PERSPECTIVES IN MAMMARY GLAND DEVELOPMENT AND BREAST CANCER RESEARCH

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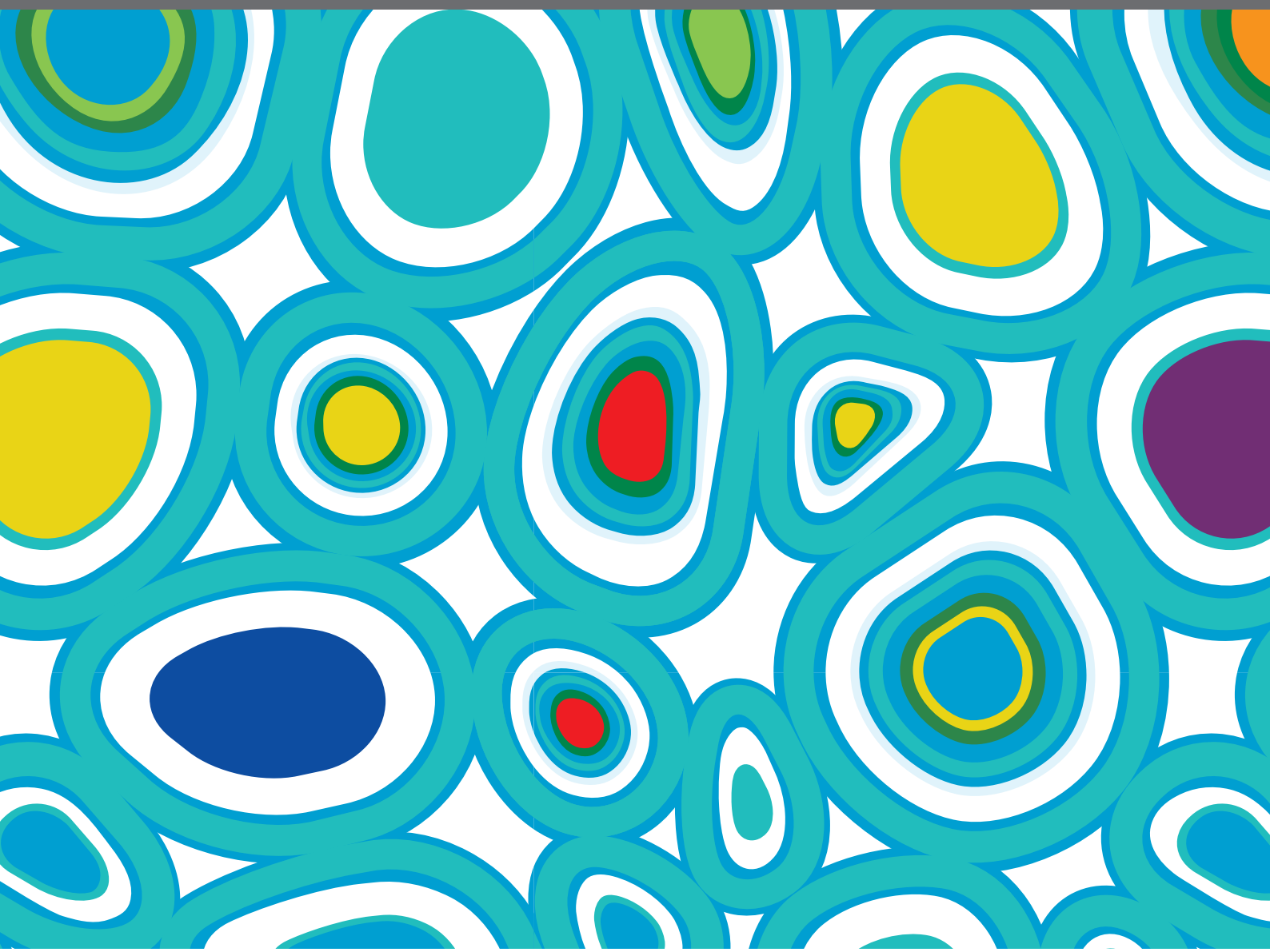
PUBLISHED IN: Frontiers in Cell and Developmental Biology



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ISSN 1664-8714
ISBN 978-2-88966-094-0
DOI 10.3389/978-2-88966-094-0

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PERSPECTIVES IN MAMMARY GLAND DEVELOPMENT AND BREAST CANCER RESEARCH

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Citation: Koledova, Z., Van Keymeulen, A., Vafaizadeh, V., Peuhu, E., eds. (2020). Perspectives in Mammary Gland Development and Breast Cancer Research. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-094-0

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frontiers in Cell and Developmental Biology

EDITORIAL
published: 25 August 2020
doi: 10.3389/fcell.2020.00719

Editorial: Perspectives in Mammary Gland Development and Breast Cancer Research

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Keywords: breast cancer, mammary gland, development, imaging, microenviroment, organoid, signaling, omics

Editorial on the Research Topic

Perspectives in Mammary Gland Development and Breast Cancer Research

Annually, researchers from around the world, who work in the field of mammary gland biology and breast cancer and related areas, have a great opportunity to meet and discuss their work at a conference organized by the *European Network of Breast Development and Cancer* (ENBDC). These meetings, entitled *Annual ENBDC Workshop: Methods in mammary gland biology and breast cancer*, are largely methodologically oriented. They enable not only presentation of the latest scientific results, but also dissemination of cutting-edge approaches and forefront technologies that have facilitated these discoveries. The latest meeting took place on the 16th to 18th of May 2019 in Weggis, Switzerland, and presented exciting findings achieved using high resolution 'omics approaches, genetic mouse models, organoids, and state-of-the-art imaging (Vafaizadeh et al., 2019). Here, we present a collection of articles based on or related to the topics of the ENBDC workshop.

Breast cancer is the most common cancer in women, annually diagnosed in more than 2.1 million women worldwide and more than 650,000 women worldwide die from this heterogeneous disease every year. To improve treatment strategies, deep understanding of breast cancer and metastasis is required. In their review, Parsons and Francavilla discuss how genomics, transcriptomics, proteomics, and metabolomics datasets, in combination with traditional breast cancer models, provide insights into breast cancer biology and enable discovery of novel therapeutic targets or biomarkers. They also emphasize the importance of transparent data sharing in data repositories to allow further meta-analysis and potential discoveries of previously unnoticed biomarkers or therapeutic targets. Waterhouse et al. further discuss the challenges of targeting driver oncogenes of triple negative breast cancer (TNBC). They suggest that identification of protein-protein interactions of TNBC oncogenes is needed to understand their functions in TNBC and to reveal novel therapeutic targets. They provide a nice overview of current and emerging agents for targeting TNBC oncogenes on cell surface, cytoplasm, and nucleus, including different genetic and epigenetic strategies for targeting transcription factors.

Three of the articles are focused on specific signaling pathways in breast cancer. van Schie and van Amerongen highlight the role of aberrant WNT-CTNNB1 signaling in human breast cancer and discuss three major gaps in this field: (i) Incomplete understanding of WNT signaling functions in normal human breast development and physiology, (ii) lack of knowledge of the extent and effect of (epi)genetic changes in the WNT pathway in different breast cancer subtypes, and

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *06 July 2020*
Accepted: *14 July 2020*
Published: *25 August 2020*

Citation:
Vafaizadeh V, Peuhu E, Van Keymeulen A and Koledova Z (2020) Editorial: Perspectives in Mammary Gland Development and Breast Cancer Research. Front. Cell Dev. Biol. 8:719. doi: 10.3389/fcell.2020.00719

(iii) lack of insight and biomarkers for selection of the correct subset of patients who might benefit from WNT pathway therapeutics. Fang et al. reviewed the roles of genes in the Fanconi Anemia pathway, which plays a central role in repairing DNA interstrand cross-links and includes the well-known DNA repair proteins BRCA1 and BRCA2. The authors describe promising strategies, like synthetic lethality, to target Fanconi Anemia pathway for breast cancer therapy. A new insight on how STRIP1, a component of the STRIPAK kinase and phosphatase complex, contributes to breast cancer regulation, is provided by Rodriguez-Cupello et al.. They observed increased viability in STRIP1-depleted breast cancer cells after chemotherapy treatment compared to control cells, and detected high induction of the CDK inhibitor p21 via MST3/4 kinases in STRIP1-depleted cells, which appeared to provide protection from treatment-induced DNA damage. These observations suggest that loss of STRIP1 can promote recurrent disease after treatment with sub-optimal doses of chemotherapy.

Microenvironment plays an important role in tissue homeostasis, cancer progression, and metastasis (Bissell and Hines, 2011). Cancer-associated stroma (CAS) is composed of different cellular and extracellular components. Understanding transcriptional reprogramming of CAS is crucial for efficient targeting of tumor progression. Spontaneous canine simple mammary tumors (CMTs) are a useful model of human breast cancer to study the reprograming of CAS in malignant carcinomas compared to benign adenomas. In her article, Markkanen provides evidence for molecular homologies in stromal tissues between canine and human mammary tumors.

Disseminated breast cancer cells can survive for a long period in a foreign environment without developing into overt metastasis (Park and Nam, 2020). Detection and eradication of these cells is imperative to avoid cancer relapse. Montagner and Sahai have assembled a very useful overview about the current *in vitro* models to study breast cancer dormancy. They highlight the challenges of development and validation of the models, discuss the role of different dormant niche components, and present the models developed for metastatic breast cancer dormancy in different tissues, such as lung or bone.

The immune checkpoint blockade (ICB) therapy (Pardoll, 2012) is one of the promising approaches in personalized breast cancer therapy. Vafaizadeh and Barekati summarized recent studies on immuno-oncology biomarkers, which are crucial for selection of responsive cancer patients to ICB, such as anti-PD1 and anti-PD-L1 antibodies, to achieve clinical benefit.

To fully understand the defects leading to breast cancer, it is essential to decipher the mechanisms that regulate normal mammary epithelial morphogenesis and homeostasis. Mammary gland consists of a branched network of epithelial tubes embedded in a complex stroma. The three-dimensional (3D) epithelial architecture is critical for proper mammary function. Therefore, to study mammary morphogenesis and dynamics, 3D cell cultures are essential (Weigelt et al., 2014; Koledova, 2017). To this end, Sumbal et al. developed a new *ex vivo* model of mammary lactation and involution using primary mouse organoids. This model can be applied to study mechanisms of physiological mammary gland lactation and involution as well as pregnancy-associated breast cancer. Budkova et al. investigated regulation of epithelial-to-mesenchymal transition (EMT), a developmental process that is often hijacked by cancer cells. They found that maternally expressed non-coding RNAs of the *DLK1-DIO3* locus are markers of EMT and that MEG3 is a novel regulator of EMT/MET in breast tissue.

Mammary stroma provides instructive signals for mammary gland morphogenesis and homeostasis (Wiseman and Werb, 2002). Macrophages are one of the cellular components of the stroma, implicated in regulation of all stages of mammary gland development (Schwertfeger et al., 2006). Using optical tissue clearing and 3D imaging of mammary tissue obtained from *Csf1r-EGFP* mice, Stewart et al. revealed stage-specific differences in macrophage abundance, localization, morphology, and association with epithelial cells. Their article provides important insights into dynamics of macrophage distribution during mammary gland development and demonstrates the need for high-resolution, multidimensional imaging approaches to study the highly dynamic mammary gland morphogenesis. The current and state-of-the-art imaging approaches, instrumental to shedding light on mammary gland ductal development, lactation, as well as tumor invasion and metastasis, are review by Lloyd-Lewis. She discusses advantages of several fluorescence light-based microscopy platforms and considers specific technical requirements for intravital imaging as well as fixed tissue processing, including clearing.

In summary, this Research Topic includes both original research articles as well as review articles and reflects the wide range of current research in the mammary gland biology and breast cancer fields. We hope that they will be of interest to a broad scientific readership.

AUTHOR CONTRIBUTIONS

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

FUNDING

AV was senior associate researcher of the FNRS. EP was supported by the Academy of Finland (grant no. 323096). ZK was supported by the Grant Agency of Masaryk University (grant no. MUNI/G/1446/2018) and by funds from the Faculty of Medicine Masaryk University to junior researcher (ZK, ROZV/28/LF/2020).

