 (CD61) were increased significantly in TGF-β1 pretreated cells compared to the control; whereas TGF-β1 pretreatment reduced VCAM1 (CD106) expression significantly compared to the control. **(E)** Analysis of cell surface adhesion molecules α_V (CD51-PE), β_1 (CD29-FITC), and β_3 (CD61-PE) integrins, and VCAM-1 (CD106-FITC) using flow cytometry after 24 h of removal of stimulus. Black and red indicates negative and control cell population; whereas green and blue represent TGF-β1 pretreated cells at immediately (t_0) and 24 h ($t_0 + 24$ h) after the removal of stimulus respectively. TGF-β1 pretreated cells exhibit higher integrin expression and lower VCAM-1 expression at 24 h after removal of the stimulus compared to control cells. Results are reported as average. ($n = 2$). Values given as mean ± s.e.m.; significance is indicated as *($p < 0.05$), **($p < 0.005$).

Mean fluorescence intensity (MFI) analysis of histograms further confirmed the trends of integrin upregulation and VCAM-1 downregulation due to TGF-β1 pretreatment (**Figure 2E**). This result correlated with the observed adhesive strengthening response.

Pretreated MSCs Maintain Adhesive Phenotype on Wound-Mimetic Substrates

To better understand the difference between pretreated MSCs and control during the initial stages of attachment in the wound bed, we used compliant semi-flexible PA substrate with stiffness (E ∼34 kPa) like fibrotic skin wound (Goffin et al., 2006; Discher et al., 2009). TGF-β1 pretreated MSCs seeded on both collagen coated glass and PA gel substrates for 3 h (t_0 + 3 h) exhibited >1.7-fold increase in adherent fraction compared to control ($p < 0.05$; **Figure 3A**). Furthermore, F-actin staining with Phalloidin revealed that the TGF-β1 pretreated cells spread more rapidly and display a well-defined cytoskeletal organization compared to its untreated counterparts (**Figure 3B**). We hypothesized that previously seen change in integrin expression may alter the traction force exerted by the MSCs on PA substrates. At 24 h (t_0 + 24 h) traction force maps of TGF-β1 pretreated MSCs displayed very different force distribution compared to control cells (**Figure 3C**). Pretreated MSCs displayed very high forces localized around the edges without any apparent polarization. Also, the magnitude of overall traction forces exerted by the TGF-β1 pretreated cells was significantly higher compared to the control cells (**Figure 3D**).

TGF-β1 Pretreated MSCs Displayed Enhanced Cell Motility *In vitro*

Control and pretreated MSCs were seeded on 48-well plate to evaluate the effect of soluble factors on cell motility. In presence of pro-migratory soluble factors PDGF (15 ng/ml) and IGF-1 (30 ng/ml), the migration coefficients were determined by tracking the cell nuclei. Both PDGF and IGF-1 enhanced random

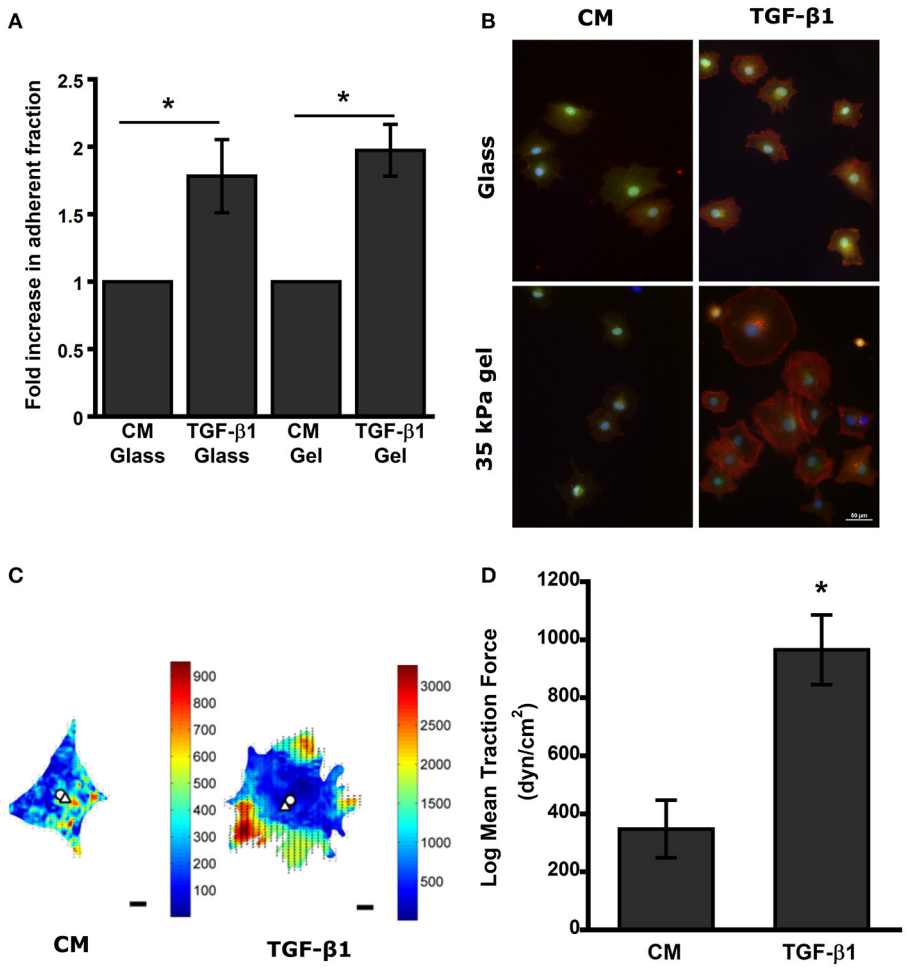

FIGURE 3 | TGF-β1 pretreated MSCs display higher adhesivity and traction force after removal of stimulus. (A) TGF-β1 pretreated cells display higher adhesivity on both glass and gel. **(B)** Pretreated MSCs (CM vs. TGF-β1) plated on glass and PA gels were washed after 3 h to remove detached cells. (Red-Actin, Blue- DAPI, Green- Calcein AM). **(C)** Traction force heat map of CM and TGF-β1 treated MSCs, **(D)** TGF-β1 pretreated MSCs exerted higher traction force compared to control after 24 h' removal of stimulus. Values given as mean ± s.e.m.; significance is indicated as *($p < 0.05$).

cell migration speed of MSCs; however, significant increase in migration was observed for TGF-β1 pretreated cells compared to control (**Figures 4A,B**).

FAK Signaling is Essential for Adhesive Strengthening and Enhanced Motility

TGF-β1 pretreatment had profound effects on *in vitro* function of MSCs including, adhesion and migration. TGF-β1 regulates cell functions via both canonical and non-canonical pathways. To further elucidate roles of key signaling pathways in enhanced cell functions, we blocked following pathways using small molecule inhibitors: (1) integrin-FAK signaling using PF573228, (2) TGF-β signaling via SMAD2/3 using SIS3 and (3) growth factor mediated PI3K signaling with Ly294002. Interestingly, only FAK inhibitor PF573228 could inhibit TGF-β1 mediated enhanced migration of MSCs as the difference in migratory activity between TGF-β1 treated cells and control were completely abrogated by FAK inhibitor (**Figures 4C,D**). To further evaluate the effects of the inhibitors on directed migration of MSCs, we used an *in vitro* scratch assay that mimics the wound site. Cells migrated toward

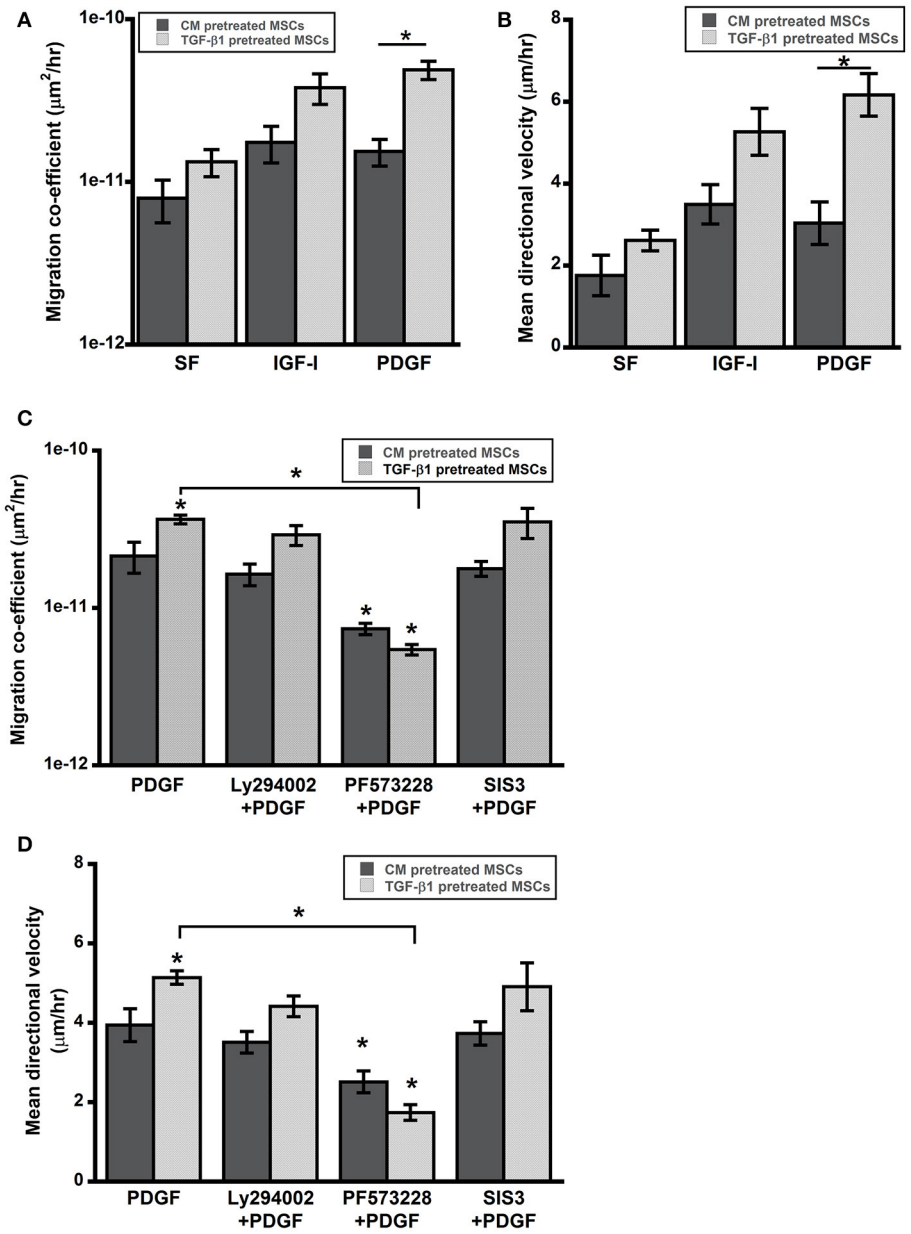

FIGURE 4 | TGF-β1 pretreated MSCs displayed enhanced cell motility. (A,B) Cell motility of pretreated MSCs was evaluated in presence of pro-migratory factors PDGF-BB and IGF-I (present in wound bed). **(C,D)** Enhanced motility of TGF-β1 pretreated MSCs in presence of PDGF (15 ng/ml) was abrogated by FAK inhibition (PF573228) but not by PI3K (Ly294002) or SMAD3 (SIS3) inhibition. Values given as mean ± s.e.m.; significance is indicated relative to control unless otherwise noted, *($p < 0.05$).

an artificially created gap to establish new cell-cell contact under the influence of PDGF (30 ng/ml). Consistent with the previous results FAK inhibitor treatment blocked MSC migration toward the gap (**Figures 5A,B**). SMAD3 inhibitor, SIS3 was not able to block TGF-β1 pretreatment dependent directed migration, implicating a SMAD2/3 independent non-canonical pathway. Next we analyzed effect of FAK and SMAD3 inhibitors on initial adhesion on TCP after 3 h and found that both pathways have significant effect in reducing adhesion (**Figure 5C**).

DISCUSSION

Wound healing is a very complex process and understanding the coordinated action of the different types of cells and niche

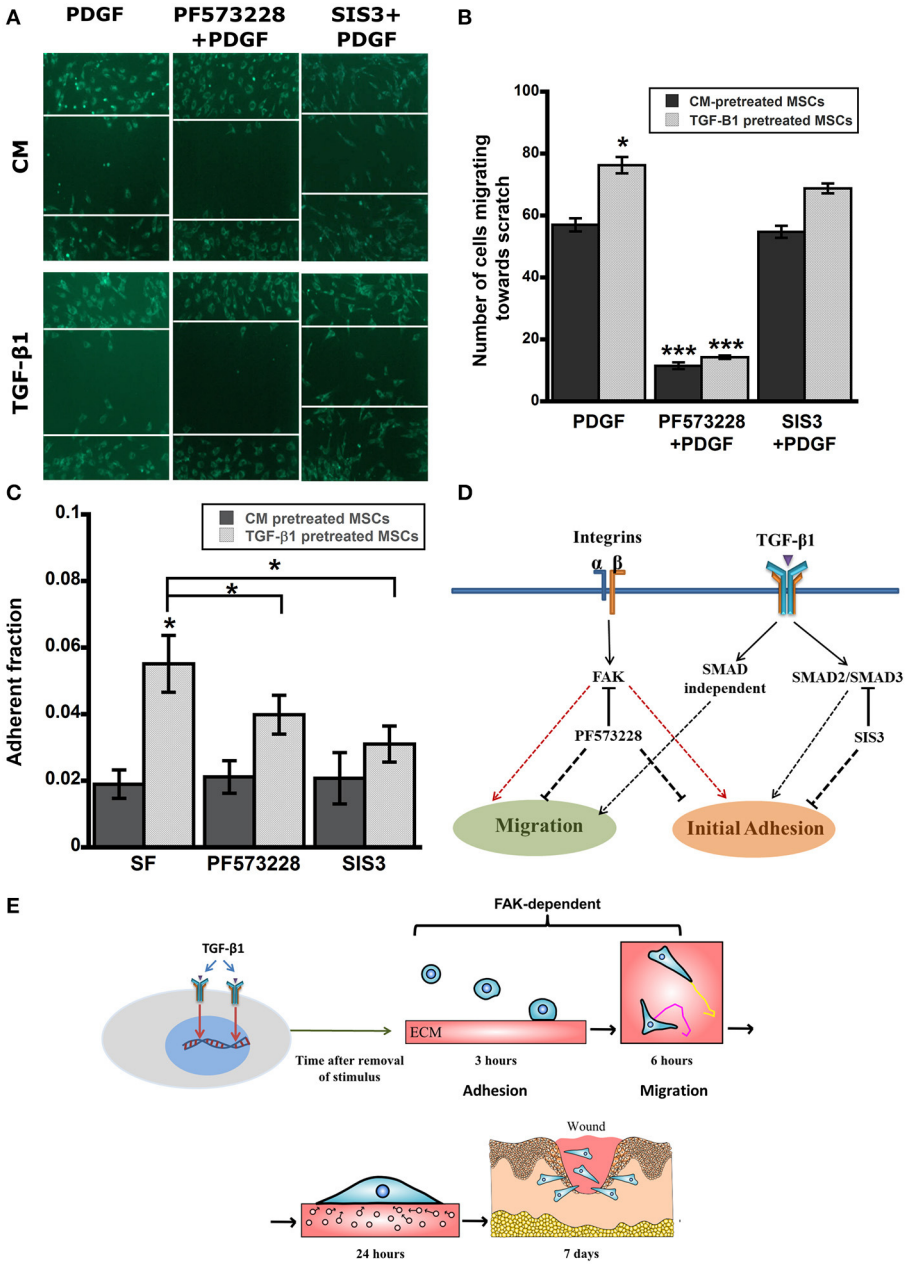

FIGURE 5 | (A) Scratch assay was used to evaluate directed migration of pretreated MSCs in presence of inhibitors (12 h). **(B)** TGF-β1 pretreated MSCs displayed higher directed cells migration toward scratch in a SMAD2/3 independent but FAK dependent manner. **(C)** Enhanced Adhesive strength of TGF-β1 pretreated MSCs was both FAK and SMAD3 dependent. Pretreated MSCs (CM vs. TGF-β1) plated on TCP were washed after 3 h to remove detached cells. **(D)** A simplified diagram demonstrating the effects TGF-β1 pretreatment on adhesion and migration via integrin-FAK and SMAD pathways. **(E)** Proposed mechanism of TGF-β1 pretreated MSCs leading toward improved *in vitro* functions and enhanced *in vivo* wound healing. Values given as mean ± s.e.m.; significance is indicated relative to control unless otherwise noted, *($p < 0.05$), ***($p < 0.0005$).

specific soluble and insoluble factors is of utmost importance for the development of modern wound care. Growth factors and bioactive materials have been used increasingly in recent times; however, on their own, there efficacy remains limited (Greer et al., 2013). Recent studies have highlighted the benefits of using mesenchymal stem cells for wound healing that holds high hope for the future of the wound care (Wu et al., 2007a; Jackson et al., 2012; Isakson et al., 2015; Duscher et al., 2016). However, characterization of MSCs with niche specific factors is still in its early stages. In this study, we have investigated interaction between mesenchymal stem cells and TGF-β1 a growth factor that plays an important role both in normal wound healing and in fibrosis(Gilbert et al., 2016; Meng et al., 2016). The current study was designed to determine if TGF-β1 induces a persistent alteration in MSC phenotype, leading to more efficient function in the wound bed *in vivo*.

Non-healing wounds are often characterized with loss or lack of recruitment of motile cells in the wound bed that are required to close the formed gaps. Even MSCs added locally to the site often form island like structure with visible necrosis (Yang et al., 2005). Previous studies with hypoxic preconditioning and soluble factor (e.g., EGF) pretreatment have shown to enhance paracrine effects of MSCs as well as increase in cell migration *in vitro* and hypothesized that these outcomes will translate to improved wound healing *in vivo* (Lee et al., 2009; Tamama et al., 2010; Kim and Sung, 2012). We found that TGF-β1 pretreatment contributed to more efficient propagation of MSCs and increased wound closure rate *in vivo* (**Figure 1**). Further, analysis of the diffusion pattern of fluorescently labeled MSCs in our study revealed a 2.5-fold increase in distribution of TGF-β1 pretreated MSCs toward the center of the wound. This result agrees well with the outcomes of *in vitro* migration studies where TGF-β1 pretreated cells displayed increased directional migration compared to control cells (**Figures 4**, **5A,B**).

To further understand the function of implanted cells, we designed a series of *in vitro* experiments to analyze the cell–matrix interactions required for adhesion and migration. Adhesion studies at 3 h ($t_0 + 3$ h) with compliable PA gels (E ∼34 kPa) representing the skin tissue stiffness indicated that TGF-β1 pretreatment increased the initial attachment to substrate (Goffin et al., 2006; Discher et al., 2009). Subsequently, a centrifuge-based assay at longer time scales (t_0 + 24 h) also displayed similar trend with the measured adherent fraction. Interestingly, pretreated MSCs displayed more than 4-fold increase in the adherent cell fraction at 24 h (t_0 + 3 h) compared to the 2-fold increase measured in our washing study after 3 h (t_0 + 3 h; **Figures 2**, **3**). These results would account for the adhesive strengthening due to changes in integrin expression and the secretion of native ECM proteins by MSCs on uncoated surface (at t_0 + 24 h; Gallant and García, 2007; Michael et al., 2009). Previous studies have demonstrated enhanced ECM and other growth factor secretion from cells in response to TGF-β1 and transcriptional activation of the ECM genes such as collagen 1, fibronectin, and laminins were upregulated in previous microarray results (Supplementary Table 1; Ghosh et al., 2014). TGF-β1 pretreatment induced change in adhesion kinetics at 3 h may translate to more successful engraftment efficiency *in vivo*, whereas the adhesive strengthening at 24 h could increase long term survival and integration in wound bed. Additionally, TGF-β1 pretreated cells were more contractile on substrates and exerted almost 3-fold higher traction force (**Figures 3C,D**).

The phenotypical changes in MSCs due to TGF-β1 pretreatment that has been reported here are quite analogous to the changes associated with myofibroblast differentiation from fibroblast in wound bed with characteristics including, increase in both αv and β1 integrin expressions, increase in ECM production, and more motile phenotype (Thannickal et al., 2003; Lygoe et al., 2004; Häkkinen et al., 2011). Our analysis confirmed that TGF-β1 pretreated MSCs maintained an increased surface α_v and β1 integrin expression (**Figures 2D,E**) which has been correlated with enhanced migration both *in vivo* and *in vitro* and can be critical for the observed changes *in vivo* (Liu et al., 2010b; Veevers-Lowe et al., 2011; Saller et al., 2012; Koivisto et al., 2014). Myofibroblasts are essential for closure of the wound gap; however, chronic inflammation may hinder fibroblast recruitment to wound site and its differentiation into myofibroblast. TGF-β1 pretreated MSCs that are added locally can act as an alternative but further studies are required to characterize the functional difference between these two cell phenotypes.

All the previous results suggest that TGF-β1 pretreatment activates an integrin-FAK mediated mechanosensitive pathway and the sustained response of the cells can be related to formation of clusters between growth factor-receptors and integrins that increases and prolongs the signaling in synergy (Alam et al., 2007; Kim et al., 2011). To examine this hypothesis, three types of small molecule inhibitors were used: (1) Ly294002, a PI3K inhibitor was used as a control to ensure that the enhanced response was not due to cell proliferation, (2) PF573228 was used to block integrin mediated FAK activation, and (3) SIS3 was utilized to inhibit TGF-β1 mediated SMAD3 signaling. Ly294002 didn't alter the motility of MSCs indicating that the difference in response between treated and untreated cells is not an artifact of growth factor mediated proliferation. FAK activation has been shown to be essential for myofibroblast differentiation and wound healing and we here report that inhibiting FAK results in complete abrogation of adhesion and migration of MSCs in agreement with previous studies (Thannickal et al., 2003; Song et al., 2010). Finally, the effect of SIS3 on adhesion and migration of MSCs were quite interesting. SIS3 could block the enhanced adhesion of pretreated MSCs; however, it could not stop the directed migration of cells toward a scratch. Previous studies have correlated SMAD3 signaling inhibition with EMT switch, enhanced reepithelization and wound contraction (Ashcroft et al., 1999). In summary, the activation of FAK via integrins is essential for both SMAD3 dependent and independent signal transduction that are responsible for sustained phenotypical changes associated with TGF-β1 pretreatment *in vitro* (**Figure 5D**). Taken together

this study suggests that TGF-β1 pretreatment of MSCs can improve their attachment rate and motility *in vivo* via activation of FAK and improves their therapeutic efficacy in wound bed (**Figure 5E**).

CONCLUSIONS

The function of *ex-vivo* expanded MSC based therapeutics has been shown to be limited after reintroduction *in vivo*. This study illustrates that pretreatment of MSCs with TGF-β1 resulted in sustained improvement in migration, and adhesion even after removal of the stimulus *in vitro*. Subsequently, these pretreated cells enhanced the *in vivo* wound healing process, which may lead to improved therapeutic efficacy. Future studies with site specific factors will be used to guide new strategies for microenvironment specific MSC based therapy.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at Georgia Institute of Technology. The protocol was approved by the Institutional Animal Care and Use Committee at Georgia Institute of Technology (PHS Assurance Number 3822-01).

AUTHOR CONTRIBUTIONS

DG, DM, and MD designed the experiments. DG and DM performed the experiments and data analysis. DG, DM, and MD prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2017.00028/full#supplementary-material

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REVIEW
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Neural Stem Cell Plasticity: Advantages in Therapy for the Injured Central Nervous System

Linda Ottoboni †, *Arianna Merlini* † *and Gianvito Martino* *

Neuroimmunology Unit, Division of Neuroscience, Institute of Experimental Neurology, San Raffaele Scientific Institute, Milan, Italy

The physiological and pathological properties of the neural germinal stem cell niche have been well-studied in the past 30 years, mainly in animals and within given limits in humans, and knowledge is available for the cyto-architectonic structure, the cellular components, the timing of development and the energetic maintenance of the niche, as well as for the therapeutic potential and the cross talk between neural and immune cells. In recent years we have gained detailed understanding of the potentiality of neural stem cells (NSCs), although we are only beginning to understand their molecular, metabolic, and epigenetic profile in physiopathology and, further, more can be invested to measure quantitatively the activity of those cells, to model *in vitro* their therapeutic responses or to predict interactions *in silico*. Information in this direction has been put forward for other organs but is still limited in the complex and very less accessible context of the brain. A comprehensive understanding of the behavior of endogenous NSCs will help to tune or model them toward a desired response in order to treat complex neurodegenerative diseases. NSCs have the ability to modulate multiple cellular functions and exploiting their plasticity might make them into potent and versatile cellular drugs.

Keywords: neural stem cells, microenvironment, plasticity, metabolism, inflammation, stroke, multiple sclerosis, aging

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Ivana Gadjanski,
University of Belgrade, Serbia

Reviewed by:
Loic P. Deleyrolle,
University of Florida, USA
Marco Tatullo,
Tecnologica Research Institute, Italy

***Correspondence:**
Gianvito Martino
martino.gianvito@hsr.it

† *These authors have contributed equally to this work.*

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INTRODUCTION

Although it has been thought for a long time that mammalian neurogenesis occurs only during embryonic and perinatal stages, young neurons are continuously incorporated into the adult brain as demonstrated by Altman and Das already in the early sixties (Altman, 1962; Altman and Das, 1965). Indeed, neural stem cells (NSCs), residing in the brain of most adult mammals in the so-called "neurogenic niches," sustain neurogenesis throughout life. It is estimated that 700 new neurons are generated every day by the neuropoietic niche in an adult human hippocampus, outlining one aspect of the plasticity/renewal capacity of NSCs (Knoth et al., 2010; Spalding et al., 2013). As for other stem cells, the specialized microenvironment of the neurogenic niche ensures not only NSC self-renewal but also differentiation, mainly into neurons. However, neurogenesis is not the only activity of NSCs in the adulthood. As a matter of fact, recent studies indicate that adult NSCs residing within the sub-ventricular zone (SVZ) might physiologically exert alternative functions to cell replacement, the so-called non-neurogenic functions (Martino et al., 2014), mainly aimed at protecting CNS homeostasis, in both physiological and pathological conditions. They regulate and are regulated by several signaling pathways (Faigle and Song, 2013) that, tuning

the evolution of progenitor proliferation, division, and migration, can *per se* also impact the composition of the niche (Preston and Sherman, 2011; Gattazzo et al., 2014). Neighboring cells, the vasculature and the cerebrospinal fluid constitute the main routes through which molecular signals reach NSCs and affect their behavior.

Overall, knowing the physiological properties of NSCs and what changes in pathological conditions opens up the possibility of exploiting NSC plasticity for preventive/therapeutic purposes.

This review will primarily focus on (i) the properties of precursors of the adult neurogenic niches of the central nervous system (CNS); (ii) the mechanisms of inter- and intra-cellular communication of NSCs and other cells, resident or not in the niche, in physio- and patho-logical conditions, with focus on multiple sclerosis (MS) and ischemic stroke, neurodegenerative disorders of the brain that unfold acute and chronic consequences.

WHAT DEFINES A NSC AND A NSC NICHE?

At the onset of murine neurogenesis, at embryonic day 9.5, the precursors in the CNS are neuroepithelial cells (NECs) that form a tube with a central canal (Taverna et al., 2014). NECs are highly proliferative and initially divide symmetrically to expand; afterwards they convert into radial glial cells (RGCs) that divide both symmetrically and asymmetrically. Basal processes of RGCs are used by newborn neurons as guiding scaffolds while they migrate away from the germinal niche toward the pial surface.

Although most CNS regions largely extinguish their NSC pool after development, discrete areas of the adult brain retain NSCs and active neurogenesis throughout life (Ming and Song, 2005, 2011). Namely, the striatal subventricular zone (SVZ) and the hippocampal dentate gyrus (DG, subgranular zone SGZ) are the most extensively characterized adult neurogenic niches. However, according to the most recent evidences, sites of neurogenesis are present also in the ependyma (Alvarez-Buylla and Lim, 2004; Bjornsson et al., 2015), near the third and fourth ventricle, in the forebrain, in the striatum, in the amygdala, in the hypothalamus, in the substantia nigra and in the subcortical white matter or spinal cord root ganglia (Bernier et al., 2002; Lie et al., 2002; Kokoeva et al., 2005; Chang et al., 2008; Ernst et al., 2014; Muratori et al., 2015; Stolp and Molnar, 2015). Proliferating cells from those regions, namely somatic NSCs, can be isolated and established as virtually perpetual cell lines in response to fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) similar to their embryonic counterparts (Temple, 2001).

In the adult neural stem cell niche, NSCs, immature neurons, supporting astrocytes, blood vessels and epithelial ciliated cells are in close contact and the vasculature with "leaky" features supports adult neurogenesis (Butti et al., 2014). In the mouse, the SVZ contains slowly dividing progenitors that can be subdivided into two types: type B1 cells, in close contact with both the cerebrospinal fluid (CSF) and the blood vessels of the SVZ, and type B2 cells, closer to the striatum (Ihrie et al., 2011). B1 cells give rise to transit amplifying cells (type C cells), located in close proximity to blood vessels, and along with B2 cells, they form a glial supportive sheath around their more differentiated progeny and migrating neuroblasts, type A cells, that originate from type C cells. Type A cells migrate tangentially to form the rostral migratory stream (RMS) to the olfactory bulb for terminal differentiation. Once in the olfactory bulb, the neuroblasts defasciculate from the stream and migrate radially to their site of terminal differentiation into neurons (Alvarez-Buylla et al., 2000). SVZ-NSCs give rise also to oligodendrocyte precursors and mature oligodendrocytes, continuously replenishing cells in the corpus callosum (Menn et al., 2006).

The primary role of the neurogenic SGZ niche instead is to generate new granule cells, primary excitatory neurons that support hippocampus-dependent cognitive functions (Zhao et al., 2008). Stem cells of the SGZ give rise to radial astrocytes that convert into immature progenitors (Type 1, the counterpart of type B in the SVZ) and eventually into neuroblasts (Type 2, the counterpart of Type C-A cells in the SVZ) (Zhao et al., 2008). Complete depletion either of type 2 or type C cells, respectively in the SGZ and SVZ, (non-radial glia like cells) stops neurogenesis (Doetsch et al., 1999; Ahn and Joyner, 2005), although, but infrequently, dividing radial glia could sustain neurogenesis as well (Seri et al., 2004). NSCs of the SGZ are also in close contact with blood vessels and endothelial cells, that act as scaffolding cells for NSCs (Palmer et al., 2000) and play a major role in directing NSC specification (Shen et al., 2004, 2008; Tavazoie et al., 2008; Kokovay et al., 2010).

This is the conventional cell classification for the mouse neural germinal center, which has been better characterized.

In humans the SVZ differs from the one in rodents because it organizes into four layers instead: the ependymal layer, the hypocellular gap, the astrocytic ribbon, and the transitional zone to the parenchyma, rich in myelin and oligodendrocytes (Quinones-Hinojosa et al., 2006). Migrating neuron-like cells can occasionally be found in the layers II and III as individual cells (Sanai et al., 2004; Quinones-Hinojosa et al., 2006). In detail, layer I consists of an ependymal monolayer lining the ventricular wall with astrocytic processes contacting the ventricular wall. Layer II, also known as the gap region, is rich in $GFAP^+$ processes, with only some neuroblasts in the anterior regions. Ependymal cells send basal processes into Layer II, making critical contacts with the underlying basal lamina. Layer II may function as the corridor for neuroblast migration. Layer III is the proliferative region of the human SVZ, with $GFAP^+/Ki67^+$ and $CD133^+$ cells. Few neuroblasts are present in the human SVZ, compared to the rodent, and are found mainly in Layer III. Some ependymal cells, which typically comprise the epithelial barrier of the ventricles, have motile cilia and are found in small clusters (4–14 cells) in Layer III (Quinones-Hinojosa et al., 2006; Kam et al., 2009). Layer IV represents the first portion of brain parenchyma away from the parietal ventricle and where the first evidence of neurons is found.

EXTRACELLULAR CUES AND INTRINSIC GENETIC PROGRAMS CONTROL THE NSC FUNCTIONS IN PHYSIOLOGICAL CONDITIONS IN THE ADULT BRAIN

Main feature of NSCs is their plasticity (Suh et al., 2009; Martino et al., 2011). The two major adult stem neurogenic niches take

advantage of different mechanisms to exploit this property, which manifests as self renewal capacity, quiescence, metabolic modulation, homing, differentiation capacity, cellular cross-talk, and immune surveillance (**Figure 1A**, **Table 1**).

The maintenance of the neurogenic niche itself and the renewal capacity depends on active intrinsic genetic (Tirone et al., 2013) and epigenetic (Liu et al., 2010; Yao et al., 2016) programs along with microenvironment-dependent specific properties. Autocrine regulators of NSC proliferation, such as transforming growth factor α (Tropepe et al., 1997; Guerra-Crespo et al., 2009) and β (Dias et al., 2014), amphyregulin, fibroblast growth factor-2 (FGF-2), insulin-like growth factor 2 (IGF2) (Marques et al., 2011) are released from specific subsets of NSCs along with leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) that promote proliferation (Emsley and Hagg, 2003; Lee et al., 2013) and sphingosine-1-phosphate (S1P) or prostaglandin D2 (PGD2) that instead maintain quiescence (Codega et al., 2014; Chaker et al., 2016). The environment-contribution to NSCs plasticity encompasses several elements as described below.

Close anatomical association between NSCs and the vascular structure (vascular niche) is preserved both in the SVZ and SGZ and vascular endothelial cells of the niche secrete Notch ligands Jagged1, Jagged2, and Delta-like-4, crucial factors for self-renewal and neurogenesis (Shen et al., 2004; Androutsellis-Theotokis et al., 2010; Lu et al., 2011). Recent evidence supports the hypothesis that the cross-talk between blood vessels and NSCs is bi-directional: NSCs can indeed provide juxtacrine and paracrine signals to drive endothelial cells (Chou and Modo, 2016) and promote angiogenesis (Hicks et al., 2013). Moreover, vascular endothelial growth factor (VEGF) is a shared cue both for angiogenesis and neurogenesis because on one side it promotes the angiogenic development of capillaries, on the other, the secretion of neurogenic molecules by proximal endothelial cells (Jin et al., 2002; Cao et al., 2004; Kim et al., 2004; Udo et al., 2008; Ruiz de Almodovar et al., 2009). Further, other cues secreted from vascular endothelial cells such as neurotrophin 3 (NT3) or betacellulin (BTC) maintain quiescence or promote proliferation of NSCs, respectively (Gomez-Gaviro et al., 2012; Delgado et al., 2014), although there is evidence of NT3 effect on differentiation (Shimazu et al., 2006), while stromal derived factor-1 (SDF1) stimulates the motility of type A, B, and C neuroblasts (Kokovay et al., 2010).

The extracellular matrix (ECM), a dynamic and complex environmental element characterized by biophysical and biochemical properties specific for each tissue and able to regulate cell behavior, represents also an essential player in stem cell niche (Gattazzo et al., 2014). Extensions of the extracellular matrix known as fractones project from the blood vessels of the subventricular plexus as thin, highly branching ECM stalks that expand into bulbs where they contact the basal surface of the ependymal layer (Mercier et al., 2002). Fractones are enriched in laminin, heparan sulfate, perlecan, nidogen, and collagens. Those associations are able to bind several growth factors, suggesting that they may play a role in concentrating, activating, and presenting trophic factors to cells within the niche (Kerever et al., 2007).

NSCs receive inputs also from other cells such as microglia which reside in close proximity to NSCs of the niche. Indeed, resting microglia secrete factors that promote NSC niche maintenance and, at the same time, astrocyte differentiation of striatal NSCs *via* the Jak/Stat3 pathway (Zhu et al., 2008). Moreover, microglia contribute to the development of cytoarchitectonic and functional differences across cortical areas of the brain, secreting growth factors, and cytokines that tightly regulate the neurogenic process (Kim and de Vellis, 2005; Harry, 2013; Su et al., 2014). Conversely, *in vitro,* microglia promote neuronal differentiation, but not maintenance or self-renewal (Walton et al., 2006). On the other side, activated microglia inhibit neurogenesis (Sierra et al., 2014) favoring gliogenesis *via* tumor necrosis factor-α (TNFα) (Carpentier and Palmer, 2009), and when exposed to interleukin 4 (IL4) and interferon-γ (IFNγ), they secrete insulin-like growth factor 1 (IGF1) and promote neuronal differentiation of NSCs (Butovsky et al., 2006). Conversely, NSCs can also influence microglia *via* VEGF that in turn modulates microglial activation, proliferation and phagocytosis (Mosher et al., 2012).

Stem plasticity is modulated by other cell types as well, namely astrocytes, residing in close proximity with NSCs both in the SVZ and in the SGZ. Their contribution to NSC proliferation is likely exploited *via* ATP release (Cao et al., 2013) while Wnt3, neurogenesin-1 (NG1), thrombospondin-1 (TSP1) as well as interleukin-1β (IL1β) and interleukin-6 (IL6) promote hippocampal neurodifferentiation (Ueki et al., 2003; Lie et al., 2005; Barkho et al., 2006; Lu and Kipnis, 2010). Of note, when FGF2-producing astrocytes age, neurogenesis is impaired (Shetty et al., 2005).

Signals arising from the ependymal and meningeal cells and released in the CSF may also influence NSC activity (Lim et al., 2000; Siegenthaler et al., 2009). Indeed, NSCs possess primary cilia which sense liquor morphogens, such as FGF2, IGF2 (effective at lower level postnatally), Wnt and Sonic Hedgehog (SHH) (Corbit et al., 2005; Rohatgi et al., 2007; Breunig et al., 2008; Kim et al., 2010; Ihrie et al., 2011; Lehtinen and Walsh, 2011) and, possibly, the CSF flow itself. The latter indeed can, *via* mechano-sensing signaling, promote proliferation and differentiation (Li et al., 2011; Arulmoli et al., 2015; Jagielska et al., 2017).