ACKNOWLEDGMENTS

We thank all contributing authors and reviewers for their contributions to the Research Topic. We also appreciate the great support received from Frontiers Team members at all times.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 24 October 2019
doi: 10.3389/fcell.2019.00250

Developmental Stage-Specific Distribution of Macrophages in Mouse Mammary Gland

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Mammary gland development begins in the embryo and continues throughout the reproductive life of female mammals. Tissue macrophages (Mϕs), dependent on signals from the Mϕ colony stimulating factor 1 receptor (CSF1R), have been shown to regulate the generation, regression and regeneration of this organ, which is central for mammalian offspring survival. However, the distribution of Mϕs in the pre- and post-natal mammary gland, as it undergoes distinct phases of development and regression, is unknown or has been inferred from immunostaining of thin tissue sections. Here, we used optical tissue clearing and 3-dimensional imaging of mammary tissue obtained from *Csf1r-EGFP* mice. Whilst tissue Mϕs were observed at all developmental phases, their abundance, morphology, localization and association with luminal and basal epithelial cells exhibited stage-specific differences. Furthermore, sexual dimorphism was observed at E14.5, when the male mammary bud is severed from the overlying epidermis. These findings provide new insights into the localization and possible functions of heterogeneous tissue Mϕ populations in mammogenesis.

Keywords: mammary gland, macrophages, development, embryonic mammary stem cells, adult mammary stem cells, stem cell niche

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *24 August 2019*
Accepted: *09 October 2019*
Published: *24 October 2019*

Citation:
Stewart TA, Hughes K, Hume DA and Davis FM (2019) Developmental Stage-Specific Distribution of Macrophages in Mouse Mammary Gland. Front. Cell Dev. Biol. 7:250. doi: 10.3389/fcell.2019.00250

INTRODUCTION

Mammary gland development is phasic, with distinct developmental periods occurring in the embryo, at puberty and during pregnancy/lactation (Watson and Khaled, 2008; Lloyd-Lewis et al., 2017). The formation of the milk lines occurs at approximately embryonic day (E) 10 in mice and within 36 h resolves into five pairs of disk-shaped thickenings known as mammary placodes (Cowin and Wysolmerski, 2010). At around E12.5, mammary placodes invaginate into the dermal mesenchyme forming the mammary buds, which later elongate and invade the fat pad precursor, creating a rudimentary epithelial tree (Cowin and Wysolmerski, 2010; Paine and Lewis, 2017; Lilja et al., 2018). During embryonic development, multipotent mammary stem cells are replaced by unipotent luminal and basal stem/progenitor cells (Lilja et al., 2018; Wuidart et al., 2018), with epithelial cell identities being resolved by E15.5 (Lilja et al., 2018).

Initial postnatal growth of the mammary epithelium is proportional to body size and it is not until puberty that ductal elongation occurs, fueled by proliferation of adult mammary stem/progenitor cells within terminal end bud (TEB) structures (Davis et al., 2016; Lloyd-Lewis et al., 2017, 2018; Paine and Lewis, 2017). Further epithelial expansion occurs during pregnancy to generate the functional (milk-producing) alveolar epithelium (Watson and Khaled, 2008; Davis et al., 2016). With the cessation of infant suckling, alveolar mammary epithelial cells undergo massive programed cell death (a process known as post-lactational involution), returning the mammary gland to a near pre-pregnant state that is capable of supporting future pregnancies (Sargeant et al., 2014; Lloyd-Lewis et al., 2017).

Mϕs are present in all adult tissues (Hume D. et al., 2019). These cells are first and foremost professional phagocytes, but also regulate tissue development, function and dysfunction (Hume, 2015; Naik et al., 2018; Yang et al., 2018). In the normal postnatal mammary gland, Mϕs regulate ductal morphogenesis during puberty (Gouon-Evans et al., 2000; Van Nguyen and Pollard, 2002; Ingman et al., 2006), alveolar budding during ovarian cycling (Chua et al., 2010), alveologenesis in pregnancy (Pollard and Hennighausen, 1994) and tissue remodeling during post-lactational involution (O'Brien et al., 2010, 2012; Hughes et al., 2012), with many of these processes being impaired in mice deficient in tissue Mϕs. Moreover, Mϕs identified by fluorescence-activated cell sorting (FACS) of disaggregated tissue were detected within the embryonic mammary gland by E16.5 and fetal-derived Mϕs were apparently retained and expanded by self-renewal in adult mammary tissue (Jäppinen et al., 2019).

With accumulating evidence demonstrating the dependence of the mammary epithelium on Mϕs at all developmental stages, it is tempting to speculate that tissue-resident Mϕs institute or influence a putative mammary stem cell niche, as has been shown for hematopoietic stem cells (Winkler et al., 2010), intestinal stem cells (Sehgal et al., 2018) and hair follicle stem cells (Castellana et al., 2014; Naik et al., 2018). Indeed, the activity of mammary "stem" or repopulating cells (defined as a subset of basal cells that are capable of recreating the bi-layered mammary epithelium upon limiting dilution transplantation) is reduced when cells are transplanted into the cleared fat pads of Mϕ-depleted recipient mice (Gyorki et al., 2009). More recently, mammary repopulating cells were shown to express a Notch ligand Delta like 1 (DLL1) and *Dll1*-conditional knockout mice showed reduced mammary repopulating activity and lower levels of F4/80$^+$ Mϕs (Chakrabarti et al., 2018). Thus, it has been suggested that DLL1-expressing basal cells activate Notch-expressing Mϕs in a reciprocal stem cell-macrophage niche (Chakrabarti et al., 2018; Kannan and Eaves, 2018). Studies revealing developmental stage-dependent distribution of Mϕs in the mammary gland, including their sites of confluence, would provide further evidence for the existence of a stem cell-macrophage niche in this organ and may help to reveal the specific and stage-dependent localization of mammary stem/progenitor cells within the dynamic, bilayered epithelium under physiological conditions. Here, we utilize a fluorescent reporter model and optical tissue clearing techniques to reveal the presence, prevalence and position of Mϕs in the mammary gland at all phases of development.

MATERIALS AND METHODS

Reagents

Neutral buffered formalin (NBF), Quadrol®, triethanolamine and 4′,6-diamidino-2-phenylindole (DAPI) dilactate were purchased from Sigma Aldrich. Normal goat serum was purchased from ThermoFisher. Urea and sucrose were purchased from Chem-Supply. Triton-X-100 was purchased from VWR International. The following primary antibodies were used for immunostaining: chicken anti-GFP (Abcam, ab13970, batch #s GR3190550-3 and -12), rat anti-F4/80 (Novus, NB600-404), rat anti-keratin 8 (DSHB, TROMA-I, batch #s 7/7/16 and 30/3/17), rabbit anti-keratin 5 (BioLegend, 905504, batch # B230397) and rabbit anti-SMA (Abcam, ab5694, batch # GR3183259-26). The following secondary antibodies were used: goat anti-chicken Alexa Fluor-488 (ThermoFisher, A21236), goat anti-rat Cy3 (ThermoFisher, A10522) and goat anti-rabbit Alexa Fluor-647 (ThermoFisher, A21245).

Animal Models

Animal experimentation was carried out in accordance with the *Australian Code for the Care and Use of Animals for Scientific Purposes* and the *Queensland Animal Care and Protection Act (2001)*, with local animal ethics committee approval. Animals were housed in individually ventilated cages with a 12 h light/dark cycle. Food and water were available *ad libitum*. *Csf1r-EGFP* (MacGreen) (Sasmono et al., 2003) mice were a kind gift from A/Prof Allison Pettit (Mater Research Institute-UQ). Mice were maintained as hemizygotes on a C57BL6/J background. C57BL6/J mice were obtained from the Animal Resources Center (Western Australia).

To obtain mammary tissue during gestation, female mice were mated and tissue harvested 14.5 days-post-coitus (mean no. embryos: 7; range: 6–9). GFP$^+$ embryos (E14.5) were also harvested and analyzed after PCR-sexing. To obtain tissue during lactation, female mice were mated, allowed to litter naturally and lactating mammary tissue harvested on day 10 of lactation. For studies during involution, females were allowed to nurse for 10 days and mammary glands harvested 96 h post forced involution. Litter sizes were not standardized (mean litter size: 7; range: 5–10). Mammary glands from pre-pubertal female GFP$^+$ mice (postnatal day 10), pubertal (6.5 weeks) and post-pubertal (12 weeks) were also harvested and analyzed. No estrus staging was performed in these studies. In all mice the 2nd, 3rd, 4th, and 5th mammary glands were excised and fixed as described above; 2nd/3rd and 5th mammary glands were preferentially selected for 3D imaging, owing to their smaller size.

CUBIC-Based Tissue Clearing and IHC

Tissue clearing was performed as previously optimized and described (Davis et al., 2016; Lloyd-Lewis et al., 2016). Briefly, mammary tissue was spread on foam biopsy pads and fixed for 6–9 h in NBF (10%). Embryos were fixed whole. For CUBIC-based clearing, tissue was immersed in Reagent 1A (Susaki et al., 2014; Lloyd-Lewis et al., 2016) at 37°C for 3 days before

washing and blocking in goat serum (10%) in PBS with Triton-X-100 (0.5%) overnight at 4°C. Tissue was incubated in primary antibody in blocking buffer for 4 days and secondary antibody in blocking buffer for 2 days at 4°C. DAPI (5 μg/mL) treatment was performed for 2–3 h at room temperature [omitted for second harmonic generation (SHG)] and tissue was immersed in modified Reagent 2 (Lloyd-Lewis et al., 2016) at 37°C for at least 24 h prior to imaging.

Immunohistochemistry (FFPE Slides)

IHC on FFPE slides was performed as previously described in detail (Stewart et al., 2019). Wholemount immunostaining using anti-GFP antibody was performed prior to processing for paraffin embedding.

Microscopy

Immunostained tissue sections were imaged using an Olympus BX63 upright epifluorescence microscope using UPlanSAPO 10 × /0.4, 20 × /0.75, 40 × /0.95, 60 × /1.35, and 100 × /1.35 objective lenses. Immunostained optically cleared tissue was imaged using an Olympus FV3000 laser scanning confocal microscope with UPLSAPO 10 × /0.40, UPLSAPO 20 × /0.75, UPLSAPO 30 × /1.05, and UPLFLN 40 × /0.75 objective lenses. 3D de-noising was performed as previously described (Boulanger et al., 2010). For SHG, images were acquired using a Mai Tai DeepSee multiphoton laser on a Zeiss 710 laser scanning inverted microscope. Visualization and image processing was performed in ImageJ (v1.52e, National Institutes of Health) (Linkert et al., 2010; Schindelin et al., 2012).

RESULTS

Mϕs Are Present in the Embryonic Bud and Early Postnatal Gland With Sexually Dimorphic Distribution

Mϕs have never been visualized in the embryonic mammary gland. A recent study by Jäppinen et al. revealed the presence of F4/80$^+$ cells in digested mammary tissue by E16.5 by flow cytometry (Jäppinen et al., 2019). However, in the absence of *in situ* imaging, it is currently unclear whether these embryonic Mϕs physically associate with the developing mammary epithelium, as has been observed in the postnatal gland.

To assess Mϕ distribution in 3-dimensions in intact mammary tissue, we used a *Csf1r-EGFP* mouse model (Sasmono et al., 2003), combined with methods for optical tissue clearing and deep tissue imaging (**Supplementary Figure S1**) (Davis et al., 2016; Lloyd-Lewis et al., 2016, 2018). In this model, green fluorescent protein (GFP) expression in tissues is restricted to monocytes and Mϕs in the developing embryo, starting with yolk sac-derived phagocytes, and in all adult tissues (Sasmono et al., 2003; Hume D. A. et al., 2019). Much lower expression in granulocytes and some B lymphocytes is detectable by FACS, but not in tissues. Multi-color fluorescence immunostaining of tissue sections from mouse spleen confirmed that the majority of GFP$^+$ cells were also positive for the Mϕ cell surface marker, F4/80 (**Supplementary Figure S2**). Previous studies using digested mammary tissue from *Csf1r-EGFP* mice analyzed by flow cytometry have shown that >90% of GFP$^+$ cells in the mammary gland react with F4/80 (Chua et al., 2010; Hodson et al., 2013).

In 3D image stacks of female *Csf1r-EGFP* embryos, Mϕs were detected in the mammary and dermal mesenchyme surrounding the mammary epithelial bud as early as E14.5 (**Figure 1A** and **Supplementary Figure S3A**). As expected (Sasmono et al., 2003), Mϕs were also present in the embryonic liver at this stage (**Figure 1B**), and it has been suggested that these fetal liver-derived Mϕs contribute extensively to the pool of tissue Mϕs present in the adult gland (Jäppinen et al., 2019). Our data show that Mϕs were positioned adjacent to the embryonic mammary epithelium around the time of lineage segregation (Lilja et al., 2018; Wuidart et al., 2018). Interestingly, although Mϕs were positioned around the embryonic bud, they were rarely observed to directly interact with the developing epithelium of female embryos (**Figure 1A** and **Supplementary Figure S3A**). In contrast, Mϕs directly contacted and invaded the mammary bud of male mice at E14.5, the developmental period when the male bud is severed from the overlying epidermis in mice and begins to regress (**Figures 1C,D** and **Supplementary Figure S3B**) (Dunbar et al., 1999; Heuberger et al., 2006; Cowin and Wysolmerski, 2010). Mammary Mϕs were also observed in the early postnatal period in female mice (**Figures 1E,F**). By this stage, however, Mϕs were positioned around and inside of this rudimentary structure, apparently interacting with the epithelium (**Figure 1E**).