Moreover, cytoarchitectonic innervation *via* GABA (γ-Aminobutyric acid)-, glutamin-, colin-, serotonin-, and dopamin-ergic neurons sustains neurogenesis in the niche (Suh et al., 2009; Song et al., 2012; Paez-Gonzalez et al., 2014; Young et al., 2014; Alunni and Bally-Cuif, 2016; Chaker et al., 2016). Conversely, it is not clear yet whether NSCs have an impact on axons and neuronal circuitry (Zhang Y. et al., 2016).

The niche is also very much dependent on and prompt to metabolic changes. While lipid metabolism maintains proliferation and neurogenesis (structural and energy support), glycolysis regulates NSCs development and differentiation (Knobloch et al., 2013). The metabolic activity strongly depends on oxidative saturation because in mammalian CNS oxygen regulates the growth and differentiation state of stem cells (De Filippis and Delia, 2011; Ivanovic and Vlaski-Lafarge, 2016; Sandvig et al., 2017). Dividing progenitor cells depend more on glycolysis, whereas differentiated progeny relies on energetically efficient oxidative phosphorylation occurring at low oxygen concentration. Apart from those, other important

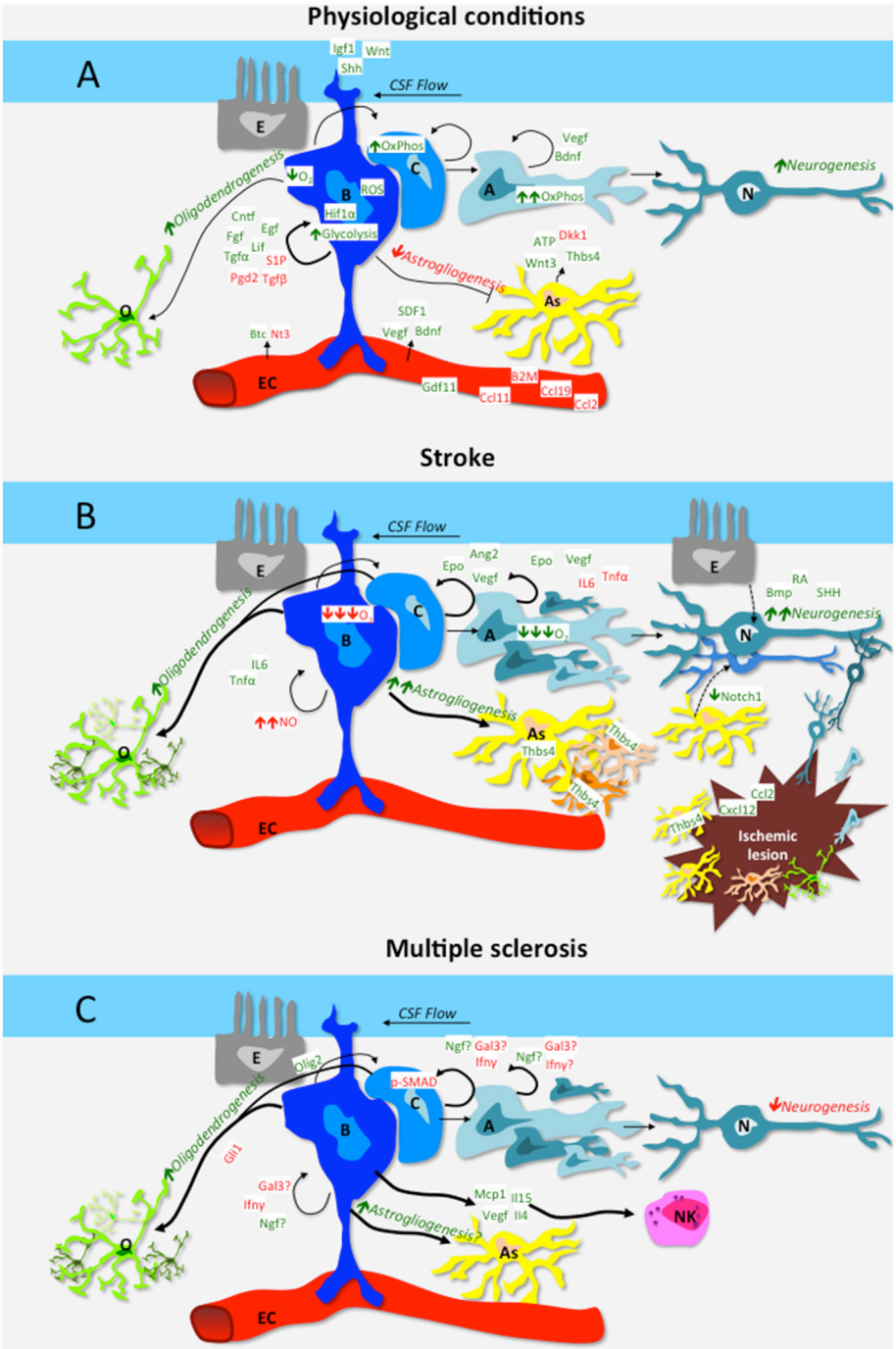

FIGURE 1 | Schematic representation of the interplay among cells of the neural stem cell niche of the subventricular zone (A,B,C) , vascular endothelial cells (EC), ependymal cells (E), differentiated oligodendrocytes (O), astrocytes (As), and neurons (N). Green is used for positive regulators of neural stem cell function, red for inhibitory regulators. Mechanisms are in italic. **(A)** depicts mechanisms and factors in physiological conditions: in steady-state, B cells self renewal is promoted by niche-derived factors such as CNTF, EGF, FGF2, LIF, PGD2, S1P, and TGFα, as well as by systemic-derived factors as VEGF, BDNF and SDF1. The cerebrospinal fluid also contributes actively to niche homeostasis *via* IGF1, Wnt and Shh that signal to B cells *via* their apical cilium. Aging increases neurogenesis-inhibitory factors such as B2M, CCL2, CCL11, CCL19, while pro-neurogeneic factors as GDF11 decrease. The hypoxic milieu of the niche favors B cell quiescence, while C and A precursors rely on oxidative phosphorylation. In steady-state, astrogliogenesis is generally inhibited, while growing astrocytes secrete both pro-neurogeneic and anti-neurogeneic mediators. Nonetheless, a basal level of oligodendrogenesis and in particular neurogenesis occurs also during the steady state. **(B)** depicts mechanisms and factors that are altered in the SVZ niche in the context of stroke. Ischemia increases Epo, Ang2 and VEGF as well as morphogens BMP, RA and SHH, which stimulate neurogenesis. Moreover, chemotactic and growth factors produced within the lesion (e.g. CXCL12, CCL2) guide newly formed glial and neuronal cells toward the ischemic area. Hypoxia and increased nitric oxide inhibit B cell cycling while low O_2 promotes precursor differentiation. Direct

(Continued)

FIGURE 1 | Continued
transdifferentiation (dashed arrow lines) from ependymal cells and astrocytes to neurons might also contribute to stroke-induced neurogenesis. Stroke *per se* increases oligodendrogenesis and astrogliogenesis as well. In particular, SVZ-derived, Thbs4 positive astrocytes are pivotal in containing tissue damage and preventing hemorrhagic transformation. **(C)** depicts mechanisms and factors that are altered in the SVZ niche in the context of MS. Neurogenesis in inhibited by IFNγ, Gal3 and upregulation of phoshorylated-SMAD (pSMAD) in neurogenic precursors. IFNγ also inhibits oligodendrogenesis *via* upregulation of Gli1. NSCs produce a wide array of soluble mediators, including IL15 that attract NK cells, which in turn contribute to the neurogenic niche dysfunction observed in MS models.

metabolic pathways are active in neural stem cells, such as (i) glycogen synthesis or glutamine/folate metabolism (Goodman and Hajihosseini, 2015); (ii) phosphatidylinositol 3-kinase/AKT (PI3K/AKT) growth factor pathway insulin-dependent; (iii) mTOR pathway nutrient-dependent (Rafalski et al., 2012); (iv) AMP-activated protein kinase (AMPK)/LKB1 pathway, sensor of intracellular adenosine monophosphate (AMP) to ATP ratios, and (v) the sirtuin pathway, metabolic sensors of NAD (nicotinamide adenine dinucleotide) level and epigenetic repressors (Folmes et al., 2012; Shyh-Chang et al., 2013).

Cell to cell contact has also been shown to play a role in exploiting the plasticity of NSCs. Astrocytes of the niche negatively control neuronal differentiation through astrocyte-secreted factors such as insulin like growth factor binding protein 6 (IGFBP6) and decorin (Barkho et al., 2006; Wilhelmsson et al., 2012), while astrocytic ephrin-B2 positively regulates proliferation (Ashton et al., 2012).

Another peculiar property of NSCs consists in their capacity to migrate where their replacement or bystander effect is needed. Indeed, endogenous NSCs migrate out of the niche at physiological rate to maintain brain homeostasis, either differentiating or releasing tropic factors (Shen et al., 2004; Kokaia et al., 2012). When transplanted during acute or chronic neuroinflammatory disorders, NSCs show remarkable pathotropism: they follow the molecular gradient of chemotactic inflammatory factors (Muller et al., 2006) and reach the damaged site where they start secreting a series of molecules (i.e., bone morphogenetic protein 4, noggin, Notch, Jagged, and SHH) to recapitulate the microenvironment of the SVZ niche (atypical ectopic perivascular niche) (Pluchino et al., 2003, 2010; Irvin et al., 2004; Stidworthy et al., 2004; Martino and Pluchino, 2006; Bonaguidi et al., 2008).

Of note, beside communication *via* soluble factors, NSCs sense and can release intracellular messengers wrapped in vesicles (Cossetti et al., 2014). Although their role in adult NSCs is almost unexplored (more is known for other types of stem cells), vesicles can transfer information in the form of mRNA, ribosomal RNA, long non-coding RNA, microRNA, DNA, protein, or lipids (Thery et al., 2002; Huang et al., 2013; Batiz et al., 2015; Kirby et al., 2015).

NSC FUNCTION IN NEUROINFLAMMATION AND NEURODEGENERATION. FOCUS ON MULTIPLE SCLEROSIS AND ISCHEMIC STROKE.

The peculiar plasticity of the CNS and of its NSCs manifests after CNS injury, when (i) proliferation and differentiation of NSCs is enhanced; (ii) striatal spiny interneurons and glutamatergic neurons are ectopically found in the injured cortex and in the striatum after stroke (Thored et al., 2007) while migrating neuroblasts become oligodendrocytes in area of demyelination after exiting the niche; (iii) ependymal cells behave as progenitors (Luo et al., 2008) and directly convert into neurons (Carlen et al., 2009); (iv) reactive astrocytes in ischemic brain injury exhibit self renewal capacity and multipotency (Buffo et al., 2008; Gabel et al., 2016).

The fact that non-neurogenic precursors can convert into neurogenic cells clearly highlights how exogenous factors can trigger plasticity within and outside of the niche (Carlen et al., 2009; Magnusson et al., 2014; Shetty and Hattiangady, 2016). Moreover, during pathology and steady-state, NSCs also exert trophic non-neurogenic functions which are crucial to maintain brain homeostasis.

In the following section we will explore how NSC niches function during CNS injury, with focus on stroke, multiple sclerosis and their animal models (**Figures 1B,C**, **Table 1**).

Effect of Oxygen Supply on Neural Stem Cell Plasticity, Connection with Stroke, and Multiple Sclerosis

In physiological conditions, neural stem cells are exposed to an oxygen concentration between 2.5 and 5.0%, which promotes NSC self-renewal *via* VEGF and erythropoietin (EPO) production induced by hypoxia inducible factor 1α, HIF1α (Pavlica et al., 2012; Li et al., 2014).

In the quiescent state, NSC mitochondria are quite immature with globular shape, do not depend much for energy on oxidative phosphorylation (OXPHOS), rather on glycolysis, with high lactate production (Zheng et al., 2016). Although glycolysis produces less ATP than mitochondrial OXPHOS, the pathway is very fast in NSCs (Ito and Suda, 2014). Their anaerobic metabolism is sustained mainly by mitochondria uncoupling protein 2 (UCP2), high level of hexokinase II and low pyruvate dehydrogenase to keep under control the production of reactive oxygen species (ROS) (Madhavan et al., 2006; Zheng et al., 2016). In this way, DNA and proteins of the cells are protected from ROS-dependent potential damage. Further, ROS, produced in limited amount in physiological conditions and normally neutralized, are also beneficial because they trigger self-renewal and neurogenesis (Le Belle et al., 2011). Conversely, differentiated cells present elongated, crystae-rich mitochondria, higher ratio of mitochondrial glucose oxidation (OXPHOS) over glycolysis as metabolic support (Zhang et al., 2014; Marcialis et al., 2016). Moreover, NSCs increase Krebs' cycle functionality and decrease lactate production, concurrent with increased number and total mitochondrial mass (Sola et al., 2013).

TABLE 1 | Evidences from the literature are schematically reported.

Molecule	Physio-pathology	Source	Outcome	References
Acetylcholine	Physiology	(ChAT) (+) neurons	↑neurogenesis (synergizing with FGF2)	Paez-Gonzalez et al., 2014
Angiopoietin 2	Stroke	SVZ neuroblasts, endothelial cells	↑NSC and neuroblast migration ↑NSC neurogenic differentiation	Cui et al., 2009; Liu et al., 2009
ANKYRIN3	Physiology	Ependymal cells	↑neuroblasts	Paez-Gonzalez et al., 2011
ATP	Physiology	Astrocytes	↑NSC proliferation	Cao et al., 2013
B2M	Aging (increases)	Blood	↓neurogenesis	Smith L. K. et al., 2015
BDNF	Physiology and stroke		↑NSC differentiation	Chen et al., 2005
Betacellulin (BTC)	Physiology	Endothelial cells	↑NSC proliferation	Gomez-Gaviro et al., 2012
BMP4	Physiology	Ependymal cells	↑glial differentiation	Gajera et al., 2010
CCL11	Aging	Blood	↓neurogenesis	Villeda et al., 2011
CNTF	Steady-state	A subtype of B cells of the SVZ, other?	↑NSC self-renewal ↓↑NSC neurogeneic differentiation	Emsley and Hagg, 2003; Lee et al., 2013
Decorin	Steady-state	Astrocytes	↓NSC neurogeneic differentiation	Barkho et al., 2006
Delta-like-4	Steady-state	Endothelial cells	↑NSC proliferation	Androutsellis-Theotokis et al., 2010
Dickkopf-1	Aging	NSCs	↓neurogenesis	Seib et al., 2013
Dopamine	Physiology	Dopaminiergic neurons	↑neurogenesis (synergizing with with EGF)	O'Keeffe et al., 2009
Ephrin-B2	Steady-state	Astrocyte	↑NSC proliferation ↑neurogenesis	Ashton et al., 2012
EPO	Hypoxia, stroke	Endothelial cells, Blood	↑NSC proliferation and survival	Pavlica et al., 2012
FGF2	Physiology	Astrocytes	↑NSC proliferation and survival	Shetty et al., 2005; Widera et al., 2006
GABA	Physiology	Young neuroblasts	↓NSC proliferation and neuronal differentiation	Liu et al., 2005
GDF11	Aging (decreases)	Blood	↑neurogenesis	Katsimpardi et al., 2014
Galectin-3	MS	SVZ	↓NSC proliferation	James et al., 2016
Glutamate	Physiology	Tissue	neuroblast survival	Platel et al., 2010
Gonadotropin- releasing hormone (GrH)	Aging	Hypothalamic cells	proliferating activity of hypothalamic NPC	Zhang et al., 2013
IFNγ	Stroke, MS, steady-state	Immune cells, NSCs	↓↑NSC proliferation ↑↓Differentiation	Pluchino et al., 2008; Li et al., 2010b; Kulkarni et al., 2016
IGF1	Steady-state, aging	Microglia, endothelial cells	↑NSC proliferation ↑NSC neuronal differentiation ↑glial development	Butovsky et al., 2006; Joseph D'Ercole and Ye, 2008; Llorens-Martin et al., 2009
IGF2	Steady-state, CNS tumor, development, aging	Cerebrospinal fluid	↑NSC proliferation	Lehtinen and Walsh, 2011
IGFBP6	Steady-state	Astrocytes	↓neurogenic differentiation	Barkho et al., 2006
IL10	Steady-state, stroke	T_{reg}	↑NSC proliferation ↓neurogenic differentiation	Perez-Asensio et al., 2013; Wang et al., 2015
IL1β	Stroke, MS	Microglia, NSCs, monocyte/macrophages?	↑↓NSC proliferation ↑NSC apoptosis ↑gliogenic differentiation	Wu et al., 2013 Widera et al., 2006; Guadagno et al., 2015
IL6	Infections, stroke	NSCs, microglia	↑NSC proliferation ↑neuroblast survival	Gallagher et al., 2013; Chucair-Elliott et al., 2014; Meng et al., 2015
Jagged1	Steady-state, MS	Astrocytes	↓neurogenesis differentiation ↑OPC proliferation	Stidworthy et al., 2004; Wilhelmsson et al., 2012
LIF	Steady-state	?	↑NSC proliferation	Bonaguidi et al., 2005

(Continued)