Mϕs Envelope and Infiltrate the Elongating Terminal End Bud During Ductal Morphogenesis

Mϕs are essential for normal ductal morphogenesis during puberty (Gouon-Evans et al., 2000; Van Nguyen and Pollard, 2002; Ingman et al., 2006). Pre-pubertal leukocyte depletion using sub-lethal γ-irradiation is associated with impaired ductal development and in Mϕ-deficient *Csf1^{op}/Csf1^{op}* mice, misshapen TEBs fail to properly invade the mammary fat pad at the rate observed in age-matched controls (Gouon-Evans et al., 2000; Van Nguyen and Pollard, 2002; Ingman et al., 2006). Previous studies analyzing Mϕ density and distribution in mouse mammary tissue sections have shown recruitment of F4/80$^+$ Mϕs to the pubertal epithelium and their convergence around the neck of TEBs (Gouon-Evans et al., 2000; Schwertfeger et al., 2006), where adult mammary stem/progenitor cells are thought to reside (Sreekumar et al., 2015; Lloyd-Lewis et al., 2017).

3D imaging of mammary tissue from pubertal *Csf1r-EGFP* mice revealed that mammary TEBs were enveloped by Mϕs, with spatial clustering observed (**Figure 2A** and **Supplementary Figure S4A**). Previous studies using the F4/80 marker indicated that Mϕs were mainly distributed at the neck of TEBs, whereas eosinophils (distinguished by their eosinic cytoplasm and bi-lobed nuclei) were concentrated at the TEB head (Gouon-Evans et al., 2000, 2002). By contrast, in this study GFP$^+$ Mϕs in both locations shared stellate morphology

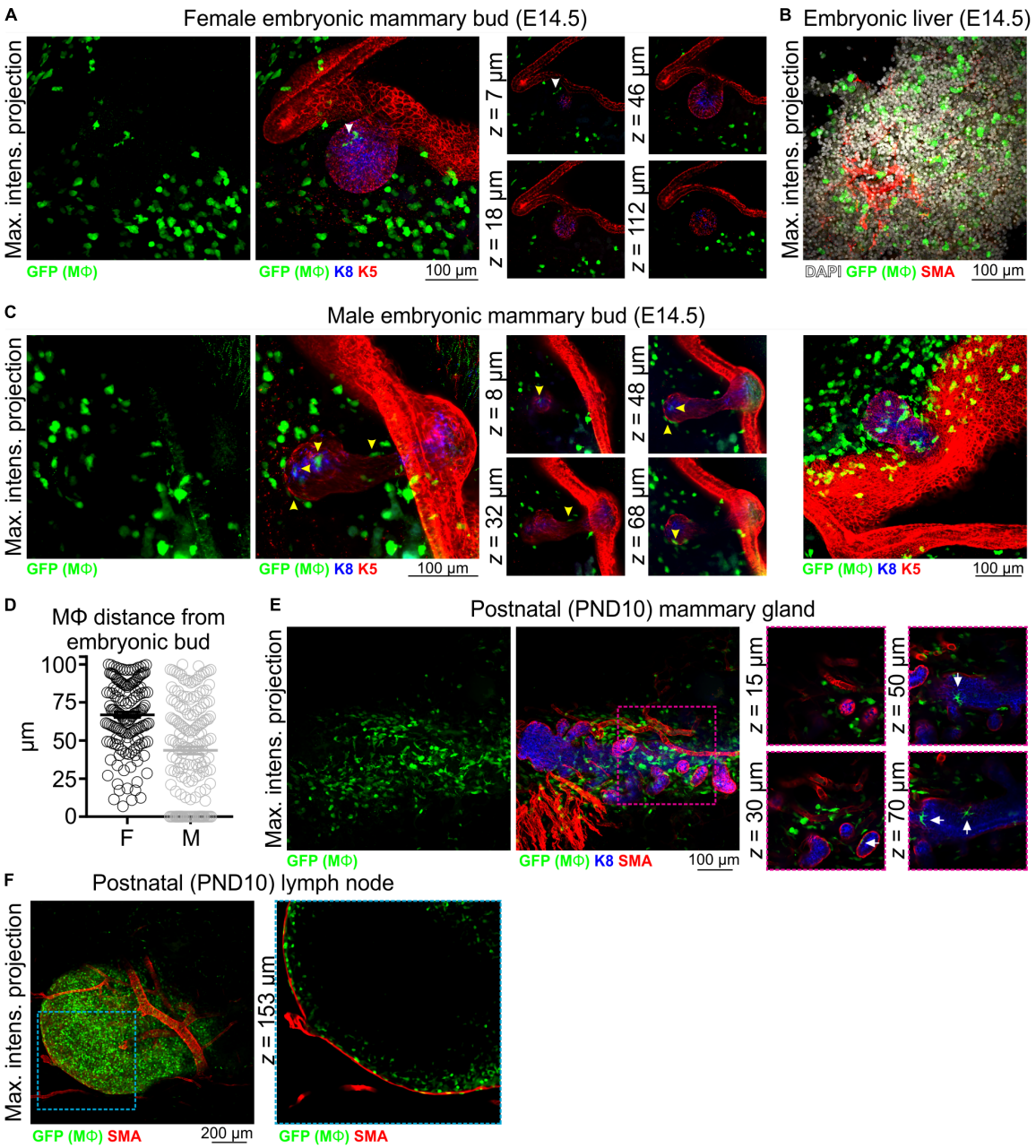

FIGURE 1 | Mϕs in the embryonic and early postnatal mouse mammary gland. Maximum intensity *z*-projection and single optical (*z*) slices of cleared tissue from **(A,B)** embryonic (E14.5) female mice and **(C)** embryonic (E14.5) male mice. **(D)** The distance of Mϕs (within a 100 µm radius) of the female and male embryonic buds. Mϕs contacting the bud or inside of the bud were assigned a value of 0; this was only observed in male embryos. **(E)** Mammary tissue from postnatal day (PND) 10 *Csf1r-EGFP* female mice. **(F)** Inguinal lymph node from PND10 mice showing subcapsular sinus Mϕs. Keratin (K) 8 immunostaining shows K8-positive luminal cells; K5 immunostaining reveals K5-expressing basal cells; smooth muscle actin (SMA) immunostaining reveals basal cells and SMA-positive vessels. White arrowhead in **(A)** points to a Mϕ that appears to be in contact with the embryonic bud in the maximum intensity projection, but is revealed to be positioned in the mammary mesenchyme above the bud in optical slices. Yellow arrowheads in **(C)** point to Mϕs that are in direct contact with the embryonic bud. Arrows in **(E)** point to Mϕs that are in contact with the PND10 mammary epithelium. Images are representative of 3 mice/embryos at each developmental stage.

(**Figure 2A** and **Supplementary Figure S4A**) and neither showed any evidence of segmented nuclei (**Supplementary Figure S4A**). A small number of mammary Mϕs were observed inside the body of TEBs (**Figure 2A**), where they may contribute to clearance of apoptotic cells from the TEB lumen (Humphreys et al., 1996; Gouon-Evans et al., 2000; Paine and Lewis, 2017). GFP$^+$ Mϕs were found along the length of the ductal epithelium in the pubertal gland (**Figure 2B** and **Supplementary Figure S4B**) and in some cases appeared to be positioned between the luminal and basal cell layers

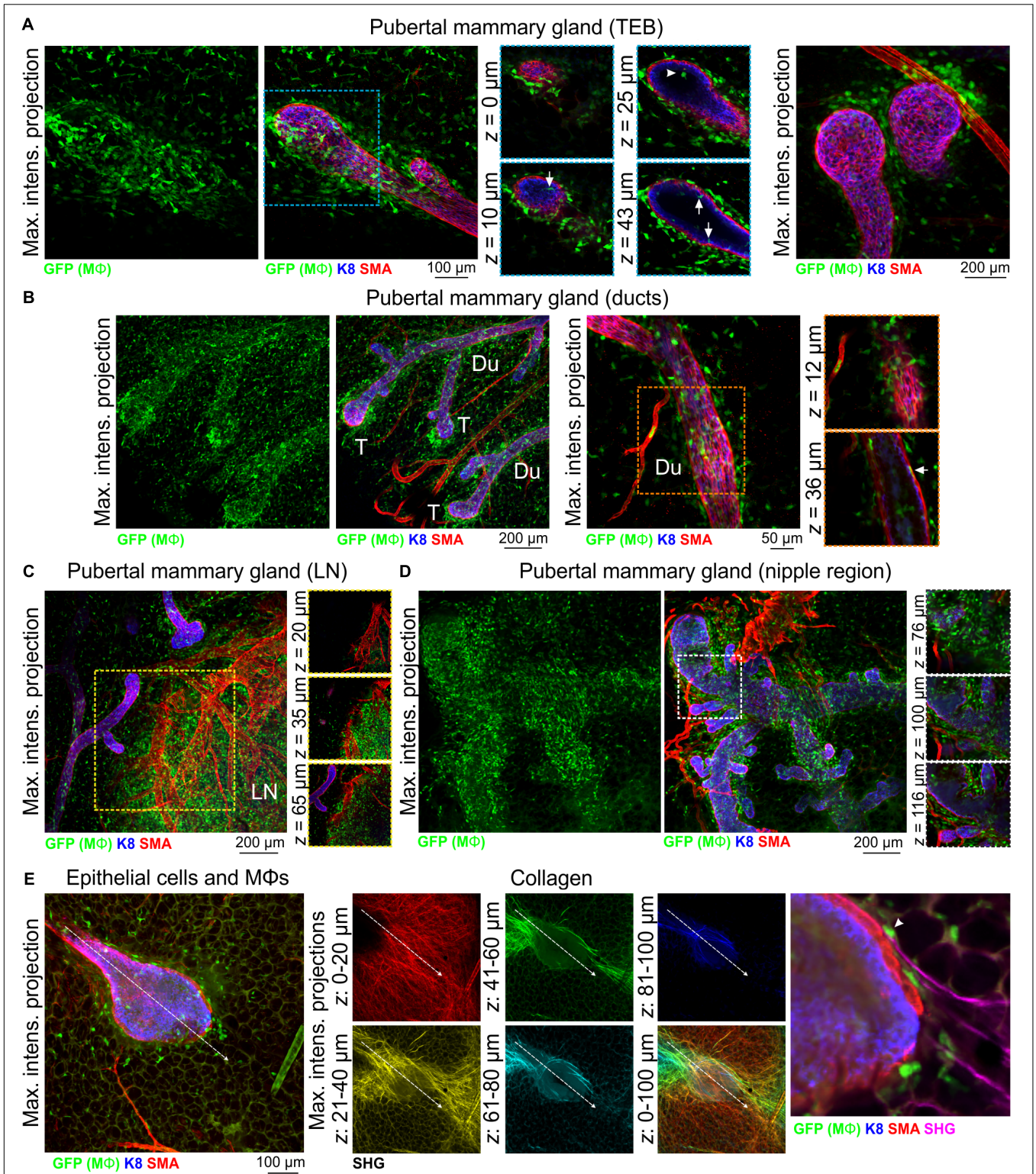

FIGURE 2 | Mϕs in the mammary glands of pubertal virgin mice. Maximum intensity *z*-projection and single optical (*z*) slices of cleared mammary tissue from pubertal (6–7 week old) *Csf1r-EGFP* mice. K8 immunostaining reveals the luminal cell layer; SMA marks the basal cell layer and SMA-positive vessels. **(A)** terminal end buds (TEBs), **(B)** ductal regions, **(C)** inguinal lymph node, and **(D)** nipple region. Arrows in **(A)** show Mϕs that have invaded the TEB epithelium and lumen (arrowhead). Arrow in **(B)** shows a Mϕ positioned between the epithelial bilayer. T, ductal tips; Du, ducts; LN, lymph node. Images are representative of 3 mice. **(E)** Second harmonic generation (SHG) showing fibrillar collagens around a TEB structure. Image stacks in middle panel are depth-coded (R-Y-G-C-B). Dashed arrow shows direction of TEB growth. Arrowhead in **(E)** shows a Mϕ interacting with collagen.

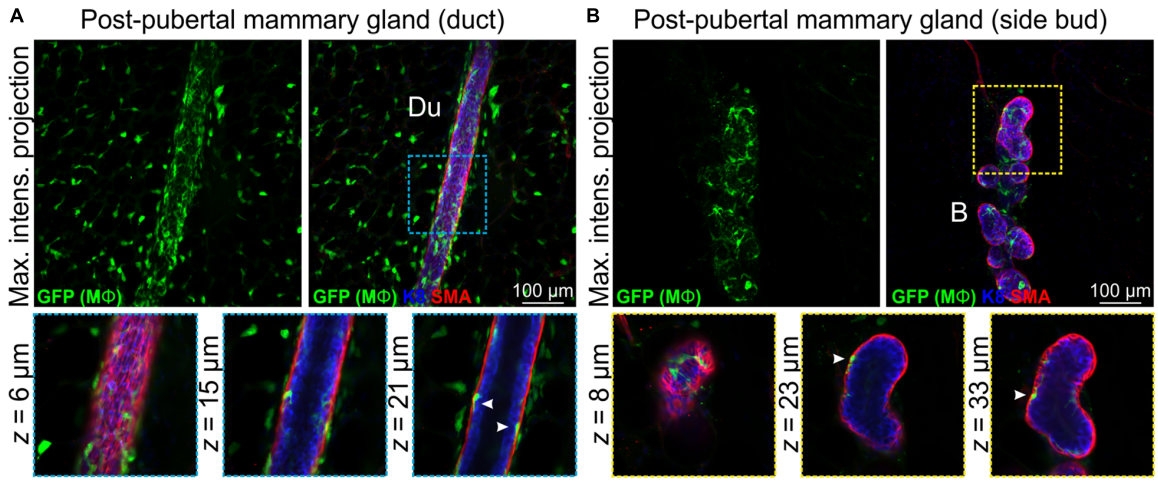

FIGURE 3 | Mϕs in the mammary glands of post-pubertal virgin mice. Maximum intensity *z*-projection and single optical (*z*) slices of cleared mammary tissue from post-pubertal (12 week-old) *Csf1r-EGFP* mice. K8 immunostaining shows luminal cells; SMA immunostaining reveals basal cells and SMA-positive vessels. **(A)** Mammary ducts and **(B)** side buds. Du, duct; B, side bud. Arrowheads show Mϕs that are positioned within the epithelial bilayer. K8 immunostaining reveals the luminal cell layer and SMA marks the basal cell layer. Images are representative of 3 mice.

(**Figure 2B**, arrow). Intraepithelial Mϕs, detected with F4/80, are a feature of ductal epithelia throughout the body (Hume D. A. et al., 1984). It is currently unclear how these interposed Mϕs affect luminal-basal cell connections [e.g., desmosomes and gap junctions (Shamir and Ewald, 2015)] and their precise function within the epithelial bilayer. GFP$^+$ cells were also dispersed throughout the mammary fat pad (**Figure 2** and **Supplementary Figure S4**; Schwertfeger et al., 2006; Chua et al., 2010) and were densely packed in the inguinal lymph node (**Figure 2C** and **Supplementary Figure S4B**) and nipple region (**Figure 2D**).

Mammary Mϕs have been shown to organize collagen into fibrillar bundles to steer TEB growth through the stromal fat pad (Ingman et al., 2006). We therefore examined fibrillar collagens with SHG (Williams et al., 2005) in tissue from *Csf1r-EGFP* mice at depth using an immersion-based optical clearing approach, which preserves endogenous fluorescence and tissue architecture (Lloyd-Lewis et al., 2016; Vigouroux et al., 2017). Although surface collagen fibers in the mammary gland were dense and multi-directional [**Figure 2E** (red)], deeper collagen fibers proximal to the growing TEB were aligned along its perimeter, extended in the direction of TEB growth and were associated with Mϕs (**Figure 2E**). These data provide further evidence that mechanical forces from the stroma guide epithelial development in the normal mammary gland (Ingman et al., 2006; Stewart et al., 2019).

Mϕs Are Intimately Associated With the Mature Ductal Epithelium

Mϕs are present in the post-pubertal mouse mammary gland at all phases of the estrus cycle, with the numbers being highest in diestrus (Chua et al., 2010). In tissue sections at all estrus stages, F4/80$^+$ cells are detectable around alveolar side buds versus ducts, where they are thought to promote the development and regression of these transient structures (Chua et al., 2010). Using 3D imaging of mammary tissue from *Csf1r-EGFP* mice, we observed similar numbers of Mϕs closely associated with mammary ducts (**Figure 3A** and **Supplementary Figure S5**) and side buds (**Figure 3B** and **Supplementary Figure S5A**). As in the pubertal epithelium, Mϕs were also positioned between the luminal and basal cell layers in mature ducts and buds (**Figures 3A,B** and **Supplementary Figure S5B**, arrowheads) with some evidence of periodicity in intraepithelial Mϕ placement (**Supplementary Figure S5B**). This is consistent with regular distributions of Mϕs in many locations throughout the body (Hume D. et al., 2019). SHG of mature ducts revealed some fibrillar collagens that were located around the ducts and vessels (**Supplementary Figure S5C**).

Mϕs Surround Alveolar Units in Gestation and Lactation

Mϕ deficient *Csf1^{op}/Csf1^{op}* female mice have compromised fertility (Pollard et al., 1991). Amongst those that do generate offspring, none are able to nurture a full litter, despite normal maternal behaviors (Pollard and Hennighausen, 1994). In-depth analyses of mammary tissue from pregnant and lactating *Csf1^{op}/Csf1^{op}* mice showed incomplete branching and precocious alveolar development (Pollard and Hennighausen, 1994) and F4/80$^+$ cells have been detected around the developing and functional alveolar units during pregnancy and late gestation (Gouon-Evans et al., 2002).

3D analysis of mammary tissue from pregnant *Csf1r-EGFP* mice (day 14.5 gestation, dG) confirmed Mϕ localization around the expanding alveolar structures (**Figure 4A** and **Supplementary Figure S6**). By lactation, Mϕs were observed immediately adjacent to alveolar basal cells, where they frequently imitated basal cell morphology (**Figures 4B,C**, white

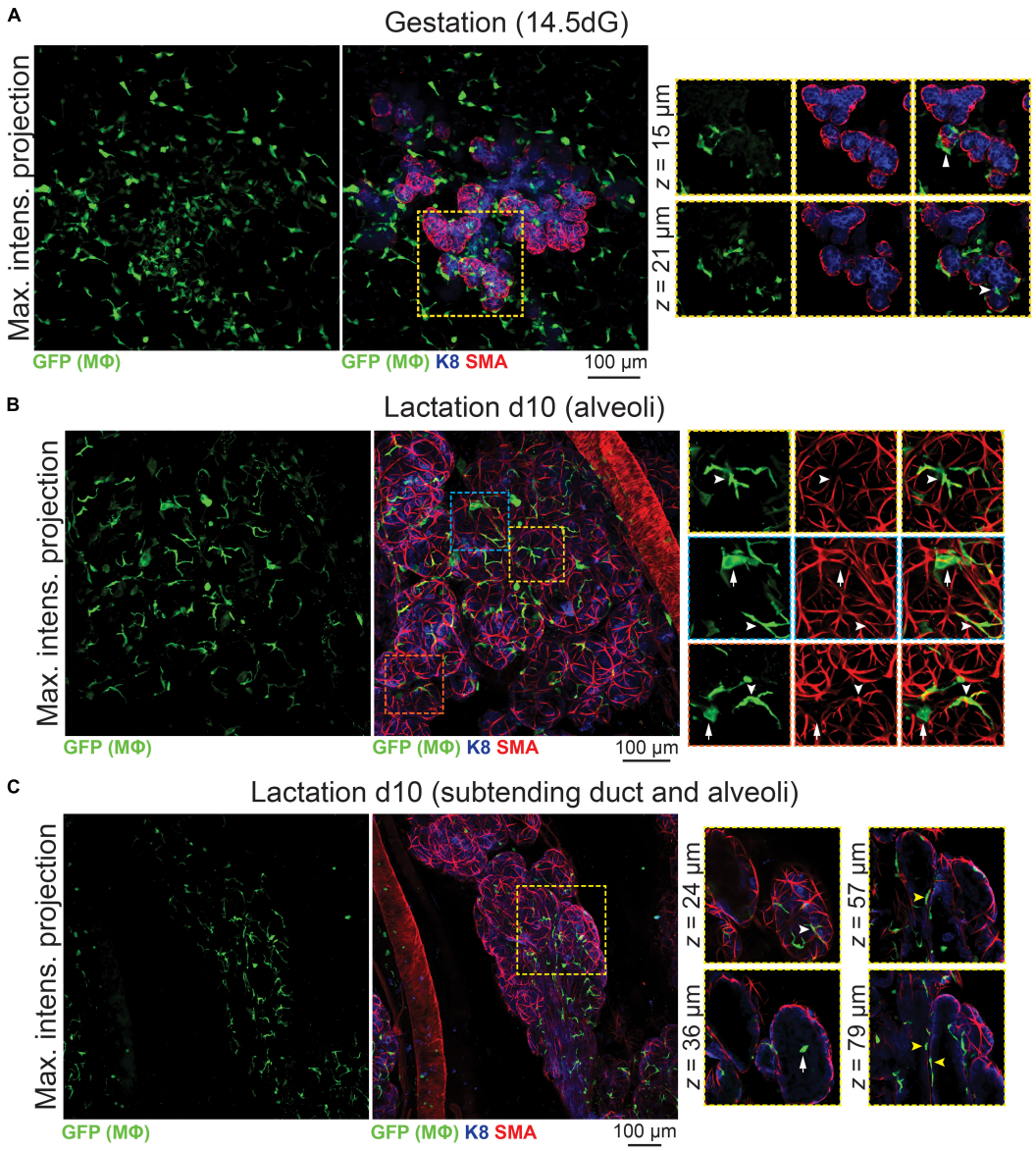

FIGURE 4 | Mϕs in the mammary glands of pregnant and lactating mice. Maximum intensity *z*-projection and single optical (*z*) slices of cleared mammary tissue from **(A)** pregnant (14.5 days gestation, dG) and **(B,C)** lactating (day 10 lactation, d10) *Csf1r-EGFP* mice. K8 immunostaining reveals K8-positive luminal cells; smooth muscle actin (SMA) marks the basal/myoepithelial cells and SMA-positive vessels. Arrowheads in **(A)** show Mϕs that are interacting with the developing alveolar epithelium. In **(B,C)**, white arrowheads show Mϕs that are aligned along basal cells (versus white arrows showing Mϕs that are not imitating basal cell morphology). Yellow arrowheads in **(C)** show Mϕs that are positioned between the ductal epithelial bilayer. Images are representative of 3 mice at each developmental stage.

arrowheads). Mϕs were also present within lactational alveoli (**Figure 4C**, arrow), consistent with their enrichment in breast milk (Field, 2005).

The Irreversible Phase of Involution Is Associated With an Increase in Mϕ Number in and Around Regressing Alveolar Structures

The number of Mϕs surrounding the mammary epithelium increases drastically from days 3–4 of involution (Lund et al., 1996; Stein et al., 2004; Hughes et al., 2012), and involution-associated Mϕs appear polarized toward tissue repair (O'Brien et al., 2010). The recruitment and polarization of Mϕs in the involuting mammary gland is regulated by epithelial *Stat3* expression (Hughes et al., 2012). Moreover, pre-weaning depletion of CSF1R-expressing cells reduces mammary epithelial cell death during post-lactational involution, an effect that can be reversed by orthotopic transplantation of bone marrow-derived Mϕs (O'Brien et al., 2012).