TABLE 1 | Continued

Molecule	Physio-pathology	Source	Outcome	References
MCP-1/CCL2	Stroke, aging, epilepsy, CNS tumors	Immune cells? Microglia? Astrocytes?	↑NSC migration ↑neuronal differentiation and neuritic formation of mesencephalic NSCs ↑glial differentiation of NT2 NSCs	Vrotsos et al., 2009; Colucci-D'Amato et al., 2015; Osman et al., 2016
Neuregulin 1 and 2	Steady-state	Neuroblasts, GFAP+ NSCs in the SVZ	↑NSC proliferation ↑neuroblast migration	Ghashghaei et al., 2006
Neurotrophin 3 (NT3)	Steady-state	Endothelial cells	↑NSC quiescence ↑neurogenic differentiation	Shimazu et al., 2006; Delgado et al., 2014
NGF	Steady-state, MS, stroke?	SVZ	↑NSC proliferation ↑neurogenic differentiation	Calza et al., 1998; Triaca et al., 2005
Noggin	Steady-state	Ependymal cells, subgranular zone	↑NSC proliferation ↑neurogenesis	Lim et al., 2000; Bonaguidi et al., 2008
Oxygen (2-5%)	Steady-state, stroke	NSCs	↑NSC self renewal ↓NSC differentiation	Santilli et al., 2010
Oxygen (<1%)	Stroke, MS	NSCs	↓NSC self renewal ↑NSC differentiation	Felfly et al., 2011
PDGF	Physiology	GFAP-positive cells	↑NSC proliferation	Jackson et al., 2006
PGD2	Steady-state	?	↓NSC proliferation	Codega et al., 2014
Retinoic acid (RA)	Stroke, steady -state	Meninges, other?	↑neurogenesis	Plane et al., 2008; Siegenthaler et al., 2009
ROS	Steady-state, stroke?	NSCs	↑NSC self renewal ↑neurogenesis	Le Belle et al., 2011
S1P	Steady-state	?	↓NSC proliferation	Codega et al., 2014
SDF-1/CXCL12	Stroke, MS, steady-state, traumatic brain injury	NSCs, meninges, endothelial cells, immune cells, tumor cells	↑NSC migration ↑NSC survival ↑NSC differentiation	Reiss et al., 2002; Imitola et al., 2004; Itoh et al., 2009; Carbajal et al., 2010; Li et al., 2012
Serotonin	Physiology	5-HT neurons	↑neurogenesis	Brezun and Daszuta, 1999
SHH	Development, steady-state, MS	Ventral forebrain neurons	↑NSC specification ↑ neurogenesis OPC differentiation	Breunig et al., 2008; Ihrie et al., 2011; Samanta et al., 2015
Surivivin	Aging	Astrocytes	↑neurogenesis	Miranda et al., 2012
TGFα	Steady-state, stroke	NSCs?	↑neurogenesis	Tropepe et al., 1997; Guerra-Crespo et al., 2009
TGFβ	Steady-state, development	NSCs	Temporal regulation of neurogenesis and potency of NSCs	Dias et al., 2014
Thbs4	Stroke	SVZ NSCs	↑SVZ-NSC astrogenesis (via Notch signaling) ↑glial scar formation	Benner et al., 2013
TNFα	Stroke, MS?	Microglia, astrocytes, monocyte/macrophages?	↑NSC proliferation ↑NSC apoptosis ↑Gliogenic differentiation	Widera et al., 2006; Guadagno et al., 2013
TSP1	Steady-state	Astrocytes	↑NSC proliferation ↑neurogenesis	Lu and Kipnis, 2010
VEGF	Steady-state, Stroke	NSCs; astrocytes; endothelial cells	↑NSC proliferation and maintenance ↑neurogenic differentiation ↑NSC migration	Kojima et al., 2010; Kirby et al., 2015
Wnt3	Steady-state	Astrocytes	↑neurogenesis	Okamoto et al., 2011

On the other side, the first consequence of the blood flow occlusion in brain ischemia is hypoxic injury that causes extensive neural cell death (Niquet et al., 2003). Nonetheless and surprisingly, hypoxia, while being detrimental in the acute phase of stroke, in a second step, contributes to promote neurogenesis (Arvidsson et al., 2002; Tonchev et al., 2003; Jin et al., 2006; Macas et al., 2006; Minger et al., 2007; Wang et al., 2011; Zhang and Chopp, 2016), to support replenishment of neurons in the RMS, migration in the region of ischemic brain injury, and growth of oligodendrocyte progenitors that disperse to the gray and white matter (Zhang et al., 2001, 2012; Parent et al., 2002; Minger et al., 2007; Li et al., 2010a; Zhang and Chopp, 2016).

Hypoxia has recently emerged as a concurrent complication of disease progression also in MS. Indeed, important lack of oxygen, occurring as a consequence of inflammation, has been measured in brain gray matter regions of MS patients, where it can *per se* be considered co-causative for neurodegeneration (Haider et al., 2014; Yang et al., 2015). Since hypoxia and inflammation are strictly linked, it is still difficult to tease apart their respective contributions in MS (Sun et al., 1998; Lassmann, 2003; Davies et al., 2013). It looks like hypoxia can occur in MS patients as a consequence of increased oxygen demand. When this request is not satisfied, hypoxia can be further detrimental for surrounding neural stem cells (Trapp and Stys, 2009).

Effect of Inflammation on Neural Stem Cell Plasticity, Connection with Stroke, and Multiple Sclerosis

It has been well-documented that the brain cannot be simplistically considered an immune privileged site (Kleine and Benes, 2006) and actually immune system activation in the brain exerts both damaging and beneficial effects on CNS functions (Martino, 2004), depending on the onset of the inflammation, on the cell types involved in the process and on the chronicity of the response (Crutcher et al., 2006; Kyritsis et al., 2012).

NSCs share with the immune system an array of secreted mediators and receptors, which are all relevant for the maintenance of the neurogenic niche and, at the same time, represent the prerequisite for the interaction of NPCs with the microenvironment, especially during neuroinflammation (De Feo et al., 2012; Kokaia et al., 2012).

Indeed, inflammation, *via* its associated cues (i.e. cytokines, chemokines, chemical species....), strongly impacts structure and function of the stem cell niche, acts directly on NSCs and affects tissue restoration/regeneration *via* microglia and astrocyte activation (Ekdahl et al., 2003; Pluchino et al., 2008; Pourabdolhossein et al., 2017).

In homeostatic conditions, microglia are ramified in shape to maintain surveillance. Upon pathogen invasion or insults, they retract the protrusions, become amoeboid, increase their migratory behavior, and secrete cytokines. IL6, TNFα, IL1β, and complement 1 subunit q (C1q) are among the most potent microglia–derived cytokines, able to compromise the niche environment, inhibit neurogenesis (Vallieres et al., 2002; Monje et al., 2003), induce oligodendrogenesis (Valerio et al., 2002) and a subtype of reactive astrocytes (Liddelow et al., 2017). Further, while NSC-derived nitric oxide synthase promotes release of small amounts of nitric oxide (NO) with neurogenic properties (Carreira et al., 2010; Luo et al., 2010), abundant NO released by microglia or astrocytes in inflammatory conditions (i) inhibits proliferation of NSCs acting on the EGF receptor (Carreira et al., 2014) or on the transcription factor complex Neuron-Restrictive Silencer Factor /RE1-Silencing Transcription factor, NRSF/REST (Bergsland et al., 2014) and (ii) promotes gliogenesis (Bergsland et al., 2014).

IFNγ, a pleiotropic cytokine, has a central role in regulating NSCs proliferation and quiescence (Kulkarni et al., 2016). In inflammatory conditions, such as experimental autoimmune encephalomyelitis (EAE), the preclinical model of MS, IFNγ dramatically decreases progenitor proliferation (Pluchino et al., 2008; Pereira et al., 2015) and inhibits the recruitment of newborn neurons to the olfactory bulb (OB). IFNγ might also play a crucial role in regulating glioma-associated oncogene homolog-1 (Gli1), a Shh-induced trasncription factor that drives the oligodendroglial fate of NSCs (Wang et al., 2008; Li et al., 2010b; Samanta et al., 2015).

The complement system also inhibits NSC activity as demonstrated with complement receptor 2 (Cr2) knock out mice that have increased basal neurogenesis (Moriyama et al., 2011). Moreover, lipid modifiers present in inflammation, such as leukotriene, can negatively regulate NSC proliferation likely *via* TNFα and IL1β (Bonizzi et al., 1999; Wu et al., 2013). Of note, montelukast, a leukotriene receptor blocker protects from this event (Marschallinger et al., 2015).

IL6 is another crucial mediator of NSC function and its induction, e.g., during maternal infection and stroke, permanently perturbs NSC proliferation and neurogenesis (Gallagher et al., 2013; Chucair-Elliott et al., 2014; Meng et al., 2015).

Conversely, the anti-inflammatory interleukin-10 (IL10) in rodents keeps NSCs in an undifferentiated proliferation state, rather than promoting differentiation (Ben-Hur et al., 2003; Perez-Asensio et al., 2013; Wang et al., 2015).

Nevertheless, as anticipated, inflammation can be also beneficial for NSCs. First, the immune system in general and T cells in particular are involved in maintaining niche neurogenesis, as indeed genetic T-cell depleted mice present compromised cognitive functions (Ziv et al., 2006). Moreover, chemokines such as monocyte chemoattractant protein 1 (MCP1) or SDF1, released in inflammatory conditions, promote migration of NSCs to site of injury and local TNFα or IL1β trigger their proliferative capacity (Widera et al., 2006). Second, injection of moderately activated microglia can regulate brain homeostasis and neurogenesis (Butovsky et al., 2006; Bachstetter et al., 2011).

Further, NSCs directly produce inflammatory chemokines (Covacu et al., 2009), promoting a feed forward loop at sites of tissue damage (Imitola et al., 2004; Belmadani et al., 2006; Widera et al., 2006; Wang et al., 2007). NSCs can also directly change inflammatory responses through immunomodulatory factors (Pluchino et al., 2005; Ben-Hur, 2008; Yong and Rivest, 2009; Butti et al., 2012) or trophic factors (Huang et al., 2014). In particular, SVZ-NSCs can protect striatal neurons from the excitotoxic damage occurring in stroke and epilepsy, releasing endogenous endocannabinoids, likely upon inflammatory *stimuli* (Butti et al., 2012). Endogenous NSCs also secrete MCP1, VEGF, IL4, and interleukin-15 (IL15), the latter known to retain NK cells in the chronic phase of EAE in mice and of MS in humans. Indeed, in the SVZ, NK cells contribute to the dysfunction of the neurogenic niche observed in EAE by killing stem cells (Liu et al., 2016). Moreover, inflammatory cytokines lead to metabolic reprogramming of the arginase pathway in NSCs ultimately impacting on the NSC mediated anti-proliferative effect on T cells (Drago et al., 2016). Further, it has been reported that human NSCs hinder the differentiation of myeloid precursor cells into

immature dendritic cells and the final maturation into functional antigen presenting cells (Pluchino et al., 2009).

In stroke, immune cell infiltration and inflammation take place as secondary events to hypoxic injury, which is responsible of the massive neuronal death. Upon brain injury, the blood-brain barrier (BBB) is disrupted, its permeability increases, migrating immature neurons associate with the angiogenic fenestrated endothelium, thus mimicking the neurogenic niche structure (Ohab et al., 2006; Ohab and Carmichael, 2008). Neutrophils and innate immune cells are the first players, although also T and B cells cross the damaged-BBB, inducing a rapid adaptive autoimmune response to neuronal antigens (Chamorro et al., 2012). As consequence of inflammation, neurogenesis is activated in stroke (Wang et al., 2011; Katajisto et al., 2015) supported by soluble factors such as bone morphogenic protein (Forni et al., 2013), retinoic acid (Plane et al., 2008), sonic hedgehog (Cheng et al., 2015), C-C motif chemokine Ligand 2 (CCL2) (Osman et al., 2016) along with SDF1 and angiopoietin-2 (Ang2) that home NSC progenitors to the site of injury (Thored et al., 2006), where they improve functional recovery (Guzman et al., 2008) and differentiate into neurons (Darsalia et al., 2007). Nevertheless, they present only limited reparative properties because matured neurons tend to die (Arvidsson et al., 2002; Marti-Fabregas et al., 2010; Kazanis et al., 2013). Moreover, given that NSCs themselves express and release respectively C-X-C motif chemokine receptor 4 (CXCR4) and SDF1, they might play a role in regulating axonal remodeling in ischemic brain. On the other hand, SVZ-derived astrocytes have a crucial role in ischemic stroke: photothrombotic cortical ischemia induces a strong gliogenic, Notch1-dependent response in the SVZ, which generates thrombospondin-4 (Thbs4)-positive astrocytes. Thbs4 astrocytes are essential components of the glial scar and their proliferation might be favored over neurogenesis after cortical injury (Benner et al., 2013). Conversely, it has also been reported that inflammation can promote neurogenesis in dormant neural progenitors in nonconventional neurogenic regions (Jiao and Chen, 2008).

Further, stroke increases generation of oligodendrocyte precursor cells (OPCs) from NSC in the ischemic brain, although it remains to be defined whether NSC-derived OPCs, beside oligodendrogenesis, help with brain repair, likely communicating with cerebrovasculature and other brain parenchyma cells (Itoh et al., 2015).

Overall, according to several evidences, inflammation in stroke can be beneficial for NSCs, nonetheless other reports, evaluating microglia function, claim that activated $ED1^+$ microglia impair basal neurogenesis (Ekdahl et al., 2003) likely *via* TNFα/TNFR1 signaling (Iosif et al., 2008; Chen and Palmer, 2013; Gebara et al., 2013). In humans, a definitive robust evidence of clinical post-stroke neurogenesis remains a matter of investigation as analysis of autopsy tissue provided positive results (Ekonomou et al., 2012) which are instead lacking when labeling newly born neurons with ^{14}C (Huttner et al., 2014).

In MS preclinical models, it is important to distinguish among experimental conditions since they influence the experimental evidence. Nonetheless, either chemically (LPC), focal (site injection of TNFα-IFNγ combined with subclinical immunization) or immune-mediated (EAE) demyelination causes reduced neuroblast proliferation in the olfactory bulb of mice, while neutralization of immune mediators, such as IFNγ and Galectin3 inflammatory cytokines, restores neurogenesis both *in vitro* (Pluchino et al., 2008; Tepavcevic et al., 2011) and *in vivo* (Monje et al., 2003; James et al., 2016). Indeed, in human samples, the SVZ of post-mortem MS brains is altered, with reduced number of neuroblasts in the SVZ of the lateral ventricle. Conversely, neuroinflammation increases neurotrophin levels (e.g., nerve growth factor-NGF) in the SVZ (Calza et al., 1998; Triaca et al., 2005), likely counteracting the inhibitory effect of proinflammatory molecules. Further, demyelination is able to boost proliferation of NSCs in the neurogenic niches promoting their differentiation (Pluchino et al., 2005; Menn et al., 2006). Only in conditions of acute cytokine exposure, either by focal intrathecal cytokine injection or as a consequence of strong microglia activation, the proliferative capacity of the niche is inhibited. In terms of gliogenic differentiation, inflammatory conditions may increase the percentage of $Olig2^+$ cells originating from the SVZ *via* modulation of bone morphogenetic protein (BMP) signaling (Tepavcevic et al., 2011). The transcription factor Gli1 seems to be a key regulator of SVZ oligodendrogenesis and may be a promising target for reparative strategies in MS, as pharmacological inhibition of Gli1 during EAE promotes remyelination and improves clinical outcome (Samanta et al., 2015).

Leveraging the regenerative potential of NSCs and their immunomodulatory and trophic functions, NSC transplant has been proposed as therapeutic strategy for inflammatory diseases of the CNS. In stroke, transplanted NSCs directly modulate neuronal circuit plasticity (Zhang et al., 2009) through the expression of developmental molecules such as guidance molecules (i.e., slit, thrombospondin-1 and -2) and trophic factor such as VEGF (Andres et al., 2011). In fact, transplanted NSCs in stroke stimulate the proliferation of endogenous neural stem cells (Zhang et al., 2011; Hassani et al., 2012; Mine et al., 2013), increase endogenous angiogenesis after stroke (Jiang et al., 2005), scavenge the neurotoxic molecules (Emsley et al., 2004) and contribute both directly and indirectly (*via* astrocytes) to glutamate clearance (Bacigaluppi et al., 2009, 2016).

In EAE, transplanted NSCs directly inhibit both T cell and myeloid cell immune responses. In the CNS, they block inflammatory cell recruitment, T cell proliferation and promote the apoptosis of brain-reactive T cells (Einstein et al., 2003; Pluchino et al., 2005). In peripheral secondary lymphoid organs of EAE mice, antigen-specific proliferation of T cells, dendritic cell antigen presentation and chemotactic gradients are impaired by NSCs transplanted either subcutaneously or intravenously. As an example, NSC-secreted LIF, on one hand, stimulates endogenous remyelination in the CNS (Laterza et al., 2013), on the other, inhibits Th17 cell differentiation in the periphery (Cao et al., 2011).

Overall, the current literature shows that a controlled use of inflammation in CNS injury could be of help in regenerative approaches when NSC proliferation needs to be boosted and inflammatory tweaking can have beneficial outcomes. An option could consist in reducing NSC sensitivity to inflammatory

mediators and, at the same time, increasing the therapeutic efficacy of NSCs when transplanting engineered cells.

Effect of Aging on Neural Stem Cell Plasticity, Connection with Stroke, and Multiple Sclerosis

Aging is a common feature of both stroke and progressive multiple sclerosis and loss of niche integrity, depletion of the stem cell pool, cellular senescence, defect in cell-cell contact in the niche or metabolic changes are all shared characteristics that contribute to the demise of the aging neurogenic niche (Oh et al., 2014).

The most significant consequence of age in the SVZ consists in the alteration of the niche cytostructure and in reduction of the neuroblast population, with reduced proliferation and neurogenic capacity (Kerever et al., 2015). On the contrary, oligodendrogenesis is substantially not affected by aging, rather oligodendrocyte recruitment and differentiation are impaired (Sim et al., 2002; Franklin and Ffrench-Constant, 2008; Conover and Todd, 2016). The latter is indeed a causal pathogenic characteristic of chronically demyelinated MS lesions in humans (Kuhlmann et al., 2008).

Concurrently, given that senescence does not spare the vasculature (Farkas and Luiten, 2001), the environment of the niche may enrich for toxic factors which further sustain exhaustion, reduce neuroplasticity and cause cognitive decline (Villeda et al., 2011). Potential therapeutic strategies to counteract the decline of adult stem cells may involve the promotion of a rejuvenating environment (Katsimpardi et al., 2014) or the prevention of premature exhaustion of long-lived self-renewing NSC populations. Neurogenic decline in aged SVZ can occur because of the accumulation of damaged DNA and both nuclear and mitochondrial DNA are susceptible to age-related changes (Bailey et al., 2004). Upon sensing DNA damage, SVZ cells upregulate the inhibitor of cyclin kinase to post-pone cell cycle entry and reduce proliferation of NSCs (Molofsky et al., 2006).

NSC age-dependent cell plasticity is also influenced by metabolic changes (Rabie et al., 2011; Chaker et al., 2015). As far as nutrient sensing pathways, growth differentiation factor-11 (GDF11), has been identified as one of the extrinsic circulating youth signals that maintain neurogenesis (Katsimpardi et al., 2014), although those findings are currently matter of further validation (Egerman et al., 2015; Smith S. C. et al., 2015). It is still unknown whether the effect is direct onto NSCs or indirect *via* improved microvascular network. IGF1, another critical growth factor, *per se* stimulates proliferation and differentiation of NSCs (Aberg, 2010), but decreases with aging (Sonntag et al., 2005). Nonetheless, it is also life–long exposure to IGF1 that with age causes decline of neurogenesis (Chaker et al., 2015). Along the same line, NAD^+ and AMP levels, which reflect cellular energy state, decline with age in the hippocampus, in parallel with reduction of differentiation and self-renewal capacity of NSCs, because their regulatory enzyme decreases (Liu et al., 2012; Stein and Imai, 2014). As far as oxygen-dependent effect, those are linked to failure in maintaining appropriate mitochondrial regulations. Indeed, accumulation of mutations in mitochondrial DNA (mtDNA) occurs during aging and comes along with abnormal accumulation of toxic by-products (Siqueira et al., 2005) because mtDNA lacks protective histones and is located close to the major source of ROS, the electron transport chain (Park and Larsson, 2011).