To further examine Mϕ number, morphology and distribution in the regressing mammary gland in 3-dimensions, we analyzed

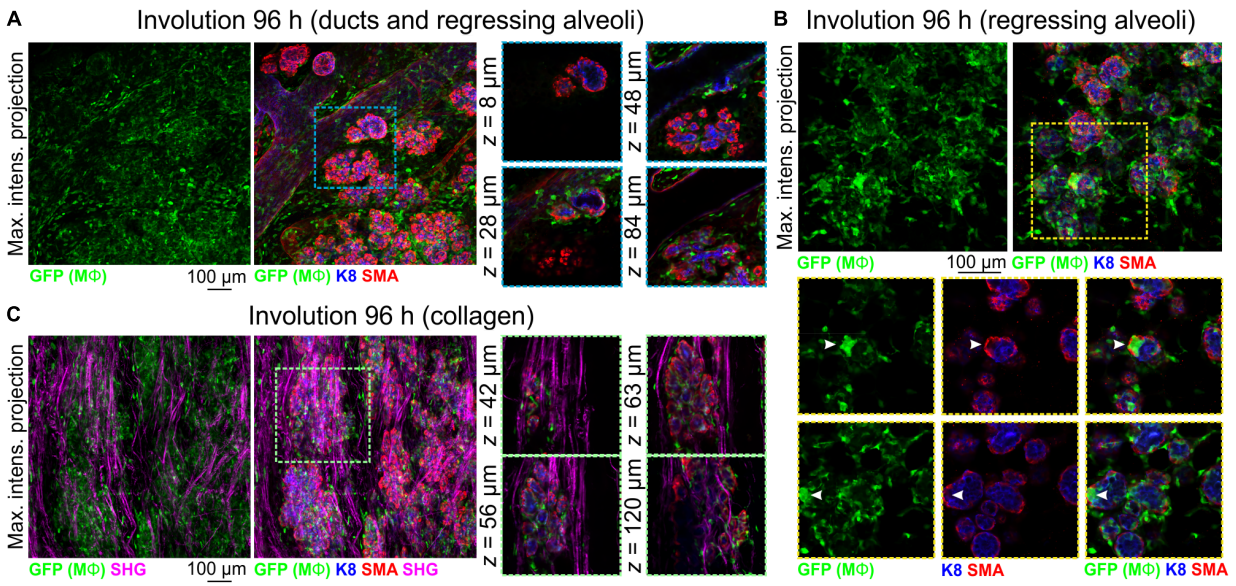

FIGURE 5 | Mϕs in the mammary glands of mice during post-lactational involution. **(A–C)** Maximum intensity *z*-projection and single optical (*z*) slices of cleared mammary tissue from *Csf1r-EGFP* mice during involution (96 h post forced weaning). K8 immunostaining shows luminal cells; SMA immunostaining reveals basal cells and SMA-positive vessels. Arrowheads in **(B)** show a cluster of GFP$^+$ Mϕs inside of collapsed alveolar units. **(C)** SHG showing fibrillar collagens surrounding regressing alveoli. Images are representative of 3 mice.

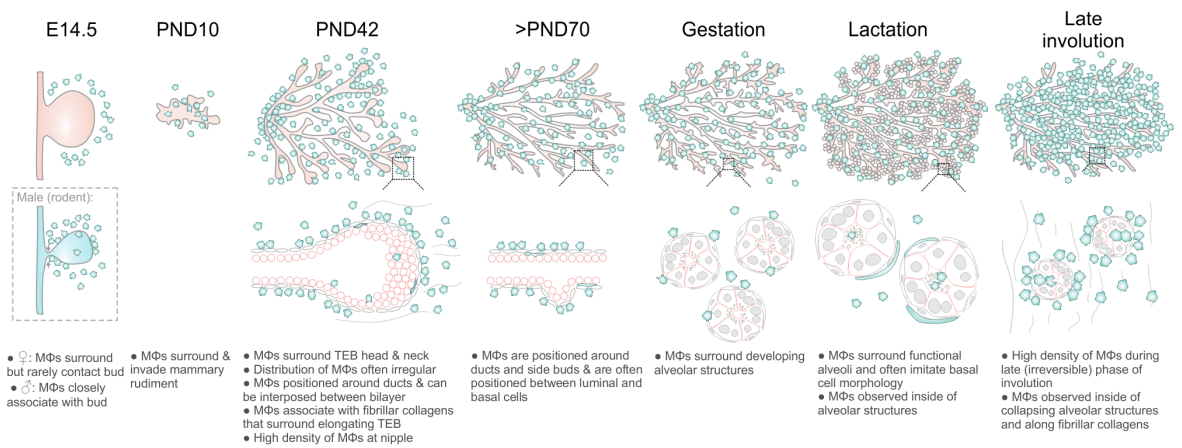

FIGURE 6 | Diagram summarizing Mϕ distribution in the mouse mammary gland during distinct phases of development and remodeling.

optically clear tissue from *Csf1r-EGFP* mice during the irreversible phase of involution. Relative to other developmental stages, Mϕ density was high at 96 h involution and Mϕs were observed around and inside ducts and regressing alveoli (**Figures 5A,B**). Large aggregates of GFP$^+$ cells, reminiscent of homotypic fusion (MacLauchlan et al., 2009), were also observed inside degenerating alveolar structures (**Figure 5B** arrowheads). Similar aggregates of GFP$^+$ Mϕs have been observed in a model of epithelial regeneration in the kidney following transient ischemia (Joo et al., 2016).

Collagen density increases during mammary gland involution and partially degraded non-fibrillar collagens have been suggested to be chemotactic for Mϕs (O'Brien et al., 2010). Intra- and interlobular fibrillar collagens were observed with SHG in *Csf1r-EGFP* mice and GFP$^+$ Mϕs were observed to be associated with collagen fibrils (**Figure 5C**).

DISCUSSION

Mϕs contribute to mammary gland development and remodeling at all developmental stages (Pollard and Hennighausen, 1994; Gouon-Evans et al., 2000; Dai et al., 2002; Van Nguyen and Pollard, 2002; Ingman et al., 2006; Chua et al., 2010; O'Brien et al., 2010, 2012; Hughes et al., 2012). The exact mechanisms by which tissue Mϕs regulate these processes are still being elucidated

(Schwertfeger et al., 2006) and may be linked to their phagocytic, trophic and/or matrix remodeling functions (Sternlicht, 2006; Pollard, 2009). A comprehensive characterization of the stage-specific physiological roles of Mϕs in the mammary gland depends upon knowledge of their precise anatomical location within this organ. In this study, we provide new insights into the allocation, morphology and distribution of Mϕs in the embryonic, pre-pubertal, pubertal, post-pubertal, pregnant, lactating and involuting mammary glands of fluorescent reporter-positive mice *in situ* in 3-dimensions (**Figure 6**). Our study yields a number of important observations that could only be revealed by multi-dimensional imaging using a tamoxifen-independent, cell type-specific fluorescent reporter model (Hume D. et al., 2019; Hume D. A. et al., 2019). Firstly, in contrast to previous reports (Gouon-Evans et al., 2000, 2002), we demonstrate that Mϕs are not concentrated at the TEB neck, although some polarity in their distribution around TEBs was observed. These findings suggest that Mϕs may regulate mammary epithelial cells both within the head and neck of the TEB structure (Paine et al., 2016). Studies performing intravital imaging of TEB dynamics in *Csf1r-EGFP* mice are an aim for the future and may help to reveal possible correlations between Mϕ density and TEB behavior (e.g., turning and bifurcation events).

Mammary Mϕs were also frequently embedded between luminal and basal cells of the ductal epithelium. This has previously been observed in mammalian ductal epithelia, including the bile duct, salivary gland, tracheobronchial gland and mammary gland using thin sections prepared from formalin-fixed paraffin-embedded or frozen tissue (Hume D. A. et al., 1984; Sun et al., 2013). Regularity in the spacing of these intraepithelial Mϕs was also noted, which may arise through mutual repulsion (Hume D. et al., 2019) and could potentially contribute to regular distribution of adjacent populations of heterogeneous luminal and basal cells (Ismail et al., 2002; Davis et al., 2016). In sum, the work presented here suggests a close functional relationship between Mϕs and ductal epithelial cells, and possible communication between morphologically related Mϕ populations. Further studies are needed to determine whether these intraepithelial Mϕs share similar gene and protein expression patterns and whether this information can be used to probe their function, retention and passage within the epithelium. Tissue Mϕs have been shown to be influenced by properties of their specific niche within each tissue (e.g., anchoring scaffolds and local cues) (Chakarov et al., 2019; Mondor et al., 2019). Single cell sequencing of isolated mammary Mϕs from *Csf1r-EGFP* mice at distinct developmental stages, as exemplified by recent studies of other tissues (Chakarov et al., 2019; Mondor et al., 2019), might help to reveal the extent of functional diversity within Mϕ populations in this organ.

We reveal that Mϕs alter their morphology at distinct developmental stages, including the transition from gestation to lactation. The localization of Mϕs around growing alveolar units during gestation and the observation that Mϕ-deficient $Csf1^{op}/Csf1^{op}$ mice exhibit precocious alveolar development, suggests that during this phase, alveolar-associated Mϕs may restrain alveologenesis. By analogy, Mϕs in the diaphragm appear to constrain the growth of lymphatic vessels and *Csf1r* mutation promoted branch formation of lymphatic sprouts (Ochsenbein et al., 2016). During lactation, Mϕs altered their anatomical position and were observed to closely imitate the morphology of adjacent, differentiated alveolar basal cells. Whether these cells specifically align themselves with oxytocin-responsive basal cells during lactation to modify basal cell function (Davis et al., 2015; Stevenson et al., 2019) or more simply to occupy the physical space that these force-exerting cells create within the alveolar epithelium (Davis, 2016; Stewart et al., 2019), remains to be seen. Such a function might be analogous to the role of a distinct population of CSF1-dependent Mϕs in the regulation of peristalsis in the muscularis externa of the intestine (Muller et al., 2014). Interestingly, in this study muscularis Mϕs and intestinal motility could be reversibly modified by lumen factors (Muller et al., 2014). Whether mammary Mϕs, positioned alongside alveolar basal cells, are capable of sampling the alveolar lumen environment to constrain basal cell-mediated alveolar contractility (e.g., in mastitis) has not yet been determined. Another possibility is that basal cell contractility may instead alter the function of alveolar Mϕs. Such an effect has been observed in the lung, another organ that is subject to cyclical mechanical stimulation, although this phenomenon was restricted to newly recruited monocytes and not the population of resident alveolar Mϕs (Solis et al., 2019). Finally, we were able to visualize for the first time tissue-resident Mϕs in the mesenchyme surrounding the mammary epithelial bud in 14.5 day-old female embryos. Intriguingly, these embryonic Mϕs rarely contacted the epithelial cells of the developing mammary bud at this stage of embryogenesis. This is in striking contrast to epithelial-Mϕ interactions in the early postnatal period, where Mϕs surround and invade the rudimentary ductal epithelium. This also contrasts with the male embryo, where Mϕs were often observed to both contact and infiltrate the epithelial bud at the time when its connection to the overlying epidermis is severed and the structure begins to regress (Dunbar et al., 1999; Heuberger et al., 2006; Cowin and Wysolmerski, 2010). At this stage, Mϕs may have an important role in clearing apoptotic epithelial and mesenchymal cells (Dunbar et al., 1999; Henson and Hume, 2006).

Mammary stem/progenitor cells are located within the mammary bud (in the embryo) and TEBs (in puberty). After ductal elongation is complete and TEBs regress, however, the location of long-lived mammary stem/progenitor cells and their putative niche remains unknown, although it has been suggested that these cells are deposited along the ductal epithelium by elongating TEBs (Davis et al., 2016; Lloyd-Lewis et al., 2017). In the 14.5 day embryo, Mϕs were positioned uniformly around, but not in contact with, the mammary bud. These data suggest that if a mammary stem cell-macrophage niche exists in the embryo around the time of lineage segregation, it operates over the scale of tens of micrometers and is fairly homogeneous. Mϕs were also positioned around pubertal TEBs, however, in contrast to the embryo, these cells contacted and infiltrated TEBs, were more densely arranged around these structures and often showed spatial clustering. Future studies combining tamoxifen-independent *Dll1-mCherry* (Chakrabarti et al., 2018) and *Csf1r-EGFP* mouse models with optical tissue clearing and 3D imaging may help to reveal the precise location of mammary

stem/progenitor cells within TEBs and the post-pubertal ductal epithelium. It should be noted, however, that whilst ductal elongation is delayed in $Csf1^{op}/Csf1^{op}$ mice, these structures are still capable of invading the fat pad and by 12 weeks of age have reached the fat pad limits (Gouon-Evans et al., 2000). These findings imply that mammary epithelial cells have mechanisms to overcome insufficiencies in niche signaling. One candidate is the alternative CSF1R ligand, IL34, which may also be expressed by mammary epithelial cells (DeNardo et al., 2011). Studies investigating the activation and roles of the CSF1R in mammary development have been thwarted by the severe postnatal phenotype of $Csf1r^{-}/Csf1r^{-}$ mice (Chitu and Stanley, 2017), but may be more amenable to study in recently described $Csf1r^{-}/Csf1r^{-}$ rats (Pridans et al., 2019). Alternatively, these findings may reflect a long-term plasticity in mammary epithelial cells (Lilja et al., 2018) and a shifting definition of "stemness" in some tissues away from a unidirectional, top-down model to a model where stemness is considered as a cell state that may be acquired or extinguished under specific microenvironmental conditions (Laplane and Solary, 2019). A closer examination of mammary cell behaviors—including lineage segregation—under conditions of Mϕ depletion may provide important insights into epithelial plasticity in this vital mammalian organ.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Queensland Health Sciences Animal Ethics Committee. In accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes and the Queensland Animal Care and Protection Act (2001).