Inflammation is another comorbidity factor associated with aging. The immune molecules C-C motif chemokine-11 (CCL11) and β2-microglobulin (B2M), as well as CCL2, C-C motif chemokine-19 (CCL19) and haptoglobin, contribute to low-grade of inflammation in aging (inflammaging) (Franceschi and Campisi, 2014), and negatively affect neurogenesis (Villeda et al., 2011; Wyss-Coray, 2016). Indeed, heterochronic parabiosis between aged and young mice rejuvenates and reverses stem cell aging in numerous tissues (Conboy et al., 2005) or administration of plasma from young mice can ameliorate cognitive impairment in aged mice (Villeda et al., 2014).

Last, it is worth mentioning that also the cellular environment of the niche sustains aging features. Astrocytes of the niche, with age, reduce release of Wnt3 (Lie et al., 2005; Okamoto et al., 2011), that in turn down regulates Survivin expression and increases release of Dickkopf-related protein 1 (DKK1), a canonical Wnt signaling inhibitor. The final effect is induced quiescence on one side (Miranda et al., 2012) and negative regulation of NSC neurogenesis (Seib et al., 2013) on the other. This is paired with physical changes of the extracellular matrix, which also affect senescence of the niche (Gattazzo et al., 2014). Conversely, it is also possible that NSCs efficiently graft the SVZ and well differentiate in both young and aged hippocampus, suggesting that advanced age of the host at the time of grafting has no major adverse effects on engraftment, migration, and differentiation of SVZ-NSCs (Shetty and Hattiangady, 2016).

Age is an independent risk factor for brain ischemia (Marinigh et al., 2010; Roger et al., 2012) and stroke models using aged animals are clinically very relevant (Popa-Wagner et al., 2007). From work on animals, it has been reported a 50% decline in neurogenesis in the SVZ of elder compared with young–adult animals (Darsalia et al., 2005; Macas et al., 2006; Ahlenius et al., 2009). Unfortunately, although endogenous neurogenesis has been observed even in aged humans, it is still not clear to what extent newly formed neurons are functionally relevant for stroke recovery in human patients. Thus, cell therapies have been implemented to address this question. An open-label, single-site, dose-escalation study was performed on 13 aged patients after ischemic stroke, transplanting by stereotaxis the neural stem cell line CTX0E03 in the ipsilateral putamen. After 2 years, improvement in the disability score scale (NIHSS) ranging from 0 to 5 (median 2) points has been recorded (Borlongan, 2016; Kalladka et al., 2016). Similarly, studies in animal models examined whether exogenous NSC delivery might restore neurogenesis in the DG of old rodents. Both embryonic and fetal derived NSCs stimulated neurogenesis in young or old animal models. Likely the effect was exerted *via* suppression of microglia/macrophage activation (Jin et al., 2011). Of note, inhibition of microglial activation has also been reported in ischemic mice following systemic delivery of mouse NSCs (Bacigaluppi et al., 2009).

Those data suggest that the problem with NSCs in ischemic stroke is to some extent cell autonomous, because the environment of the aged brain does not preclude NSC engraftment.

In MS, loss of myelin, a specialized membrane produced by oligodendrocytes, essential for normal CNS function, is a characteristic feature. Aging impairs not only the neurogenic but also the oligodendrogenic properties of the brain germinal niches as a consequence of longer cell cycle length of NSCs and of their progeny, loss of growth factors and upregulation of inhibitors (Hamilton et al., 2013). Iron accumulation (Andersen et al., 2014), linked in cascade to oxidative alteration and to mitochondrial dysfunction (Mahad et al., 2015) are other recognized characteristics of neurodegeneration in progressive aged MS individuals. A direct impact also on neural stem cells has not been experimentally proven (Crabbe et al., 2010) but can reasonable be speculated.

In the context of demyelination, work by Crawford et al. demonstrated responsiveness of OPCs to demyelination, although remyelination and susceptibility to aging is regionally dependent: in chemically-induced demyelination of the corpus callosum in aged animals, dorsal SVZ-NSCs are efficiently recruited to lesions and their differentiation into OPCs is impaired, whereas ventral NSCs are recruited more slowly but differentiate rapidly into OPCs; similarly, while in aged animals the percentage of differentiated OPCs from dorsal NSCs is half of the one seen in young animals, the percentage of ventral NSCs differentiating into OPCs remained constant (Crawford et al., 2016). This evidence has significant implications for the progressive form of MS, for which age is the current best associated risk factor (Minden et al., 2004; Scalfari et al., 2011). As disease progresses with age, repair, remyelination and other physiological functions become less robust. Animal models have shown that while OPC recruitment toward lesions remains intact, differentiation into myelinating oligodendrocytes decreases with age with a slower and less effective remyelination process compared to young rats (Rist and Franklin, 2008; Ruckh et al., 2012). Similarly, elder human subjects showed reduced remyelinating capabilities. Multiple hypotheses can explain these findings, including either extrinsic factors or intrinsic characteristics such as epigenetic changes known to be modulated during aging and thus to impact OPC maturation (Rist and Franklin, 2008).

HOW DO NEURAL PRECURSORS FROM INDUCED PLURIPOTENT STEM CELLS STAND?

The technology of somatic cell reprogramming (induced pluripotent stem cells, iPSCs) that developed in the last 10 years (Takahashi et al., 2007), represents an invaluable alternative strategy to obtain, in large scale and indefinitely, neural precursors, unfolding new research and clinical avenues. Expandable intermediate neural precursors can be obtained with published protocols (Falk et al., 2012; Reinhardt et al., 2013) and the cells resemble most of the specific properties of endogenous plastic cells. To treat neurodegenerative conditions, both iPSC-derived NSCs or terminally differentiated neurons have been transplanted in preclinical models. Early on, it has been demonstrated that those cells are able to survive and adequately integrate into the host brain (Oki et al., 2012; Tornero et al., 2013; Qi et al., 2017), and recently it was reported that transplanted human iPSC-derived cortical neurons can be incorporated also into injured cortical circuits and could contribute to functional recovery (Tornero et al., 2017). Beside reparative activity, transplanted cells can improve the brain functional outcome, directly waking up brain plasticity *via* their bystander effects. Indeed, transplanted cells promote the formation of new synapses among existing neurons or preserve axonal integrity releasing trophic factor for myelinating cells as described in ischemic stroke and in a preclinical model of MS (Oki et al., 2012; Espuny-Camacho et al., 2013; Laterza et al., 2013; Tornero et al., 2013).

In addition, the release of soluble factors by (neural) stem cells along with cell-cell interactions supports angiogenesis that, beside self organizing blood vessels (Kusuma et al., 2013), guarantees trophic support for proper integration of neuronal cells and sustains synaptogenesis. Further, the bystander effect is exploited also in terms of (i) inhibition of neutrophil and peripheral dendritic cell activation and recruitment, (ii) inhibition of effector T- and B-cells, (iii) attenuation of BBB damage, or (iv) stimulation of the M2 microglial phenotype (Drago et al., 2013). Similar evidence of the (neuro)-protective, regenerative, and angiogenic properties of iPSCs is not available yet from their complete secretome, nonetheless it can be reasonably predicted.

As of now, while work by Jensen et al. reported no iPSC-mediated improvement in behavioral function in stroke (Jensen et al., 2013), several others, as previously mentioned, show that also direct iPSC transplantation improves neurological score, motor, sensory and cognitive functions, already within the first week after transplantation, when migration, maturation, synaptic integration, and paracrine release into the host brain is completed (Oki et al., 2012; Polentes et al., 2012; Tornero et al., 2013, 2017; Yuan et al., 2013; Tatarishvili et al., 2014).

Similarly, transplant of iPSC-derived neural precursors in the MS context dramatically reduced T cell infiltration and ameliorated white matter damage in treated EAE mice (Laterza et al., 2013; Zhang C. et al., 2016), both *via* improved differentiation toward cells of the oligo-glial lineage and *via* soluble factor release (Laterza et al., 2013).

Experimental validation in humans, both for stroke and MS, could be useful in the future but regulatory guidelines for safe clinical trial are awaited.

CAN WE GUIDE NEURAL STEM CELL PLASTICITY TOWARD A DESIRED RESPONSE?

Taking advantage of their plasticity, stem cells can generate, repair, and change nervous system functions. Fine-tuning of the inflammatory responses or proper modulation of the hypoxic

and aging conditions should provide a permissive milieu for an effective reparative process. In this sense, the perspective that aged hippocampus licenses efficient and functional engraftment (Shetty and Hattiangady, 2016) puts forward the possibility that grafted niches can continuously generate new neurons and glia which, in turn, are expected to improve the plasticity and function of aged brain tissue.

As engineering and reprogramming approaches advance, transplant of exogenous human NSCs are optimized and better understanding of NSC regulation in physio- pathological conditions is achieved, it will be possible to fully take advantage of the therapeutic potential of those cells and successfully exploit their plasticity.

So far, it is possible to (i) engineer NSCs to deliver extrinsic stem cell fate determinants or intrinsic factors that allow the development of biomimetic environmental cues to control stem cell fates (Michailidou et al., 2014; Karimi et al., 2016); (ii) culture and expand stem cells outside of their native environments (Komura et al., 2015); (iii) perform localized delivery of microspheres to specifically differentiate endogenous stem cells (Gomez et al., 2015); (iv) engineer somatic cells to obtain induced NSCs with reparative functions (Hargus et al., 2014); (v) engineer human stem cells to obtain indefinitely expandable lines for clinical purposes (Pollock et al., 2006); (vi) snapshot the metabolic profile of cells in physio-pathological conditions (Bystrom et al., 2014; Dislich et al., 2015).

Systems biology will likely represent a fundamental tool to discover novel drugs to modulate NSC plasticity, as it can integrate and analyze in a quantitative manner several aspects of NSC behavior (e.g., differentiation potential and self-renewal) (Wells et al., 2013; Hussein et al., 2014).

CONCLUSIONS AND FUTURE OUTLOOK

Neural progenitor cells represent an underdeveloped therapeutic resource in clinical settings. Although their paracrine and endocrine bystander potential is well-known, much of their plasticity still needs to be properly manipulated for therapeutic purposes.

On one end, cells can be expanded and bioengineering approaches are becoming available to further potentiate their availability (Bressan et al., 2014; Bouzid et al., 2016; Gardin et al., 2016). For instance, *in vitro* preconditioning with low oxygen tension might enhance NSC survival as it mimics the oxygen supply normally found in brain tissues (Hawkins et al., 2013; Sandvig et al., 2017). Nonetheless, it is this same plasticity that could threaten their therapeutic capacity, because the manipulation of cells themselves or of the trophic microenvironment, might induce unwanted side-effects, such as senescence from over-stimulation. Thus, it might be worth increasing the knowledge on (i) the influence of cell metabolism on NSC behavior during CNS diseases; (ii) the *ex-vivo* maintenance of NSCs; (iii) the most suitable window time for transplant to best leverage graft survival and disease specific environmental conditions. Indeed engraftment efficiency strongly correlates with therapeutic benefits.

Furthermore, this additional knowledge should shed light on the age-related decline of the stem cell niche, which contributes to neurodegeneration in both chronic and acute diseases. Last, careful analysis of the microenvironment is warranted to overcome complications and to improve the efficacy of cell therapy approaches.

AUTHOR CONTRIBUTIONS

LO, AM wrote and approved the manuscript. GM, developed the concept wrote and approved the manuscript.

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REVIEW
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Use of Imaging Techniques to Illuminate Dynamics of Hematopoietic Stem Cells and Their Niches

Takayuki Morikawa and Keiyo Takubo *

Department of Stem Cell Biology, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan

Continuous generation of blood cells over an organism's lifetime is supported by hematopoietic stem/progenitor cells (HSPCs) capable of producing all hematopoietic cell subtypes. Adult mammalian HSPCs are localized to bone marrow and regulated by their neighboring microenvironment, or "niche." Because interactions of HSPCs with their niches are highly dynamic and complex, the recent development of imaging technologies provides a powerful new tool to understand stem cell/niche biology. In this review, we discuss recent advances in our understanding of dynamic HSPC/niche interactions during development, homeostasis, disease states or aging with a focus on studies advanced by imaging analysis. We also summarize methods to visualize HSPCs and niche cells *in vivo*, including use of HSPC reporter mice and chemical probes. Findings emerging from these investigations could suggest novel therapies for diseases and aging.

Keywords: hematopoietic stem cell, niche, imaging, bone marrow, hematopoiesis

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AO Foundation, Switzerland

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César Nombela Arrieta,
University of Zurich, Switzerland
Julien Y. Bertrand,
Université de Genève, Switzerland
Eirini Trompouki,
Max Planck Institute of Immunobiology and Epigenetics, Germany

***Correspondence:**
Keiyo Takubo
keiyot@gmail.com

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INTRODUCTION

In mammals, a lifetime supply of mature blood cells by a process known as hematopoiesis is maintained by differentiation and proliferation of hematopoietic stem/progenitor cells (HSPCs) in response to physiological or pathological stimuli. Removal of aging hematopoietic cells by phagocytes is a physiological stimulus for blood cell generation, while massive loss of mature blood cells due to infection, inflammation or bleeding functions as a pathological stimulus for hematopoiesis. Both types of stimuli alter gene expression and/or post-transcriptional events that prompt cell cycle activation or changes in cell fate decisions by hematopoietic stem cells (HSCs) to produce more fate-restricted progenitors. Those cells then produce mature blood cells to supply lost populations. Based on analysis of the hematopoietic system, which emerges from HSCs, one trillion blood cells are reportedly produced daily in an average human weighting 70 kg under physiological conditions (Ogawa, 1993). The detailed analysis of spatiotemporal regulation of hematopoiesis could foster development of novel therapies and diagnostics for infection, immunological disease, and hematological malignancies.

Use of imaging techniques has revealed that hematopoietic activities in both steady state and pathological conditions are dynamic and that their sequence is regulated spatiotemporally by interaction with the niche. Further development and application of imaging techniques, including *in vivo* HSC labeling, has revealed critical details relevant to the biology of the hematopoietic system (Kataoka et al., 2011; Chen et al., 2012; Koechlein et al., 2016; Sawai et al., 2016). Here, we review recent advances relevant to *in vivo* and *in vitro* imaging analysis of HSCs and their niches and discuss future directions.

HSC VISUALIZATION

Labeling Strategies Useful for HSC Tracking

Flow cytometry is commonly used to identify and purify HSCs in bone marrow. In this method, bone marrow cells stained by fluorophore-labeled antibodies that recognize HSC cell surface markers are sorted and injected into immunosuppressed mice. Consequently, donor HSCs engraft in bone marrow, enabling prospective identification and isolation of HSCs that exhibit self-renewal and multi-differentiation capacity *in vivo*. However, this method cannot provide spatial and temporal information relevant to HSC dynamics with the niche, an analysis that requires bone marrow dissection. The direct visualization of bone marrow is required to analyze HSCs in the context of the niche.

Microscopic analysis has helped define HSC niche structure (**Table 1**): briefly, confocal microscopy is used to scan bone marrow sections stained immunohistochemically and provides clear image at high speed (Joseph et al., 2013). Whereas it is hard to obtain images from deep part of tissue by using confocal microscopy, the light sheet microscopy allows us to visualize the deep portion of bone marrow (Chen et al., 2016; Greenbaum et al., 2017). Intravital deep imaging enabled by multi-photon microscopy has allowed analysis of cellular and oxygen dynamics in murine calvarial bone marrow.

Classically, labeling of HSPCs by fluorescent dyes, including carboxyfluorescein succinimidyl ester (CFSE), has been used to track transplanted HSPCs in bone marrow, and methods used to detect transplanted fluorophore-labeled HSPCs include flow cytometry, confocal microscopy, or multi-photon microscopy. Given that fluorophore-labeled cells lose fluorescence at each cell division (Weston and Parish, 1990; Lyons and Parish, 1994), fluorescence intensity also reflects the cell division history of transplanted cells over time (Takizawa et al., 2011). Insertion of intravital flexible microprobe into mouse femoral bone reveals that transplanted CFSE-labeled HSCs associate with vascularized structures in the femoral head (Lewandowski et al., 2010).

HSPC labeling requires HSPC isolation and incubation with dyes *ex vivo* prior to transplantation, and therefore this method allows analysis of only short-term dynamics after transplantation. Various transgenic reporter zebrafish and mice have been established to obtain spatial and temporal information relevant to normal dynamics of HSPCs by imaging analysis (**Table 2**). For example, promoter/enhancers of genes expressed primarily in murine HSCs (such as Evi1, Hoxb5, Pdzk1ip1, or Musashi2) are utilized to drive expression of fluorescent protein reporter genes (Kataoka et al., 2011; Chen et al., 2012; Koechlein et al., 2016; Sawai et al., 2016). Reporter mice enabling detection of HSCs and endothelial cells (ECs) have also been used to identify HSCs in bone marrow (Gazit et al., 2014; Acar et al., 2015). Although discrepancies in location between endogenous factors and reporter constructs occasionally occur, transgenic animals harboring reporters are powerful tools useful to visualize HSPCs in various hematopoietic organs, including bone marrow.

Imaging of HSC Movement and Location

Transgenic reporter mice have made it possible to detect HSCs and track their fate *in vitro* and *in vivo* based on fluorescence

TABLE 1 | Listed are advantages and disadvantages of major options for imaging the HSC niche (Lieschke and Currie, 2007; Joseph et al., 2013).

Equipment	Advantages	Disadvantages	Possible outcome
Electron microscope	Very high resolution	Unsuitable for *in vivo* imaging	Ultrastructural features of HSC niche
Confocal microscope	High resolution High scan speed	Limited observing depths Photo-bleaching effect Phototoxic impact	Positional relationship between HSPC and niche cells
Multi-photon microscopy	Deeper observation depth Minimum photo-bleaching effect Lower phototoxicity	Limited scan speed Expense	Dynamics of HSPCs and niche in bone marrow
Light sheet microscopy	Excellent observation depth High scan speed Minimum photo-bleaching effect Lower phototoxicity	Unsuitable for tissue with strong light scattering property	Conformation of niche structure in whole bone marrow
TARGETS			
In vitro	Many tissues can be subjected to observation Numerous types of factors can be visualized	Physiological properties may not be revealed	Microstructure of HSC niche in long bone
In vivo	Biological responses can be observed	Limited observable regions	Pathophysiological phenomenon in the HSC niche
DIMENSION			
2D	Distance can be measured	Unsuitable for structural understanding of bone marrow	Distance between HSPC and niche cells
3D	Tissue geometry is easy to understand	Limited temporal resolution	Shape and alignment of HSPCs and niche cells
SPECIES			
Mouse	Various transgenic lines for HSPCs and niche cells are available	Poor tissue transparency	HSPC/niche interactions in bone marrow
Zebrafish	Higher optical clarity More rapid life cycle	Anatomical similarity to terrestrial mammals is limited	HSPC/niche interactions during development

TABLE 2 | Examples of key studies using reporter mice to detect HSPCs.

Driver element	Reporter	Methods			Analysis	References
Zebrafish CD41 (Tg)	GFP	Zebrafish	*In vivo*	Imaging Clonal fate mapping	Confocal microscopy Flow cytometry	Henninger et al., 2017
Zebrafish runx1 (Tg)	GFP mCherry	Zebrafish	*In vivo*	Imaging	Confocal microscopy Flow cytometry	Tamplin et al., 2015
Zebrafish runx1 (Tg)	GFP	Zebrafish	*In vivo*	Imaging	Confocal microscopy Flow cytometry	Hall et al., 2012
Zebrafish CD41 (Tg)	GFP	Zebrafish	*In vivo*	Imaging	Confocal microscopy	Kissa and Herbomel, 2010
Mouse Msi2 (KI)	eGFP	Mouse	*In vivo*	Imaging	Confocal microscopy	Koechlein et al., 2016
Mouse Hoxb5 (KI)	Tri-mCherry	Mouse	*In vivo* *In vitro*	Transplantation Tissue clearing	Flow cytometry Lightsheet microscopy	Chen et al., 2016
Mouse Pdzk1ip1 (Tg)	GFP	Mouse	*In vitro*	Doxycycline chase Transplantation	Flow cytometry	Sawai et al., 2016
Human CD34-tTA (Tg)	H2B-GFP	Mouse	*In vivo*	Doxycycline chase Transplantation	Flow cytometry	Bernitz et al., 2016
Mouse α-catulin (KI)	GFP	Mouse	*In vitro*	Tissue clearing Immunostaining	Confocal microscopy Multi-photon microscopy	Acar et al., 2015
Mouse Fdg5 (KI)	mCherry	Mouse	*In vivo*	Transplantation	Flow cytometry	Gazit et al., 2014
Mouse Vwf (Tg)	eGFP	Mouse	*In vivo*	Transplantation	Flow cytometry	Sanjuan-Pla et al., 2013
Mouse Scl-tTA (Tg)	H2B-GFP	Mouse	*In vivo* *In vitro*	Doxycycline chase Immunostaining	Flow cytometry Confocal microscopy	Sugimura et al., 2012
Mouse Evi1 (KI)	GFP	Mouse	*In vivo*	Transplantation	Flow cytometry	Kataoka et al., 2011
Mouse Ly6a (Tg)	GFP	Mouse	*Ex vivo*	Imaging	Confocal microscopy	Boisset et al., 2010
Mouse Scl-tTA (Tg)	H2B-GFP	Mouse	*In vivo*	Doxycycline chase	Flow cytometry	Wilson et al., 2008

Tg, Transgenic; KI, Knock-in; tTA, Tetracycline-controlled transactivator protein.

imaging. For instance, mice created using knock-in of a reporter driven by the RNA-binding protein Musashi2 (Msi2) enabled confocal laser scanning microscopy analysis of HSPC movement in calvarial bone marrow (Koechlein et al., 2016); that study revealed that HSPCs residing near vessels migrate toward close proximity to endosteum (**Figure 1**).

Also, GFP knock-in into the α-catulin gene, which is dominantly expressed in HSCs, allowed detection of HSCs in the niche (Acar et al., 2015). Use of these mice combined with techniques to clear bone and bone marrow has provided microscopic evidence that the HSC niche is perisinusoidal in bone marrow (Acar et al., 2015).

Tracking of HSC Division

In addition to the HSC-specific promoter/enhancer-based labeling techniques, the non-dividing phenotype of highly primitive HSCs has been exploited to analyze and purify HSCs. Retaining of 5-bromo-2-deoxyuridine (BrdU) by long-term quiescent HSCs serves as a way to detect this cell type (Wilson et al., 2008). However, non-dividing cells that retain the BrdU label can be identified only after fixation, which kills cells, and this approach is not suitable to isolate living, quiescent HSCs for further analysis.

To resolve this difficulty, a tetracycline (Tet)-inducible expression system employing a histone H2B/fluorescent protein fusion gene was developed (Wilson et al., 2008; Foudi et al.,

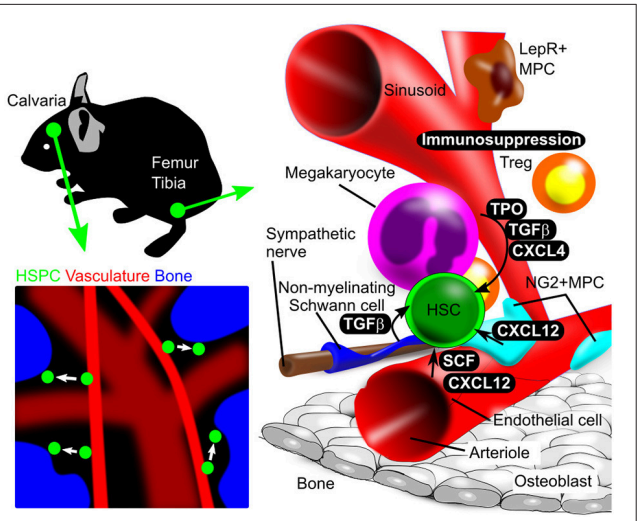

FIGURE 1 | Illustration of *in vivo* and *in vitro* bone marrow imaging. (Upper left panel) Calvarial bone marrow subjected to *in vivo* imaging. Use of reporter mice and *in vivo* staining allows HSPC detection in calvarial bone marrow. (Lower left panel) Intravenous injection of fluorescent dye (red) and second harmonics generation (blue), respectively, identify blood vessels and bone. HSPC behavior is analyzed using a chemical or genetic fluorescent reporter (green). (Right panel) Schematic showing femoral and tibial bone marrow, including HSPCs and niche cells, as revealed by immunostaining. Niche components and their spatial relationships can be observed by imaging analysis.

2009; Sugimura et al., 2012; Bernitz et al., 2016; Säwén et al., 2016). This system is based on the idea that mature hematopoietic cells and HSPCs express the basic helix-loop-helix transcription factor stem cell leukemia (Scl, also known as Tal1), a factor that regulates embryonic and adult hematopoiesis by HSC production and maintenance (Robb et al., 1995; Shivdasani et al., 1995; Mikkola et al., 2003).

A knock-in mouse line harboring the tetracycline transactivator (tTA) under control of endogenous Scl could mark Ter119$^+$ erythroid cells, Gr-1$^+$ granulocytes, CD41$^+$ megakaryocytes and lineage marker (Lin)-negative c-Kit$^+$ HSPCs (Bockamp et al., 2006). This line is then crossed to a transgenic line expressing the histone H2B-GFP fusion protein under control of a tetracycline-responsive regulatory element (TRE). In Scl-tTA::TRE-H2B-GFP double heterozygous mice, greater than 80% of HSC/MPPs express GFP at high levels. In one analysis, after 70 days of doxycycline chase, Lin$^-$ GFP^{bright} cells were highly enriched for non-cycling HSCs (Wilson et al., 2008). Using this system, non-dividing GFP^{bright} HSCs and niche cells were visualized by confocal microscopy, and HSCs were seen in contact with N-cadherin-positive osteoblasts and these osteoblasts activate non-canonical Wnt signaling in the HSC niche (Sugimura et al., 2012). Another seminal study using the hCD34-tTA::TRE-H2B-GFP line showed that HSCs reach a state of complete dormancy after four self-renewal events (Bernitz et al., 2016). The identity of factors that regulate spatiotemporal dynamics of HSC division over this time is a topic for future investigation.

As noted above, while simultaneous identification of multiple cell types remains imperfect due to the limited number of fluorescent dyes applicable to a single experiment, the emergence of novel imaging technologies has facilitated analysis of HSPC movement and molecular interactions in the niche.

USE OF IMAGING TECHNOLOGIES TO ANALYZE THE HSC NICHE DURING DEVELOPMENT

Imaging technology can reveal spatiotemporal cellular interactions and dynamics in the HSC niche. In particular, transgenic animal lines expressing fluorescent proteins in niche cells enable visualization of HSPC interaction with the hematopoietic microenvironment (**Table 3**). Zebrafish are now a powerful model in which to define mechanisms relevant to hematopoiesis and characterize HSC interactions with the microenvironment that govern development (Lieschke and Currie, 2007). In zebrafish, hematopoietic cell lineages are derived from posterior lateral mesoderm (PLM) cells, and Notch signaling between PLM cells and their scaffold, somitic cells, is required for hematopoietic development (Kobayashi et al., 2014). Using two-dimensional (2D) time-lapse live imaging of zebrafish embryo has revealed that HSCs are derived directly from aortic endothelium during development (Bertrand et al., 2010; Kissa and Herbomel, 2010). HSPCs then enter the bloodstream and arrive at the endothelial network in the ventral region of the embryo called the caudal hematopoietic tissue (CHT). HSPCs subsequently attach to the endothelium and remodel the CHT vascular plexus to form a surrounding pocket serving as a site for HSPC division (Tamplin et al., 2015).

Zebrafish models have also been useful to define embryonic HSC niche function. For example, nitric oxide production in the aorta-gonad-mesonephros (AGM) region is critical for a larval hematopoietic response to bacterial infection, as shown by studies using three-dimensional (3D) confocal live imaging (Hall et al., 2012). Since zebrafish embryos are relatively easy to manipulate, some have employed clonal mapping using multi-color genetic labeling and reported evidence suggesting that a limited number of HSC clones contributes to life-long hematopoiesis (Henninger et al., 2017). While wild-type zebrafish embryos are of high clarity, a transgenic line is now available with a body transparent enough for imaging analysis in adult fish (White et al., 2008).

In mammals, bone marrow HSCs are derived from embryonic hemogenic ECs in AGM. Fetal HSCs from AGM migrate to fetal liver (FL) or spleen and then expand their number. Analysis of Ly6a (Sca-1)-GFP transgenic mice, in which HSPCs are GFP-positive (Ma et al., 2002), combined with 3D confocal microscopy, has revealed the precise timing of HSC emerging can be visualized at the embryonic aortic endothelium (Boisset et al., 2010). Confocal microscopy-based 3D imaging of Ly6a-GFP embryos has revealed that HSPCs from FL interact with ECs (Tamplin et al., 2015). By improving sample preparation and imaging technology, longer time-lapse imaging of developmental stages will provide a more complete picture of HSC migration between organs.

Other imaging analysis has suggested that portal vessel-associated pericytes serve as critical HSC niche components in mouse FL (Khan et al., 2016). Specifically, in mice at birth, portal vessels change from a Neuropilin-1$^+$Ephrin-B2$^+$ artery to EphB4$^+$ vein phenotype, resulting in pericyte loss and HSC release from FL. Perivascular lodgment of HSPCs induces active remodeling of the perivascular niche to promote HSPC expansion and maintenance in FL during development.

Post-natal hematopoiesis in mammals occurs mainly in bone marrow. Essential processes of bone development and ossification precede bone marrow development and begin embryonically. In the case of long bones, mineralization of cartilage is followed by blood vessel invasion of the central region of that tissue. Blood then perfuses bones, and actively dividing HSPCs arrive as early as E16.5 in mice, as revealed by 2D immunohistochemical analysis (Coskun et al., 2014). A recent study using *in vitro* imaging system reported that these HSPCs in fetal bone marrow switch from actively-dividing to quiescent, a transition mediated by osteoblast activity, as loss of osteolineage cells in *Osx*$^{-/-}$ mice perturbs induction of HSPC quiescence (Coskun et al., 2014). Another study reports active division of murine HSCs in bone marrow until 3 weeks of age, but after 4 weeks HSCs stop dividing and become quiescent (Bowie et al., 2006). However, molecular and environmental cues that induce these phenotypic changes remain unclear.

TABLE 3 | Examples of key studies using reporter mice to detect niche cells by genetically expressing fluorescent protein.

Driver element	Reporter	Target cell	Model		Analysis	References
Zebrafish kdrl (Tg) Zebrafish cxcl12a (Tg)	GFP, mCherry DsRed2	ECs Stromal cells	Zebrafish	*In vivo*	Confocal microscopy Flow cytometry	Tamplin et al., 2015
Zebrafish kdrl (Tg) Zebrafish fli1 (Tg)	GFP, DsRed mCherry	ECs ECs	Zebrafish	*In vivo* *In vitro*	Confocal microscopy Flow cytometry	Kobayashi et al., 2014
Zebrafish kdrl (Tg)	mCherry	ECs	Zebrafish	*In vivo*	Confocal microscopy Flow cytometry	Hall et al., 2012
Zebrafish kdrl (Tg)	GFP, dTomato	ECs	Zebrafish	*In vivo*	Confocal microscopy	Kissa and Herbomel, 2010
Zebrafish kdrl (Tg)	mCherry	ECs	Zebrafish	*In vivo*	Confocal microscopy Flow cytometry	Bertrand et al., 2010
Mouse Efnb2 (Tg) Mouse Flk1 (Tg)	GFP GFP	ECs ECs	Mouse	*In vivo*	Multi-photon microscopy	Bixel et al., 2017
Mouse Sca-1 (Tg) Rat nestin (Tg)	EGFP EGFP	ECs ECs	Mouse	*In vivo* *In vitro*	Confocal microscopy Multi-photon microscopy	Itkin et al., 2016
Rat nestin (Tg)	GFP	MSCs	Mouse	*In vivo*	Multi-photon microscopy	Spencer et al., 2014
Mouse Cxcl12 (KI)	GFP	MPCs	Mouse	*In vitro*	Confocal microscopy	Greenbaum et al., 2013
Rat nestin (Tg)	GFP	MSCs	Mouse	*In vitro*	Confocal microscopy	Kunisaki et al., 2013
Mouse CxclL12 (KI) Mouse Scf (KI) Rat Col2.3 (Tg)	DsRed GFP GFP	EC PVSCs Perivascular cells Osteoblasts	Mouse	*In vitro*	Confocal microscopy	Ding and Morrison, 2013
Mouse Scf (KI) Rat nestin (Tg) Rat nestin-cre (Tg) Rat Col2.3-cre (Tg) Mouse Lepr-cre (KI)	GFP GFP Cherry loxP-EYFP loxP-EYFP loxP-EYFP	Perivascular cells PVSCs PVSCs Osteoblasts PVSCs	Mouse	*In vitro*	Confocal microscopy	Ding et al., 2012
Mouse Foxp3 (KI)	GFP	T_{reg}s	Mouse	*In vivo*	Multi-photon microscopy	Fujisaki et al., 2011
Mouse Cxcl12 (KI)	GFP	CAR cells	Mouse	*In vitro*	Confocal microscopy	Omatsu et al., 2010
Rat Col2.3 (Tg)	GFP	Osteoblasts	Mouse	*In vivo*	Multi-photon microscopy	Lo Celso et al., 2009
Mouse Vegfr2 (KI) Rat Col2.3 (Tg)	GFP GFP	Sinusoidal ECs Osteoblasts	Mouse	*In vitro*	Confocal microscopy	Hooper et al., 2009

Many of these lines have been used for imaging studies. Tg, Transgenic; KI, Knock-in; EC, Endothelial cells; PVSCs, Perivascular stromal cells; CAR cells, CXCL12-abundant reticular cells.

THE ADULT HSC NICHE

Structural and Regional Analysis of the Adult HSC Niche

Imaging analysis has demonstrated complex interactions between HSC and niche cells, as illustrated in **Figure 1**. In adult mouse bone marrow, the perivascular region is the major HSC niche and is composed of various cell types that function in HSC maintenance. To understand how HSC and various niche cells interact, it is crucial to know the histological structure and properties of bone marrow including vasculature components.

The types of blood vessels in bone marrow are described as follows. Arterial blood flow in bone marrow is mainly supplied by nutrient vessels that penetrate cortical bone. These vessels merge and then form the central artery of bone marrow. Arterioles branch from the central artery toward cortical bone and anastomose with the sinusoid. Transition zone vessels connect arterioles and sinusoidal vessels. Sinusoidal vessels then connect with the central vein, and blood flows from bone marrow through the nutrient vein (Li et al., 2009; Acar et al., 2015; Morikawa and Takubo, 2016). These vessels are classified by

morphological or cellular characteristics revealed by imaging analysis.

Based on imaging analyses of bone marrow, both arteriolar and sinusoidal regions serve as HSC niches (Nombela-Arrieta et al., 2013). Functionally, arteriolar niche cells promote HSC quiescence and sinusoids represent a proliferative HSC niche (Kunisaki et al., 2013). *In vivo* imaging is now an essential not only to track cell movement but to obtain information relevant to blood flow and vascular permeability in bone marrow. Sinusoid exhibits higher vascular permeability than do arteries or arterioles, a property important for bidirectional trafficking of HSCs and differentiated cells between bone marrow and the circulation (Itkin et al., 2016). 3D vascular structural analysis and blood flow measurement using multi-photon laser microscopy suggest that sinusoidal blood flow and shear stress are lower than that seen in the arteriole (Bixel et al., 2017). This study shows that blood flow profiles modulate HSPC homing in the bone marrow vasculature and employs calvaria and femur for *in vivo* imaging and FACS analysis, respectively. Since it is known that hematopoiesis continues in flat bone predominantly in aged human, hematological differences exhibited by these bones are particular interest in future studies. Because the impact of anesthesia or surgical stress on hematopoiesis remains unclear, it is important to carefully interpret results from intravital imaging analysis.

Imaging in mouse has also identified a function of the endosteal region as a regulatory environment for HSCs. For example, *ex vivo* imaging of mouse bone reveals that engrafting HSCs are maintained in the endosteal region after irradiation (Xie et al., 2009). Furthermore, *in vivo* imaging shows that transplanted HSCs dive into close proximity to endosteum (Lo Celso et al., 2009), supporting the idea that the latter functions in HSC homing to damaged bone marrow.

Interaction between Niche Cells and HSPCs in Adult Bone Marrow

Endothelial Cells

Bone marrow endothelium expresses the adhesion molecule E-selectin, playing role in the homing and engraftment of circulating HSPCs (Hidalgo et al., 2002; Katayama et al., 2003). Imaging techniques provides evidence that perisinusoidal HSC proliferation is stimulated by cellular interactions with E-selectin expressed on ECs (Winkler et al., 2012). Sinusoidal ECs also express vascular endothelial cell growth factor (VEGF) receptor 2, and VEGF signaling is required to reconstitute hematopoiesis and maintain HSCs after myeloablation (Hooper et al., 2009). Moreover, Notch ligand secreted by sinusoidal ECs promotes HSC proliferation (Butler et al., 2010).

Confocal microscopy of bone marrow from cytokine stem cell factor (Scf)-GFP knock-in mice revealed that ECs, which form the inner lumen of blood vessels, function in HSC maintenance by producing SCF (Ding et al., 2012).

Mesenchymal Stromal Cells

Mesenchymal Stromal cells (MSCs), which are associated with sinusoidal ECs, have been proposed as niche cells, as they produce factors important to maintain HSCs, such as SCF and CXCL12 (Omatsu et al., 2010; Ding and Morrison, 2013; Greenbaum et al., 2013). Mice engineered to harbor fluorescent reporters at the Scf or Cxcl12 loci provide support that MSCs highly express both genes and are required for HSC the maintenance in bone marrow. The application of tissue clearing methods to bone analysis has increased light transmission of tissue harboring fluorescent protein tags. Tissue clearing and whole bone marrow imaging by using light sheet microscopy of α-catulin-GFP mice demonstrates that in perisinusoidal regions, HSCs reside primarily with MSCs, which highly express the leptin receptor and Cxcl12 (Acar et al., 2015). In support of this finding, others have applied a tissue clearing method to bone marrow plugs of Hoxb5-Tri-mCherry mice, in which HSCs are specifically marked (Chen et al., 2016). In this analysis, Hoxb5^{+} HSCs are localized to the perivascular localization of bone marrow. Most of these HSCs are quiescent (Chen et al., 2016). Additional advances in tissue clearing techniques in mice now enable whole body imaging (Tainaka et al., 2014). These types of methodologies could allow analysis of HSPC distribution throughout the entire body.