AUTHOR CONTRIBUTIONS

FD and TS performed all the experiments. FD, DH, and TS, conceived and designed the experiments. TS, KH, DH, and FD analyzed the results. FD wrote the manuscript. DH, KH, and TS edited the manuscript.

FUNDING

This work was supported by the National Health and Medical Research Council (1141008 and 1138214 to FD) and the Mater Foundation (Equity Trustees/AE Hingeley Trust).

ACKNOWLEDGMENTS

We acknowledge the Translational Research Institute (TRI) for the research space, equipment and core facilities that enabled this research. We thank the UQ Biological Resource staff for animal care and husbandry; Mr. Alex Stevenson for laboratory management and ordering of consumables; A/Prof. Allison Pettit and Dr. Katharine Irvine for their helpful comments on the manuscript; Dr. Jerome Boulanger (MRC Laboratory of Molecular Biology) for the 3D de-noising algorithm; and Mr. Eric Pizzani (Translational Research Institute) for research computing support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2019.00250/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 17 December 2019
doi: 10.3389/fcell.2019.00348

Know Thy Model: Charting Molecular Homology in Stromal Reprogramming Between Canine and Human Mammary Tumors

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Spontaneous canine simple mammary tumors (CMTs) are often viewed as models of human breast cancer. Cancer-associated stroma (CAS) is central for initiation and progression of human cancer, and is likely to play a key role in canine tumors as well. Until recently, however, canine CAS in general, and in CMT in particular, lacked detailed characterization and it remained unclear how canine and human CAS compare. This void in knowledge regarding canine CAS and the resulting lack of unbiased cross-species analysis of molecular homologies and differences undermined the validity of the canine model for human disease. To assess stromal reprogramming in canine breast tumors, we have recently established a protocol to specifically isolate and analyze CAS and matched normal stroma from archival, formalin-fixed paraffin embedded (FFPE) clinical tumor samples using laser-capture microdissection followed by next-generation RNA-sequencing. Using this approach, we have analyzed stromal reprogramming in both malignant canine mammary carcinomas (mCAs) as well as benign canine mammary adenomas in a series of studies. Our results demonstrate strong stromal reprogramming in CMTs and identify high-grade molecular homology between human and canine CAS. Here, I aim to give a short background on the value of comparative oncology in general, and spontaneous CMT in particular. This will be followed by a concise review of the current knowledge of stromal reprogramming in both malignant canine mCA and benign adenoma. Finally, I will conclude with insights on highly conserved aspects of stromal reprogramming between CMT and human breast cancer that accentuate the relevance of CAS in CMT as a model for the human disease.

Keywords: laser-capture microdissection, RNA sequencing, canine mammary carcinoma, canine mammary adenoma, breast cancer, tumor stroma, tumor microenvironment, comparative oncology

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *24 September 2019*
Accepted: *03 December 2019*
Published: *17 December 2019*

Citation:
Markkanen E (2019) Know Thy Model: Charting Molecular Homology in Stromal Reprogramming Between Canine and Human Mammary Tumors. Front. Cell Dev. Biol. 7:348. doi: 10.3389/fcell.2019.00348

INTRODUCTION

The majority of all cancers derive from corrupted epithelial cells that give rise to tumor cells that disregard the tissue boundaries of their natural habitat. Yet, these epithelial tumor cells are not living in an isolated environment, and – far from being self-sufficient – heavily depend on their microenvironment for survival and growth (Hanahan and Coussens, 2012). This microenvironment, also called cancer-associated stroma (CAS), consists of a heterogeneous

mixture of different non-tumor cells (among them fibroblasts, immune cells, vascular cells, adipocytes, and others), as well as extracellular matrix (ECM). CAS has been abundantly shown to play a key role in initiation and progression of a wide variety of tumors, and its manifold roles in tumor biology have been widely documented [e.g., reviewed in Bissell and Hines (2011); Bussard et al. (2016), Hanahan and Coussens (2012); Kalluri (2016), and Quail and Joyce (2013)]. Nevertheless, the field is still far from completely understanding the mechanisms by which CAS influences tumor biology, the molecular players that are involved, and the intricacies of the cross-talk between CAS and tumor cells.

Due to the closely related pathophysiology, naturally occurring cancers in the domestic dog are progressively leveraged as a valuable source of information to better understand the biology behind tumor development and possibly find novel anti-cancer treatments (Karlsson and Lindblad-Toh, 2008; Gardner et al., 2015; Rogers, 2015). While increasing efforts have been focused on analysis of the molecular aspects of tumor cells in canine cancers and their comparison with aberrations in human tumor cells, canine CAS greatly lacks characterization. Hence, it remains completely unclear how canine and human CAS compare. Given the central importance of CAS for the biology of human cancer, this striking shortage of data on canine CAS and the resulting lack of unbiased cross-species analysis of molecular homologies and differences threaten to undermine the validity of the canine model for human disease.

Among various other tumor types, naturally occurring canine mammary tumors (CMTs) are viewed as excellent translational model for human breast cancer. Recent work by my group has begun to assess stromal reprogramming in spontaneous CMTs by next-generation RNA-sequencing (RNAseq) in both malignant canine mammary carcinomas (mCAs) and benign canine mammary adenomas, and to probe the extent of molecular homology between human and canine CAS. In the following, I aim to give a short background on the value of comparative oncology in general, and spontaneous CMT in particular. This will be followed by a concise review of the current knowledge of stromal reprogramming in both malignant canine mCA as well as benign adenoma. Finally, I will conclude with insights on highly conserved aspects of stromal reprogramming between CMT and human breast cancer that accentuate the relevance of CAS in CMT as a model for the human disease.

NATURALLY OCCURRING TUMORS IN DOGS AS TRANSLATIONAL MODELS FOR HUMAN CANCER

A plethora of *in vitro* and *in vivo* models have been used over the last century to gain insights into cancer biology. While these models have undoubtedly been highly informative in many aspects and lead to various scientific breakthroughs, the inherent limit in most of the used models is their inability to fully replicate the conditions and mirror the complexity of spontaneously developing patient tumors (Karlsson and Lindblad-Toh, 2008; Uva et al., 2009; Rowell et al., 2011; Liu et al., 2014; Schiffman and Breen, 2015). The field of comparative oncology aims to address these shortcomings by widening the research focus from classical rodent models toward spontaneous tumors that develop in other animals, such as the domestic dog. This additional perspective is perceived as a chance to complement and enhance our understanding of complex diseases, such as cancer, as the comparison of tumor development and risk factors across species provides the opportunity to discover basic mechanisms of tumorigenesis (Karlsson and Lindblad-Toh, 2008; Paoloni and Khanna, 2008; Gordon et al., 2009; Schiffman and Breen, 2015). Due to the many similarities shared between dogs and humans, the domestic dog is considered one of the best examples for comparative oncology. Firstly, the number of genes in dogs and humans are comparable, and evolutionarily conserved alterations in the genome are shared between these species (Bejerano et al., 2004; Karlsson and Lindblad-Toh, 2008; Rivera and Euler von, 2011; Rowell et al., 2011; Schiffman and Breen, 2015). Cancer in both species develops spontaneously with similar pathophysiology, and often manifests in similar clinical presentation and histology. As such, development of spontaneous tumors in dogs has strong parallels with the natural progression of cancer development in humans, and is considered a better proxy of tumor biology than animal models with induced tumorigenesis. The higher life expectancy compared to rodent models and the same environmental factors that dogs and humans are exposed to, combined with the fact that dogs often receive a high level of healthcare, further strengthen the value of comparatively analyzing canine and human cancers. Also, as a result of inbreeding and high degrees of consanguinity, certain breeds of dogs have been shown to carry genetic predispositions for certain cancer types, facilitating the discovery of risk alleles responsible for the disease [reviewed in Schiffman and Breen (2015)]. Altogether, these insights emphasize the potential of the dog as models for human cancer and offer the possibility to overcome limits of xenograft and genetically engineered rodent models leading to improved understanding of tumor biology and biomarker discovery. The interested reader is further referred for a more detailed discussion to several excellent reviews on the subject (Rowell et al., 2011; Schiffman and Breen, 2015).

CANINE MAMMARY TUMORS AS A MODEL FOR HUMAN BREAST CANCER

Among many different cancer types, especially CMTs have garnered attention as useful models for human breast cancer (Sorenmo et al., 2009; Queiroga et al., 2011b; Abadie et al., 2017; Nguyen et al., 2017). In relative terms with regards to life expectancy (i.e., converting "dog years" into "human years"), the age of onset is comparable between women and bitches. The incidence of CMTs starts to increase after the age of 6 years (the equivalent of age 40 years in humans) and peaks between 8 and 14 years (humans age 50–70 years) (Queiroga et al., 2011b; Rowell et al., 2011; Bundesamt für Statistik, 2015; Bray et al., 2018; World Health Organization, 2018). Furthermore,

it is the most frequent cancer diagnosed both among female dogs as well as women suffering from cancer (Sorenmo et al., 2009; Lahkhani et al., 2012; Liu et al., 2014; Bray et al., 2018). A retrospective study on canine tumors in Switzerland between 1955 and 2008 found that 20.5% of all canine tumors were located in the mammary gland (Grüntzig et al., 2015). A retrospective study in Italy between 1985 and 2002 even found 70% of all tumors of female dogs to be located to the mammary gland (Merlo et al., 2008). On a global level, CMTs occur in >40% of female dogs and show and annual incidence rate varying between 192 and 205/100,000 dogs, comparable to human data with incidence rates of 125/100,000 women in the United States, and 144/100,000 women in Switzerland (Queiroga et al., 2011b; Sleeckx et al., 2011; Liu et al., 2014; Bundesamt für Statistik, 2015). Interestingly, CMT incidence is lower in the United States than in other countries like Sweden, presumably because dogs tend to get neutered at an early age in the United States and therefore receive less gestagen preparations for heat prevention (Sleeckx et al., 2011).

The anatomy of the normal mammary gland is similar in dogs and women. The alveoli and ducts of the mammary gland consist of luminal epithelial cells lined by myoepithelial cells and are separated from the surrounding connective tissue by the basement membrane (Liu et al., 2014; Santos and Matos, 2015). In both species tumor formation is seen as a dynamic process starting from benign hyperplastic lesions that can evolve into a carcinoma *in situ*. In a further step, these tumors can become invasive, which is marked by the disruption of the basement membrane and potential seeding of metastases (Gilbertson et al., 1983; Burstein et al., 2004; Simpson et al., 2005; Sorenmo et al., 2009). On a molecular level, many of the key alterations in human breast cancer are faithfully recapitulated in CMTs, including germline mutations in BRCA1, BRCA2, and TP53 that are associated with an enhanced risk of hereditary cancer in humans (Liu et al., 2014; Matos and Santos, 2015; Santos and Matos, 2015; Schiffman and Breen, 2015). And finally, besides clinical factors such as tumor size, lymph node involvement, and clinical stage, the prognostic value of histo-pathological aspects such as tumor type and grade, and molecular subtypes (luminal A, luminal B, HER2-enriched, and basal-like) is conserved between canine and human breast cancer (Rivera et al., 2009; Queiroga et al., 2011b; Sleeckx et al., 2011; Lahkhani et al., 2012; Rasotto et al., 2012, 2017; Im et al., 2013; Pena et al., 2013; Nguyen et al., 2017). However, assessment of molecular subtypes is still limited to research purposes and not routinely applied in CMTs (Sleeckx et al., 2011; Rasotto et al., 2017).

Altogether, the similarities between CMTs and human breast cancer suggest wide-ranging homologies in tumor biology. Canine studies offer the opportunity to find novel biomarkers not only for veterinary use, but also to benefit human patients. Comparing the same disease in two different species additionally helps differentiating the molecular "drivers" of the disease from mere "passengers," as key pathways should be conserved between species. Finally, clinical trials in dogs can be conducted in a shorter period than human studies, due to a reduced lifespan and associated earlier manifestation of cancer (Karlsson and Lindblad-Toh, 2008).