Analysis using Nestin-GFP transgenic mice indicates that arterioles are associated with Nestin-GFP^{bright} perivascular stromal cells (Kunisaki et al., 2013). These cells have MSC properties *ex vivo*, highly express the pericyte marker NG2, and reside close to HSCs. Analysis of Nestin-GFP transgenic mice also shows that Nestin-GFP^{dim} cells associate with sinusoids (Kunisaki et al., 2013). Nestin-GFP^{bright} cells are more quiescent than Nestin-GFP^{dim} cells and highly express HSC niche factors. The periarteriolar niche may maintain HSCs in a more primitive state than those in the sinusoidal niche (Kunisaki et al., 2013).

Neurons and Non-myelinating Schwann Cells

Immunohistochemical analysis shows that the periarteriolar niche, which harbors Nestin-GFP^{bright}, cells, is innervated by sympathetic neurons (Méndez-Ferrer et al., 2008). Bone marrow sympathetic nerves release noradrenaline from terminals, an activity that reduces Cxcl12 expression in bone marrow stroma cells. As a result, sympathetic signaling activated by G-CSF promotes HSC release from the niche (Katayama et al., 2006; Méndez-Ferrer et al., 2008). The periarteriolar sympathetic nerve fibers are ensheathed by non-myelinating Schwann cells that activate a latent form TGF-β to maintain HSC quiescence (Yamazaki et al., 2011). 2D confocal imaging of bone marrow reveals that non-myelinating Schwann cells colocalize with HSCs and run parallel to arterioles and sympathetic nerves (Yamazaki et al., 2011; Itkin et al., 2016). These observations support the idea that the periarteriolar region forms a neurovascular-stromal unit that regulates HSC dynamics *in vivo*. Also, intravital imaging of the steps of that migration of G-CSF-stimulated HSPC mobilization from the niche is an area for further investigation.

Hematopoietic Cells

In addition to mesenchymal lineage cells, hematopoietic cells function as HSC niche cells. While platelet production is a major function of megakaryocytes, they also produce niche factors, among them, Cxcl4, TGF-β, and thrombopoietin, in bone marrow. Confocal microscopy, whole-mount imaging and

computational modeling suggest that megakaryocytes and HSCs co-localize (Bruns et al., 2014; Nakamura-Ishizu et al., 2014; Zhao et al., 2014).

Macrophages are critical for G-CSF-induced mobilization of HSCs and are considered a niche cell (Winkler et al., 2010; Chow et al., 2011). Confocal microscopic analysis reveals that macrophages reside in the vicinity of Nestin$^+$ MSC niche cells, and crosstalk between these two cell types enhances HSC retention in the niche.

Regulatory T (T_{reg}) cells suppress immune responses. Survival time of transplanted allogenic HSPCs in T_{reg} cell-depleted mice is shorter than that seen in intact mice as revealed by analysis of FoxP3-GFP reporter mice (Fujisaki et al., 2011). T_{reg} cells suppress immune responses at the HSC niche. *In vivo* imaging analysis using multi-photon microscopy also reveals spatial interactions between T_{reg} cells colocalizing with HSPCs (Fujisaki et al., 2011).

Non-cellular Elements

Non-cellular elements also serve as HSC niche factors. Studies using computer simulations of pO_2 distribution suggest that the hematopoietic compartment is relatively hypoxic (Chow et al., 2001), a condition that maintains HSCs by various mechanisms, including lowering levels of reactive oxygen species (ROS). Imaging has been used to assess the relationship between hypoxia and HSCs stemness. Imaging analysis using oxygen-sensing chemical probes now provides better understanding of molecular oxygen distribution in bone marrow. When incorporated into hypoxic tissues, pimonidazole, a hypoxia probe, can be detected by immunohistochemistry or flow cytometry with anti-pimonidazole antibodies. Using this technique, the HSPCs in bone marrow were found to be hypoxic (Takubo et al., 2010; Nombela-Arrieta et al., 2013).

Improved tissue clearing techniques combined with 3D imaging of thick bone marrow sections confirm that the hypoxic property of HSPCs is independent of their distance from the vasculature (Nombela-Arrieta et al., 2013). HSCs utilize the cellular hypoxia-response system to maintain quiescence and glycolytic metabolic properties (Takubo et al., 2010, 2013). Direct analysis of the bone marrow niche using a phosphorescence lifetime-based O_2 sensing technique and intravital microscopy suggests that (i) bone marrow extracellular space is generally hypoxic and (ii) pO_2 in the periosteum region, where arterioles reside, is higher than in the peri-sinusoidal region located far from the endosteum (Spencer et al., 2014). This study provided local pO_2 information at different regions of bone marrow. Additional dynamic analysis of 2D/3D oxygen distribution in bone marrow and other organs will be required to fully understand how hypoxia maintains stemness of HSC.

USE OF IMAGING TO ANALYZE LEUKEMIA, INFECTION, AND AGE-RELATED EVENTS IN THE HSC NICHE

In vivo imaging of bone marrow using a custom-built fluorescence confocal/multiphoton microscope revealed that pre-B-cell acute lymphoblastic leukemia (ALL) cells preferentially home to bone marrow vessels that express the adhesion molecule E-selectin and Cxcl12 (Sipkins et al., 2005). ALL cells also locally metastasize to Cxcl12-expressing vascular niche cells (Colmone et al., 2008). ALL cells also alter niche cell properties, decrease Cxcl12 production and induce SCF overexpression in bone marrow. *In vivo* time-lapse imaging of the T-ALL niche also reveals that T-ALL cells directly induce osteoblast shrinking and blebbing (Hawkins et al., 2016). Acute myelogenous leukemia and myeloproliferative neoplasms remodel the bone marrow microenvironment by disrupting niche cells, such as MSCs, neurons and Schwann cells (Arranz et al., 2014; Hanoun et al., 2014). As part of the host defense system, immune cells are consumed during infection, activating hematopoietic stem cells to supply blood cells (King and Goodell, 2011). Toll-like receptors and interferon receptors on HSPCs sense infection stress and activate a myeloid differentiation pathway called "emergency myelopoiesis" (Nagai et al., 2006). HSPCs also directly recognize the bacterial product bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) through the innate immune sensor STING. Activation of the c-di-GMP/STING pathway mobilizes HSPCs to peripheral blood (Kobayashi et al., 2015). c-di-GMP also suppresses expression of niche factors (namely, Cxcl12, SCF and Angiopoietin-1) in various non-hematopoietic niche cells. c-di-GMP induces expansion of the sinusoidal area of bone marrow, as revealed by 2D immunohistochemical analysis. Furthermore, *in vivo* time-lapse imaging during acute infection shows that HSC motility is more significantly activated after infection than in steady state (Rashidi et al., 2014). Infectious stress induces HSPC niche remodeling and facilitates HSPC mobilization.

Physiological aging also changes properties of the HSPC niche. Confocal imaging techniques have revealed that bone marrow arteries covered with the α-smooth muscle actin$^+$ cells decrease in number and become more permeable with aging in mice (Kusumbe et al., 2016). Imaging analysis of animal models of hematological disease or aging will provide pathophysiological insights with potential therapeutic application.

CONCLUSION

The dynamics of hematopoiesis are tightly regulated by HSPCs and their niches within the bone marrow. Imaging techniques provide novel methods to define spatiotemporal regulation of complex multicellular microenvironments like bone marrow that every year we know more and more (Joseph et al., 2013). Although various methodological and technological hurdles remain, use of diverse techniques brings increasing insight into HSC interaction with niche cells and reveals how hematopoietic homeostasis is achieved in a dynamic manner.

Here, we have provided examples of imaging-based investigation of various hematopoietic activities, including developmental, physiological and pathological conditions and aging. Studies discussed here focus not only on stem cell location but on properties of the niche environment, such as local oxygen conditions. We anticipate that visualization of HSC cellular status in the niche will define additional mechanisms underlying hematopoiesis and leukemogenesis and potentially suggest novel

therapies for blood cell diseases. Achieving this aim will require development of novel chemical and genetic probes of the cell cycle, metabolism, and signaling status and application of those methods to HSPC biology.

AUTHOR CONTRIBUTIONS

TM and KT wrote the manuscript; and KT conceived and supervised the project.

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MCT1 in Invasive Ductal Carcinoma: Monocarboxylate Metabolism and Aggressive Breast Cancer

*Jennifer M. Johnson[1], Paolo Cotzia[2], Roberto Fratamico[1], Lekha Mikkilineni[3], Jason Chen[1], Daniele Colombo[4], Mehri Mollaee[5], Diana Whitaker-Menezes[1], Marina Domingo-Vidal[1], Zhao Lin[1], Tingting Zhan[6], Madalina Tuluc[5], Juan Palazzo[5], Ruth C. Birbe[7] and Ubaldo E. Martinez-Outschoorn[1]**

[1] Department of Medical Oncology, Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA, [2] Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY, USA, [3] Medical Oncology, National Cancer Institute, Bethesda, MD, USA, [4] Pathology, University of Rome Tor Vergata, Rome, Italy, [5] Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA, USA, [6] Division of Biostatistics, Department of Pharmacology and Experimental Therapeutics, Thomas Jefferson University, Philadelphia, PA, USA, [7] Department of Pathology, Cooper University Hospital, Camden, NJ, USA

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Edited by:
Jelena Krstić,
Institute for Medical Research, Serbia

Reviewed by:
Hebao Yuan,
University of Michigan, USA
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Università degli Studi della Campania "Luigi Vanvitelli" Caserta, Italy

***Correspondence:**
Ubaldo E. Martinez-Outschoorn
ubaldo.martinez-outschoorn@jefferson.edu

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Introduction: Monocarboxylate transporter 1 (MCT1) is an importer of monocarboxylates such as lactate and pyruvate and a marker of mitochondrial metabolism. MCT1 is highly expressed in a subgroup of cancer cells to allow for catabolite uptake from the tumor microenvironment to support mitochondrial metabolism. We studied the protein expression of MCT1 in a broad group of breast invasive ductal carcinoma specimens to determine its association with breast cancer subtypes and outcomes.

Methods: MCT1 expression was evaluated by immunohistochemistry on tissue micro-arrays (TMA) obtained through our tumor bank. Two hundred and fifty-seven cases were analyzed: 180 cases were estrogen receptor and/or progesterone receptor positive (ER+ and/or PR+), 62 cases were human epidermal growth factor receptor 2 positive (HER2+), and 56 cases were triple negative breast cancers (TNBC). MCT1 expression was quantified by digital pathology with Aperio software. The intensity of the staining was measured on a continuous scale (0-black to 255-bright white) using a co-localization algorithm. Statistical analysis was performed using a linear mixed model.

Results: High MCT1 expression was more commonly found in TNBC compared to ER+ and/or PR+ and compared to HER-2+ ($p < 0.001$). Tumors with an *in-situ* component were less likely to stain strongly for MCT1 ($p < 0.05$). High nuclear grade was associated with higher MCT1 staining ($p < 0.01$). Higher T stage tumors were noted to have a higher expression of MCT1 ($p < 0.05$). High MCT1 staining in cancer cells was associated with shorter progression free survival, increased risk of recurrence, and larger size independent of TNBC status ($p < 0.05$).

Conclusion: MCT1 expression, which is a marker of high catabolite uptake and mitochondrial metabolism, is associated with recurrence in breast invasive ductal carcinoma. MCT1 expression as quantified with digital image analysis may be useful as a prognostic biomarker and to design clinical trials using MCT1 inhibitors.

Keywords: triple negative breast cancer, glycolysis, oxidative phosphorylation, lactic acid, tumor microenvironment

INTRODUCTION

Breast cancer remains the most common cancer diagnosed and the second most common cause of cancer-related death in US women in 2016 despite advances in early detection and novel treatments (Siegel et al., 2016). Risk-stratification of breast cancer is predominantly based on the presence or absence of the hormone receptors for estrogen (ER) and progesterone (PR), overexpression of human epidermal growth factor receptor 2 (HER2), clinical staging, and in some cases, selected gene expression profiles. ER and HER2 are both prognostic biomarkers and are used to predict response to antiestrogen drugs and HER2 inhibitors.

Human breast cancer has a different metabolic rate compared to normal breast tissue. Tumors frequently have very high levels of lactate in their microenvironment, produced by aerobic glycolysis. Otto Warburg hypothesized that cancer cells contained dysfunctional mitochondria leading to their inability to utilize oxidative phosphorylation to generate ATP and forcing them to use aerobic glycolysis (Koppenol et al., 2011). We now know that in many cases of human breast cancer, mitochondria are not dysfunctional and in fact some cancer cells have very high mitochondrial oxidative phosphorylation (OXPHOS) (Martinez-Outschoorn et al., 2017). Glycolysis is more energetically inefficient compared to OXPHOS and why cells within tumors would utilize glycolysis has remained a paradox (Vander Heiden et al., 2009).

Detailed characterization of breast tumor metabolism has revealed that there is intratumoral metabolic heterogeneity with some cells being glycolytic and generating lactic acid, while others have high mitochondrial metabolism. Metabolic heterogeneity might explain the apparent tumor metabolism paradox since it increases energetic efficiency (Martinez-Outschoorn et al., 2017). Metabolic heterogeneity can be induced in experimental models of breast cancer by oxidative stress, which damages the mitochondria of intratumoral stromal cells and induces a metabolic switch to aerobic glycolysis (Pavlides et al., 2009; Martinez-Outschoorn et al., 2012). Conversely high antioxidant activity via activation of the pentose phosphate pathway in a subgroup of carcinoma cells allows these cells to maintain high mitochondrial oxidative phosphorylation metabolism (OXPHOS) (Ko et al., 2016). Lactate is one of the links between these two intratumoral compartments since it can be produced by glycolytic tumor stromal cells and then taken up by carcinoma cells to be utilized for mitochondrial OXPHOS and ATP production (DeNicola and Cantley, 2015).

Monocarboxylate transporters (MCTs) play a key role in this symbiotic relationship between carcinoma cells and other cells of the tumor microenvironment since they regulate the release and uptake of lactic acid and the extracellular pH of the tumor (Martinez-Outschoorn et al., 2017). Metabolic reprogramming of the cancer stroma may provide a compensatory mechanism for cancer cells to survive in an energetically efficient manner in the harsh tumor environment. Lactate is mainly taken up by cancer and non-cancer cells via monocarboxylate transporter 1 (MCT1) (Pinheiro et al., 2010a, 2012; Jones and Morris, 2016). Cancer cells frequently express MCT1 (Peiris-Pages et al., 2016).

MCT1 has been correlated with increased disease aggressiveness across various solid malignancies. Previous work has shown that MCT1 is expressed in human breast, ovarian, cervical, lung, and colorectal cancers, highlighting its importance as a potential marker and therapeutic target across multiple tumor types (Pinheiro et al., 2010b). Pinhero et al. have also specifically showed an increase in MCT1 expression in basal-like breast cancer (BLBC) (Pinheiro et al., 2010a). Cytokeratin 5 is positive in BLBC and the majority of BLBC are triple negative breast cancers since they are negative for ER, PR, and HER2 (Fadare and Tavassoli, 2008).

MCT1 is associated with aggressive disease in models of breast, gastrointestinal, and squamous cell carcinomas (Koukourakis et al., 2006; Bonuccelli et al., 2010; Pinheiro et al., 2010a; Martinez-Outschoorn et al., 2011). In breast cancer models, fibroblasts surrounding malignant cells demonstrate low caveolin 1 expression, a loss which enhances aerobic glycolysis in these cells, with concurrent increased mitochondrial activity and high expression of MCT1 transporter in the epithelial cancer cells with uptake of catabolites (Bonuccelli et al., 2010). In a study carried out by Oliveira et al. expression of MCT1 and MCT4 was present in 90% of GISTs, findings concordant with the high degree of glycolytic metabolism in these tumor types (de Oliveira et al., 2012). Also, MCT1 was found in both the carcinoma cell compartment as well as proliferating basal stem cells in head and neck cancers, underscoring its importance in cell proliferation (Curry et al., 2013).

We hypothesized that altered tumor metabolism with high monocarboxylate uptake in carcinoma cells is a feature of aggressive breast cancers and that higher MCT1 expression will be found in cancer cells of this clinical subtype. This is in keeping with the observations of others that MCT1 is expressed in basal-like breast cancer. To evaluate this hypothesis, we stained breast cancer tissue microarrays totaling 257 patients for MCT1 and

Abbreviations: MCT, monocarboxylate transporter; TNBC, triple negative breast cancer; ER, estrogen receptor; PR, progesterone receptor; BLBC, basal like breast cancer; CK, cytokeratin; ATP, adenosine triphosphate; GIST, gastrointestinal stromal tumor; AJCC, American Joint Committee on Cancer; ISH, *in-situ* hybridization.

digitally analyzed the expression patterns. We have furthermore investigated the value of MCT1 as a prognostic marker in this cohort.

MATERIALS AND METHODS

Subjects

The Institutional Review Board at Thomas Jefferson University approved the protocol for this study. Samples of breast cancer were obtained from 257 subjects at Thomas Jefferson University Hospital. Patient data were collected including age, sex, staging by AJCC Version 7 criteria, size of the primary tumor, number of positive lymph nodes, grade, histologic subtypes, mitotic index, Ki67, lymphovascular invasion, resection margins (if applicable), treatment including the use of chemotherapy, radiation therapy, and hormonal therapy, recurrence, and vital status (Edge et al., 2010).

With respect to hormone receptor status, >1% of cells positive by immunohistochemistry were consider to be positive for both estrogen receptor and progesterone receptor status. For HER2 status, the current guidelines of 3+ staining by immunohistochemistry with >30% of invasive tumor cells showing staining, ISH positive based on single probe average HER2 copy number of >= 6 signals/cell, or ISH positive based on dual probe HER2 to CEP17 ratio >= 2.0 were used as cut-offs.

Samples and Immunohistochemistry

A total of 532 human samples of breast carcinoma were studied to evaluate the metabolism of cancer cells within the tumor samples representative of 257 distinct patients. When possible, samples were run in duplicate or triplicate based on the amount of tissue present in the remaining pathology specimens.

Samples were stained by immunohistochemistry for MCT1. All cancer present on a slide and its dominant staining pattern were considered when determining the percent of immune-positive cancer cells in a sample. Human tissues for analysis were fixed in neutral buffered formalin and then embedded in paraffin. Sections (4 μm) were dewaxed, rehydrated through graded ethanols, and antigen retrieval was performed on the Ventana Discovery ULTRA staining platform using Discovery CCI (Ventana cat#950-500) for a total application time of 64 min, followed by MCT1 antibody incubation for 45 min. Secondary immunostaining used a Horseradish Peroxidase (HRP) multimer cocktail (Ventana cat#760-500) and immune complexes were visualized using the ultraView Universal DAB (diaminobenzidine tetrahydrochloride) Detection Kit (Ventana cat#760-500). Slides were then washed with a Tris based reaction buffer (Ventana cat#950-300) and stained with Hematoxylin II (Ventana cat #790-2208) for 8 min.

Quantitative analysis of MCT1 was also performed employing digital pathology with Aperio software. Tissue sections were scanned on a ScanScope™ XT with an average scan time of 120 s (compression quality 70). Images were analyzed using the Color Deconvolution, the Colocalization, and the Membrane Aperio Image Analysis tool. For the Color Deconvolution and Colocalization, analysis areas of staining were color separated from hematoxylin counter-stained sections and the intensity of the staining was measured on a continuous scale from 0 (black) to 255 (bright white). For the membrane analysis, the algorithm detects the membrane staining for the individual tumor cells in the selected regions and quantifies the intensity and completeness of the membrane staining. Tumor cells are individually classified as 0, 1+, 2+, and 3+ based on their membrane staining intensity and completeness. A tumor cell is classified 1+ when there is only partial membrane staining or weak membrane staining. A tumor cell is classified 2+ when there is moderate and complete membrane staining. A tumor cell is classified 3+ when there is intense and complete membrane staining. For each sample the whole tumor area was analyzed. Tumor specimens were considered "positive" for MCT1, when greater than or equal to 30% of the cells analyzed stained at an intensity of 2+ or greater, as previously published (Curry et al., 2013).

Statistical Methods

The expression of MCT1 in human breast specimens was determined as above. Associations between estrogen receptor, progesterone receptor, and HER2 expression and the membrane expression of MCT1 defined as the percentage of cells analyzed expressing 2+ or greater stain intensity were performed using multivariate linear regression with adjustment on heteroskedasticity. Associations of MCT1 expression with race, menopausal status, histologic and nuclear grade, mitotic score, histologic subtypes, Ki67 scoring, tumor size, and the presence of lymphovascular invasion were performed using simple linear regression analysis. Hazard ratios for both risk of recurrence and overall survival were performed using multivariate cox proportional hazard ratios.

RESULTS

Baseline Patient Characteristics

Our patients included 257 individuals with a diagnosis of invasive breast cancer treated at Thomas Jefferson University Hospital between the years of 2000 and 2008. Characteristics of these patients are shown in **Table 1**. The average patient age was 57.2 with a range from 26.9 to 97.8 years. We collected self-identified information on race of the patients, which included 168 white, 66 black, 10 Asian, 3 Hispanic, and 10 samples where the race is unknown. Menopausal status was determined by the report of the patients through review of their medical oncologist's office notes. Of the 99 patients for whom this information was recorded, 22 were premenopausal, 22 were listed as peri-menopausal, and 55 were postmenopausal at the time of their initial diagnosis.

With respect to tumor characteristics, 116 patients had T1 tumors, 95 were T2, 16 were T3, 13 were T4, and 17 were unknown. In 124 patients their lymph nodes were negative, 112 were positive, and 21 were unknown. Only 8 patients had metastatic disease at the time of diagnosis. The ER, PR, and HER2 characteristics of the subjects are also provided in **Table 1**.

Association with Receptor Status

Individually, ER, PR, and HER2 positivity were all negatively associated with MCT1 expression. There is an association between ER negative status and high MCT1 expression and between HER2 negative status and high MCT1 expression ($p < 0.003$ and 0.045, respectively). There was a trend between PR

TABLE 1 | Characteristics of the samples included in the breast tissue microarray.

Age	57.2 (26.9–97.8)
RACE (SELF-IDENTIFIED)	
White, non-hispanic	168
Black	66
Asian	10
Hispanic	3
Unknown	10
MENOPAUSAL STATUS	
Pre-menopausal	22
Peri-menopausal	22
Post-menopausal	55
Unknown	158
HISTOLOGY	
IDC	254
ILC	4
LN or Metastasis	7
STAGE-TUMOR	
T1	116
T2	95
T3	16
T4	13
Tx	17
STAGE-NODAL	
N0	124
N1	98
N2	13
N3	1
Nx	21
STAGE-METASTASES	
M0	195
M1	8
Mx	54
RECEPTOR PROFILE COMPOSITES	
ER and/or PR+ HER2-	138
ER and/or PR+ HER2+	42
ER and/or PR– HER2+	20
TNBC	56

IDC, Invasive Ductal Carcinoma; ILD, Invasive Lobular Carcinoma; LN, Lymph Node; ER/PR, Estrogen/Progesterone Receptor; PR, Progesterone Receptor; HER2, Human Epidermal Growth Factor Receptor 2 status.

TABLE 2 | Clinico-pathological correlations with MCT1 staining in epithelial cancer cells.

Characteristic	**Estimated difference**	***p*-value**
RACE (RELATIVE TO WHITE)		
Black	−0.03	0.875
Asian	0.18	0.169
Hispanic	0.07	0.953
Pre-menopausal vs. post-menopausal	0.12	0.052
ER+	−0.16	0.002
PR+	−0.08	0.089
HER2+	−0.08	0.044
Triple negative	0.27	< 0.001
Size	Odds ratio 0.03	0.002
High mitotic score	0.13	< 0.001
High nuclear grade	0.16	< 0.001
Ki67	0.37	0.715
LVI	−0.01	0.833
PRESENCE OF HISTOLOGIC SUBTYPES		
Apocrine	−0.02	0.891
Colloid	−0.06	0.660
Comedo	−0.02	0.685
Cribriform	−0.08	0.092
Lobular	0.09	0.179
Metaplastic	−0.05	0.771
Micropapillary	−0.02	0.815
Mixed	−0.33	0.159
Neuroendocrine	−0.11	0.633
Pleomorphic	0.13	0.275
Solid	−0.11	0.008
Tubular	0.09	0.510
In situ component identified in the sample	−0.09	0.043

ER, estrogen receptor; PR, progesterone receptor; HER2, Human Epidermal Growth Factor Receptor 2; LVI, Lymphovascular Invasion.

negative status and high MCT1 expression but this did not reach the level of statistical significance ($p < 0.09$; **Table 2**). Thus, we further investigated TNBC (ER negative, PR negative, and HER2 negative tumors) and found an association between high MCT1 expression and TNBC compared to the other subtypes with an estimated difference of 27% ($p < 0.001$; **Table 2**).

Correlation of Histologic Features with MCT1 Expression

In addition to receptor status, we also investigated other histologic features. Tumors with an identifiable *in situ* component were less likely to stain strongly for MCT1 ($p = 0.043$; **Table 2**). We therefore also reviewed the presence of different histologic subtypes for potential correlation with MCT1 expression (**Figure 1**). There was no statistically significant correlation with apocrine features ($p = 0.891$), colloid ($p = 0.660$), comedo ($p = 0.685$), cribriform ($p = 0.092$), lobular ($p = 0.179$), metaplastic ($p = 0.771$), micropapillary ($p = 0.815$), mixed histologies ($p = 0.159$), neuroendocrine ($p = 0.633$), pleomorphic ($p = 0.13$), or tubular subtypes ($p = 0.510$). It is important to note that only a small number of samples had any of these features. In contrast, the presence of a solid subtype was associated with lower MCT1 staining with an estimated difference of 11% ($p = 0.008$, $N = 82$; **Table 2**).

High nuclear grade, defined as tumor samples scored as a 3 vs. those scored as either 1 or 2 was significantly associated with higher MCT1 staining with a *p*-value of <0.001. Similarly, a high mitotic score (score of 3 vs. those with scores of 1 and 2) was also associated with higher MCT1 staining (grade 3 vs. grades 1 and 2) with $p < 0.001$. Conversely, the presence of lymphovascular invasion and Ki67 rates scored by the pathologists were not

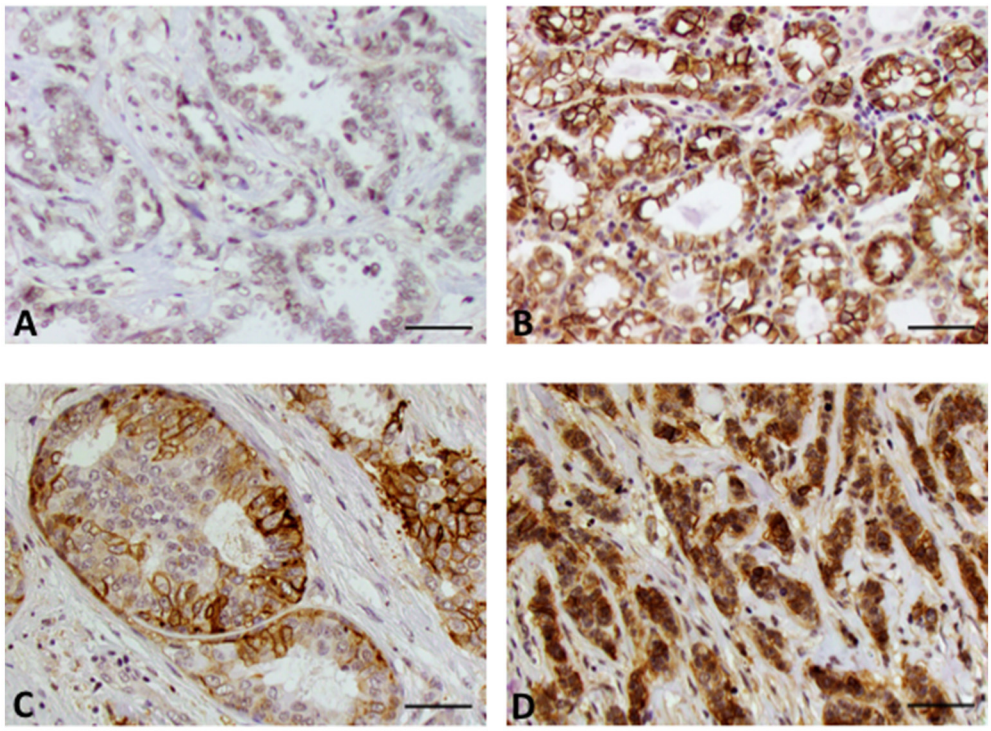

FIGURE 1 | Expression of MCT1 in invasive breast cancer. (A) Invasive ductal carcinoma grade 2. **(B)** Invasive ductal carcinoma grade 3. **(C)** Cribriform pattern. **(D)** Invasive lobular carcinoma grade 3. Note that the grade 2 invasive ductal carcinoma has lower MCT1 expression compared to the other subtypes. Original magnification 20x (scale bar 50 μM).

associated with MCT1 expression ($p = 0.833$ and $p = 0.715$, respectively; **Table 2**).

Correlation of Clinical Features with MCT1 Expression

We also reviewed patient characteristics with regards to MCT1 expression. These included age, self-identified race, menopausal status, tumor, and lymph node stage by AJCC criteria, and tumor size (**Table 2**). No statistically significant differences were noted in MCT1 expression patterns between racial groups. However, MCT1 staining was 12% higher in premenopausal women's tumors as compared to their post-menopausal counterparts ($p < 0.053$). Younger age was statistically associated with higher MCT1 staining $p < 0.001$ but the magnitude of this difference was estimated at only 3% with a confidence interval from 1 to 5%. Thus, this is likely of little clinical significance.

Comparisons were made both by individually comparing T1a, 1b, 1c, 2, 3, 4a, 4b, and 4d and by comparing all T1, 2, 3, and 4 samples. Higher T stage tumors were noted to have a higher expression of MCT1, as summarized in **Table 3**. Tumor size, considered as a continuous variable, was also positively associated with MCT1 expression with an odds ratio of 0.03 and *p*-value of 0.002 (**Table 2**). Nodal status was not associated with MCT1 expression.

Outcome Correlations Data

At the time of the collection of our data, survival data was available with an average follow up time of 8.04 years. Seventy-three patients recurred by the time data was collected at an average of 4.37 years after diagnosis. Higher MCT1 staining was predictive of recurrence with a hazard ratio of 2.82 and a *p*-value of 0.024 (**Table 4**, **Figure 2**). Of the 257 patients presented here, 196 were alive at the time of data analysis. The overall survival of the sum of all patients was 5.68 years. MCT1 staining trended toward an association with overall survival but this was not statistically significant, with a hazard ratio of 1.89 and *p*- value of 0.171 (**Table 4**).

DISCUSSION

Triple Negative Breast Cancer Is Associated with Higher Expression of MCT1

Our work has shown a statistically significant association between ER negative, HER2-negative, and triple negative breast cancer and high MCT1 expression. This is in keeping with the observation by McCleland et al. that 26% of their 31 triple negative samples stained either 2+ or 3+ for MCT1 by IHC (McCleland et al., 2012). Pre-menopausal patients

TABLE 3 | T stage correlation with MCT1 staining.

AJCC Clinical Stage	vs. T1	vs. T2	vs. T3	vs. T4
T1		−0.05 ($p < 0.18$)	0.19 ($p < 0.001$)	0.21 ($p < 0.001$)
T2			0.14 ($p < 0.04$)	0.16 ($p < 0.02$)
T3				0.02 ($p < 0.99$)

TABLE 4 | Correlation of MCT1 staining with recurrence and overall survival using a Cox Regression Model.

Outcome measure	Hazard ratio	*p*-value
Recurrence	2.82	0.024
Overall survival	1.89	0.171

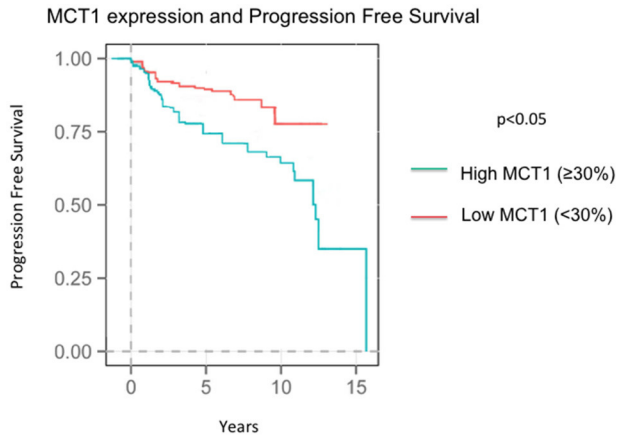

FIGURE 2 | Progression Free Survival in patients expressing high MCT1 (≥30%) and low MCT1 (<30%).

(relative to their post-menopausal counterparts) also had a higher proportion of cells expressing MCT1, although this may in fact be due to the fact that triple negative disease is more prevalent in pre-menopausal females. Identifying a metabolic signature that is associated with these aggressive features provides an additional insight into this category of breast cancer.

MCT1 Expression Is Associated with Other Poor Prognostic Markers

Triple negative breast cancer is known to be associated with African-American race, younger age at diagnosis, higher clinical stage, higher grade, higher mitotic indices, and pre-menopausal status (Carey et al., 2007). Similarly, MCT1 staining was associated with younger age, higher grade, higher mitotic index, and menopausal status. These are all features of more aggressive disease regardless of their association with triple negative breast cancer. Race and clinical stage were not associated.

We have also investigated histologic subtypes as previous work within triple negative breast cancer has suggested a difference in aggressiveness based upon these characteristics (Brower et al., 1995; Jensen and Page, 2003; Go et al., 2010). With the exception of the solid subtype (−0.11, $p = 0.008$) there were no associations found within our cohort of patients. This is in contrast to other studies showing that the comedo and pure papillary subtypes may be associated with poor prognostic markers and increased invasion on biopsy (Brower et al., 1995; Jensen and Page, 2003; Go et al., 2010). A limitation of this study may be the small numbers of cases of each subtype with the exception of the solid subtype. We also do not have information of gene expression profiling of these tumors in order to determine if gene expression features are associated with MCT1 expression.

There was an association between MCT1 positivity and larger tumor size, suggesting that MCT1 promotes increased carcinoma cell survival and growth ($p = 0.002$). Studies in other cancers have also demonstrated a correlation between high MCT1 staining and advanced tumor stage, particularly in head and neck squamous cell carcinomas (Curry et al., 2013), gastrointestinal cancers (de Oliveira et al., 2012), prostate cancer (Pertega-Gomes et al., 2014), and urothelial cancer (Choi et al., 2014).

MCT1 Expression Is Associated with Poor Outcomes

Increased MCT1 staining was associated with a higher recurrence rate with a HR of 2.62 ($p = 0.024$). In addition, the association between a higher percentage of cells staining for MCT1 and death had a hazard ration of 1.89, however this result was not statistically significant ($p = 0.171$). When we investigated whether the associations with higher risk of recurrence was driven by TNBC vs. MCT1 expression, MCT1 expression has an independent contribution to the chance of recurrence.

One potential explanation is that recurrence of disease is more likely when the stroma is metabolically primed to host tumor cells. If chemotherapy failed to eradicate the entirety of the tumor burden, small satellite colonies may more easily settle in soil that has metabolically shifted to serve these tumor cells, thus providing another level of chemo-resistance not previously delineated. To prevent recurrence in high-risk, triple negative disease, it may be necessary to target this soil. MCT1 not only provides diagnostic value but it could serve as an important therapeutic target in the future where chemotherapy would target not only the tumor cell but its stromal energy supply as well. MCT4, a lactate-pyruvate shuttle found in stromal cells, could be MCT1's counterpart therapeutic target. Thus, TNBCs may be further described not only as having a distinct molecular subtype but potentially a distinct metabolic phenotype that can aid in diagnosis and prognostication of this group of tumors.

Relevance and Future Directions

The current study is consistent with previous work revealing an association between MCT1 and aggressive breast cancer. MCT1 expression in a TNBC *in vitro* model is associated with cell migration (Gray et al., 2016). Also, Pinheiro et al. have discovered an association between MCT1 expression and up-regulation of basal-associated markers such as CK5, CK14, and vimentin and an inverse relationship of MCT1 to ER and PR expression (Pinheiro et al., 2012). We have discovered that MCT1 is associated with poor outcomes irrespective of breast cancer subtype. MCT1 expression in carcinoma cells may improve risk-stratification of breast cancers. For example, high MCT1 staining

may be a marker of a subgroup of very aggressive breast cancers. Inhibitors of MCT1 are in development and may prove to be active in this disease. Our results will need to be evaluated prospectively to confirm the role of MCT1 as a prognostic and predictive biomarker in breast cancer.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Declaration of Helsinki. The protocol was approved by the Thomas Jefferson University Institutional Review Committee. Samples were de-identified and no consent was required from participants.

AUTHOR CONTRIBUTIONS

JJ, PC, RB, and UM were involved in the study design and concept. JJ, PC, RF, LM, JC, DC, MM, DW, MD, ZL, MT, JP, TZ, RB were involved in data acquisition. JJ, PC, RF, LM, JC, DC, TZ, MT, JP, RB, and UM were involved in data analysis and interpretation. JJ, RF, LM, and JC drafted the manuscript. JJ and UM edited the manuscript.

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