LARGELY UNCHARTED TERRITORY: STROMAL REPROGRAMMING IN CANINE MAMMARY TUMORS

While the importance of CAS in cancer initiation and progression is becoming increasingly clear, data regarding the molecular composition of CAS in canine cancer overall, and CMTs in particular are sparse. Traditionally, analysis of tumor samples by RT-qPCR or next-generation sequencing approaches is performed in bulk. While highly informative, the major drawback of bulk tissue analysis is the fact that results reflect the mixture of all cells present in the sample, not discriminating between epithelial cancer cells and other non-neoplastic cells. The avoidance of inclusion of samples "too rich in stroma" is usually attempted by setting a cut-off value for stromal content as exclusion criterium. Nevertheless, results from such bulk analyses clearly are a conglomerate of highly varying amounts of different cell populations present at sampling. Thus, this approach heavily complicates the correct attribution of observed changes either to the cancer cells or to the stromal cells. To date, most studies investigating CMT on a molecular level, such as by sequencing or microarray analysis, have analyzed tumor tissue in bulk (e.g., Uva et al., 2009; Klopfleisch et al., 2010, 2011; Liu et al., 2015; Bulkowska et al., 2017). Accordingly, specific analysis of CAS in CMTs has thus far been restricted to just single markers that were analyzed predominantly through immunohistochemistry (IHC). CAS is composed of various different cellular and extracellular components that have been shown to exhibit strong influence on the hallmarks of tumor cells (Hanahan and Coussens, 2012). Of these, in CMTs, most attention has been focused on the roles of cancer-associated fibroblasts (CAFs), a few components of the ECM, a subset of infiltrating immune cells, as well as single markers for angiogenesis. In the following, I will attempt to shortly summarize the currently available data on the state of these components in CAS of CMTs.

Cancer-Associated Fibroblasts and the Extracellular Matrix

Cancer-associated fibroblasts are a heterogeneous population of activated fibroblastic cells that present the most abundant cell fraction in CAS and strongly influence tumor development and progression (Chen and Song, 2019). CAFs often stain positively for alpha smooth muscle actin (αSMA), a marker for myofibroblast activation, and αSMA expression has been associated with reactive tumor stroma in human breast cancer and other tumors. As such, upregulation of αSMA is often used as marker for CAS, and enhanced expression of αSMA in human breast cancer is associated with poor prognosis (Sappino et al., 1988; Elenbaas and Weinberg, 2001; Yazhou et al., 2004; Surowiak et al., 2006, 2007; Yamashita et al., 2012). In CMTs, αSMA-positive myofibroblasts have been detected in CAS of adenomas and carcinomas, but not in normal breast tissue, increasing in abundance with higher tumor grade and histopathological signs of invasion

and metastasis, and significantly related to poor prognosis (Yoshimura et al., 2011).

The main function of fibroblasts is to maintain the integrity of the ECM as structural support for cells and organs. As activated fibroblasts, CAFs strongly influence CAS composition and architecture through production and remodeling of ECM. Changes in collagen density and fiber organization have been associated with tumor grade and overall survival in CMTs (Case et al., 2017). Expression of the ECM molecule Tenascin-C (Tn-C), most likely produced by myofibroblasts, increased from benign adenomas to malignant carcinomas, and with signs of invasion and metastasis (Faustino et al., 2002; Yoshimura et al., 2011, 2014). Versican is another component of the ECM whose expression has been found to increase with malignancy and invasiveness of the tumor cells (Damasceno et al., 2016). Interestingly, the stroma of malignant tumors has been described to increasingly express the known multidrug resistance-causing transporters P-glycoprotein (PGP) and breast cancer resistance protein (BCRP), suggesting a role for the tumor stroma in the development therapeutic resistance (Levi et al., 2016). The most thoroughly investigated ECM-remodeling enzymes in CMTs belong to the group of ECM-degrading proteases including matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) and their inhibitors. These play a key role in ECM homeostasis in human breast cancer (Chen and Song, 2019). In CMTs, MMP2, MMP9, MMP14, and uPA levels increase from healthy tissue to benign to malignant CMTs, and are associated with increasing histological grade, signs of invasion, and early death from CMT, and present good prognostic factors (Papparella et al., 1997, 2002; Yokota et al., 2001; Hirayama et al., 2002; Kawai et al., 2006; Vinothini et al., 2009; Aresu et al., 2011; Lamp et al., 2011; Santos et al., 2011b, 2012, 2013; Santos and Matos, 2015). Unfortunately it is not always clear where exactly these MMPs were expressed, as some of the analyses were performed on bulk tumor tissue. Nevertheless, it is evident that MMP activity is mainly focused on the ECM, and a subset of these studies detected their expression also in fibroblasts close to the invasive tumor cells. Interestingly, MMP13 expression decreased significantly between benign and malignant CMT (Aresu et al., 2011).

The picture is slightly less clear for the MMP inhibitors: most reports have found TIMPs 1-3 and RECK to be highly expressed in malignant carcinomas (Papparella et al., 1997; Yokota et al., 2001; Hirayama et al., 2002; Kawai et al., 2006; Santos et al., 2011a). One report found expression of TIMP-2 to decrease in tumor tissue compared to controls, and also to decrease from grade I to grade III tumors (Vinothini et al., 2009), while Aresu et al. (2011) did not find statistically significant differences in TIMP-2 (nor TIMP-1, TIMP-3, or RECK) levels between benign and malignant tumors.

In summary, understanding of fibroblast activation and ECM remodeling in CMTs has thus far mainly focused on αSMA-positive myofibroblasts, and expression of Tn-C, MMPs, and their inhibitors. While these analyses have yielded interesting data also regarding similarities to CAS in human breast cancer, the understanding of both fibroblast activation and ECM remodeling in CMT remains extremely limited to date.

Infiltrating Immune Cells

Infiltration of immune cells into tumors has been a longstanding area of interest in tumor biology, and the cellular composition of the immune infiltrate is clearly linked to disease outcome in CMTs (Gilbertson et al., 1983; MacEwen, 1990; Kim et al., 2013). High levels of CD4+ and CD3+ T-cells have been associated with metastasizing tumors and shorter overall survival (Estrela-Lima et al., 2010; Saeki et al., 2012; Carvalho et al., 2015a,b, 2016b). More detailed assessment of the localization of immune infiltrates with respect to the tumor cells found that tumor-infiltrating CD3+ T-lymphocytes were significantly more frequent in benign than malignant tumors, and conversely, peripheral CD3+ cells were more frequent in malignant than benign tumors (Carvalho et al., 2011). Furthermore, high number of neutrophils were associated with aggressive CMTs, while in contrast high amounts of plasma cells, macrophages, and CD8+ T-cells, together with low numbers of CD4+ T-cells, were associated with less aggressive tumors (de Souza et al., 2018). Taken together, these results suggest a strong role for T-lymphocytes in progression of CMTs, and also highlight that it is important to assess not only abundance of immune cells, but also identify their subtypes and define their exact localization within the tumor, as infiltrating immune cells that shielded from reaching the tumor cells cannot achieve immune control, and might really do more damage by fueling tumor-promoting inflammation instead. This is in line with current concepts of immunologically hot vs. cold tumors in humans (Galon and Bruni, 2019). Accordingly, presence of Foxp3+ regulatory T-cells (T-regs) and myeloid-derived suppressor cells (MDSCs) positively correlated with adverse prognostic factors, such as high histological grade, lymphatic invasion, and metastasis (Król et al., 2011a; Kim et al., 2012; Carvalho et al., 2016a; Mucha et al., 2016; Sakai et al., 2018). These findings support the concept that immune suppression through T-regs and MDSCs might contribute significantly to CMT progression.

Several studies have shown a strong correlation between high levels of tumor-associated macrophages (TAMs) and indicators of malignancy, metastasis, as well as worse overall survival in CMT (Restucci et al., 2002; Król et al., 2011b; Raposo et al., 2012, 2013; Lim et al., 2015; Carvalho et al., 2016b; Reis dos et al., 2019). All of these studies detected TAMs based on IHC detection of MAC387, but unfortunately did not attempt further subtyping of the macrophages into (antitumoral) M1 or (pro-tumorigenic) M2 phenotype. More detailed data regarding M1/M2 polarization of TAMs in CMT have been recently emerging, demonstrating significantly higher numbers of M2-TAMs in malignant CMTs while benign tumors harbored M1-TAMs, suggesting a M1-to-M2 shift of TAMs in malignant CMTs (Monteiro et al., 2018; Seung et al., 2018). While these results are highly interesting, there remains some controversy regarding whether CD204 represents a useful IHC marker for M2-polarized macrophages in dogs that awaits clarification (Belluco, 2018).

In summary, striking parallels between canine and human CAS with respect to the effect of the type of immune cell

that strongly determines the effect on tumor progression are beginning to emerge. The interested reader wishing to further extend on parallels of CMT with human breast cancer in terms of tumor-associated inflammation is referred to a recent review on the topic (Carvalho et al., 2016c). Despite this progress, the field is still far from a complete understanding of the effects of different immune cells on the clinical course and prognosis of the disease and more detailed insights are needed to further clarify many of the outstanding questions. Further detailed insights into immune components in CAS of CMTs, ideally also with regards to molecular subtypes, are highly anticipated.

Angiogenesis

Sustained angiogenesis represents one of the core hallmarks of cancer (Hanahan and Weinberg, 2011). A series of studies has assessed the contribution of blood vessel supply to the biology of CMTs. Indeed, in analogy to human breast cancer, increased microvessel density (MVD) correlated with malignancy and metastasis (Graham and Myers, 1999; Restucci et al., 2000; Millanta et al., 2006; Lavalle et al., 2009; Al-Dissi et al., 2010; Queiroga et al., 2011a; Carvalho et al., 2013; Sleeckx et al., 2014; Diessler et al., 2016; Anjos Dos et al., 2019).

A plethora of different molecules are involved in controlling the rate and extent of angiogenesis. Among the best studied ones are vascular endothelial growth factors (VEGFs) that regulate formation, function, and maintenance of vasculature (Simons et al., 2016). In most studies, VEGF expression in CMT has been closely correlated with metastasis to lymph nodes, clinical stage, tumor grade, and malignancy (Qiu et al., 2008; Vinothini et al., 2009; Clemente et al., 2010, 2013; Millanta et al., 2010; Klopfleisch et al., 2011; Queiroga et al., 2011a; Carvalho et al., 2015a, 2016b; Moschetta et al., 2015; Mucha et al., 2016). However, a few reports have failed to see such an association (Millanta et al., 2006; Santos et al., 2010, 2014). Unfortunately, most of these studies have not differentiated between VEGF isoforms, which would be an interesting additional information. Interestingly, there is evidence for a strong link between immune cells, such as TAMs, CD3+ T-cells, FoxP3+ T-regs, and mast cells, and VEGF expression with increasing malignancy, suggesting that immune cells influence tumor angiogenesis through secretion of VEGF (Restucci et al., 2002; Im et al., 2011; Raposo et al., 2013; Carvalho et al., 2015a, 2016a). Likewise, expression of VEGFR-2, the main signaling VEGF receptor in vascular endothelial cells, in endothelial cells within the tumor tissue increased with malignancy, histological grade, and lymph node metastases, implicating VEGF and VEGFR-2 in angiogenesis in CMTs (Restucci et al., 2004; Diessler et al., 2016; Anjos Dos et al., 2019). One study failed to find a connection between VEGFR-2 expression and histologic grade (Al-Dissi et al., 2010). Interestingly, a positive association between expression of VEGFR-2 and stromal MMP9 has been described, indicating a link between ECM remodeling and endothelial cell activation (Santos et al., 2014).

While expanding our understanding of CAS in CMTs, all these studies have only investigated a very limited number of targets, mostly due to methodological limitations. When information is available as to whether a molecule is expressed in the tumor cells or rather one of the stromal components, it has been mostly obtained through IHC analysis, whereas other approaches have relied on bulk tumor analysis. A major draw-back of the targeted analyses is that one can only analyze targets that are known *a priori*, which precludes unbiased identification of novel molecules of interest. Furthermore, the limited number of targets that can be interrogated through most of these approaches makes it impossible to gain a more wide-angled perspective of changes in molecular networks underlying stromal reprogramming in CMTs. As a direct consequence, unbiased cross-species analyses of molecular homologies and differences in CAS between species have therefore been precluded to date. Due to these limitations, it remains largely unknown to what extent stromal reprogramming in canine and human breast cancer are comparable, and what the molecular similarities and differences are. A better understanding of the biology of CAS in canine breast cancer is imperative to both understand how CAS influences growth and progression of CMTs as well as understand whether canine breast cancer really is comparable to the human disease in all of its aspects.

TOWARD A MORE COMPREHENSIVE PICTURE OF STROMAL REPROGRAMMING IN CMTs

Driven by the lack of detailed characterization of stromal reprogramming in CMTs caused by technical limitations described above, we established a workflow to isolate subsections of formalin-fixed paraffin-embedded clinical tumor samples by laser-capture microdissection, and analyze gene-expression changes therein. In a first study, we isolated CAS and matched "normal" stroma (i.e., stroma isolated adjacent to unaltered mammary glands) from FFPE specimen of 13 cases of canine simple mCA, and analyzed the expression of seven well-described CAS-markers in human mCA (PDGFRβ, MMP2, Col1α1, FAP, ACTA2/αSMA, CXCL12/SDF1, and IL6) by RT-qPCR (Ettlin et al., 2017). Our results demonstrated that ACTA2, COL1A1, and FAP were upregulated in canine CAS, while PDGFRB, MMP2, and IL6 expression did not significantly change between normal stroma and CAS. CXCL12 expression was downregulated in CAS compared to normal stroma. IHC validation of these results revealed upregulation of αSMA, FAP, PDGFRB, and Cav-1, while SDF1, MMP2, and FGF2 expression did not change. These findings not only suggested the presence of molecular similarities in CAS biology between canine and human mCA, but also revealed some differences. While interesting, this RT-qPCR-based approach had two major limitations: (i) the targets of interest have to be defined *a priori*, which precludes an unbiased analysis of the samples, and (ii) to the small amount of RNA that can be extracted from small LCM-subsections of FFPE strongly limits the number of RT-qPCR reactions that can be run, thus strongly restricting the number of targets that can be analyzed per sample. To overcome these problems, we further optimized the RNA extraction protocol for the LCM samples of FFPE tissue in a way that increased the average yield per sample between 8- and 12-fold and allowed us to perform next-generation RNAseq (Amini et al., 2017). An overview of

the entire workflow is depicted in **Figure 1**. Thus far, we have successfully applied this novel approach to analyze stromal reprogramming in several different cohorts of clinical samples, including malignant canine mCA and benign canine mammary adenomas. In the following, I will shortly summarize the main findings from these analyses.

Stromal Reprogramming in Canine Simple Mammary Carcinoma

To begin to understand stromal reprogramming in canine simple mCA on a transcriptome-wide scale, we analyzed matched CAS and normal stroma from 15 clinical cases using our LCM-RNAseq pipeline (Amini et al., 2019a). Strikingly, differential gene expression changes clearly differed between normal stroma and CAS, with 884 significantly deregulated genes. Strongest changes were found in the genes involved in the immune system, cell adhesion and differentiation, ECM organization, and angiogenesis. Clearly, all of these processes are strongly associated with stromal biology, further validating our analytical approach. Unsupervised clustering of samples based on the landscape of immune and stromal cells present in the samples again clearly separated CAS and normal stroma, and revealed strong increases in mesenchymal stem cells, gamma delta T-cells, macrophages, plasmoid dendritic cells, and natural killer T-cells in CAS. These results provide evidence for wide-ranging stromal reprogramming in canine mCA, enabling for the first time a detailed molecular analysis of CAS in canine mCA. We envisage these data to significantly support the understanding of the biology of canine mCA.

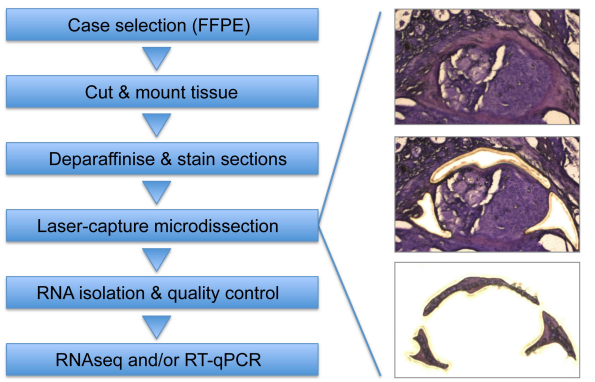

FIGURE 1 | Workflow to isolate and analyze areas of interest from FFPE tissue specimens. After selection of appropriate FFPE specimen, tissue is cut into 10 μm sections and mounted on PEN membrane glass slides (Ettlin et al., 2017). Slides are deparaffinized and stained using Cresyl Fast Violet (Amini et al., 2017), followed by LCM to isolate areas of interest (e.g., CAS). Whenever possible, matched control tissue (e.g., normal stroma) is isolated from the same section to minimize differences in sample processing. RNA isolation is performed using a sonication-based protocol combined with protease digestion (Amini et al., 2017), followed by RNA quality control. Analysis of isolated RNA can be performed by RNAseq [routinely using 4 ng RNA as input per run (Amini et al., 2019a)], by RT-qPCR (Ettlin et al., 2017), and possibly other applications that allow RNA analysis.

Stromal Reprogramming in Canine Simple Mammary Adenoma

While canine simple mCAs are classified as malignant epithelial neoplasms that infiltrate the surrounding tissue, canine simple mammary adenomas represent benign, well-demarcated, and non-infiltrative tumors that generally contain only very little fibrovascular supporting stroma (Goldschmidt et al., 2011). To date, it remains unclear whether and to what extent stromal reprogramming occurs in these naturally occurring benign tumors of the mammary gland. In fact, stromal reprogramming in human breast cancer has been studied during progression from *in situ* to *invasive* human mCA, in pregnancy-associated breast cancer, in response to therapeutic radiation, and in inflammatory breast cancer (Finak et al., 2006, 2008; Boersma et al., 2007; Casey et al., 2008; Ma et al., 2009; Westbury et al., 2009; Planche et al., 2011; Knudsen et al., 2012; Vargas et al., 2012; Harvell et al., 2013). However, we are not aware of any published dataset regarding stromal reprogramming in naturally occurring benign tumors of the mammary gland. Since CAS has been shown to have important roles in determining the growth and progression of different tumor types, we hypothesized that differences in stromal reprogramming between benign adenomas and malignant mCA could contribute to the clinical behavior of these two tumor types. To begin to understand stromal reprogramming in naturally occurring benign tumors, we thus applied our approach to isolate and analyze CAS and normal stroma from FFPE tissue sections to 13 cases of canine simple mammary adenoma (Amini et al., 2019b). We observed clear separation of normal stroma and CAS samples, and identified 193 genes to be significantly deregulated between the two entities. The strongest changes occurred in processes related to cell adhesion, immune system, proliferation and growth, differentiation, and ECM and collagen organization. Hence, these results demonstrate that substantial stromal reprogramming occurs also in small, benign tumors of the mammary gland.

Having previously characterized stromal reprogramming in canine mCA, we then sought to understand commonalities and differences in stromal reprogramming between benign mammary adenomas and malignant mCA. Our analyses showed that CAS in benign adenomas is clearly distinct from malignant mCA. Furthermore, adenoma-derived stroma was much more similar to normal stroma than CAS from mCA, suggesting gradual changes from normal to benign to malignant CAS to occur during the development of tumors. Nevertheless, we also identified commonly regulated genes in CAS of both benign and malignant tumors. This comparative dataset allowed us to interrogate for the first time the transcriptional levels of targets that have been implicated in stromal reprogramming of canine breast tumors thus far (see the sections "Cancer-Associated Fibroblasts and the Extracellular Matrix," "Infiltrating Immune Cells," and "Angiogenesis" for details). **Figure 2** intends to give a schematic overview of changes in mRNA abundance of these targets between normal stroma, CAS in adenoma, and CAS in carcinoma as detected by our RNAseq approach (Amini et al., 2019b). These data give rise to several interesting observations: (i) some targets, such as αSMA, Tn-C, and VEGFA, show changes in

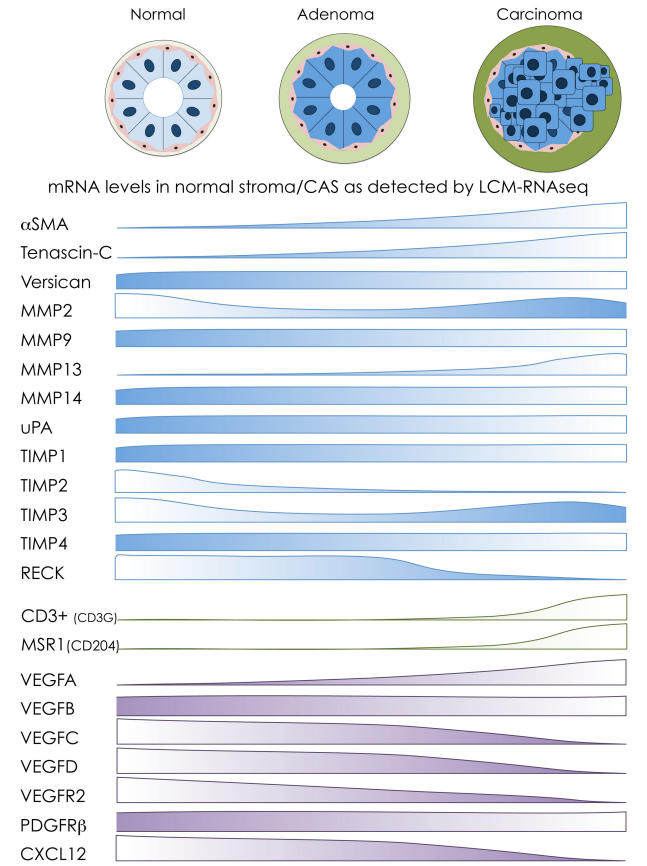

FIGURE 2 | Overview of transcriptomic changes in selected markers during stromal reprogramming in canine mammary adenomas and carcinomas. Schematic overview of transcriptomic changes of selected CAS-related markers based on our transcriptomic analyses of normal stroma, CAS from adenoma, and CAS from carcinoma isolated from canine breast tumor specimens (Amini et al., 2019b). Protein names on the left are targets whose expression in canine tumors has been investigated on protein level mostly by IHC (for details and references, see the main text). The bars next to the protein names indicate changes in RNA abundance of the respective targets between stroma from normal glands **(left)**, stroma from adenomas **(middle)**, and stroma from carcinomas **(right)**, as detected by LCM-RNAseq. Targets associated with fibroblast activation and remodeling of extracellular matrix are depicted in blue, markers of immune cells are colored in green, and targets involved in angiogenesis are shown in purple.

mRNA levels that mirror closely results obtained on protein level, with increasing abundance from normal stroma to adenoma to carcinoma; (ii) a number of targets whose expression has been positively correlated with increasing malignancy (e.g., versican, MMP9, and MMP14) show no changes in stromal mRNA abundance between the three entities; (iii) there are some genes whose expression is opposite of what would be expected from literature regarding protein levels (e.g., MMP2, MMP13, TIMP3, and VEGFR2); and (iv) one of the advantages of RNAseq-based analysis is differentiation between closely related isoforms, e.g., changes in VEGF that are very specific to the different isoforms of the protein. Differences in mRNA versus protein levels can be explained by two different mechanisms: either the main source of the protein in question is not the stroma itself, but rather the tumor cells which release the product into their surroundings. Or, if indeed it is produced by stromal cells, the increase in protein production is due to post-transcriptional regulatory mechanisms that do not impinge on mRNA transcription. To further clarify these aspects, it would be interesting to compare RNAseq data from the tumor cells to that of the respective stroma, or to analyze the different tumor compartments using proteomic analysis pipelines.

Highly Conserved Stromal Reprogramming Between Canine and Human Mammary Carcinoma

Our next aim was to understand to what extent stromal reprogramming in canine and human mCA is comparable. We reasoned that if stromal reprogramming in the two species shared high levels of homology, this should result in a similar expression pattern of differentially regulated genes between normal stroma and CAS in both. We assessed this using several different approaches. Firstly, juxtaposition of our canine CAS dataset to a similar human dataset revealed that genes upregulated in the canine dataset were on average also upregulated in the human dataset, and likewise genes downregulated in the canine dataset were also downregulated in the human dataset. Secondly, we ranked the samples in the TCGA breast cancer subset (that contains >1000 human tumor samples) according to stromal enrichment scores (i.e., according to how much stroma they contain) to compare our canine-derived stromal signature with. By doing so, we found the canine-derived stromal signature to be highly positively correlated with the enrichment of human-derived stromal signature of the TCGA breast cancer subset. The commonly perturbed pathways between canine and human CAS included angiogenesis, epithelial mesenchymal transition, glycolysis, pathways involved immune response, and others. And finally, we demonstrated that the high level of molecular homology between canine and human stromal reprogramming manifested in a prognostic value of the canine CAS signature, with upregulated genes in canine CAS highly enriched among adversely prognostic genes in humans, and upregulated genes in canine normal stroma highly enriched among favorably prognostic genes in humans. In conclusion, these results clearly demonstrated that stromal reprogramming in canine and human mCA shares significant molecular homology. This homology derives from conservation of key signaling pathways which underlie the prognostic value of stromal gene expression changes in both canine and human mCA. Hence, these findings clearly emphasize the value of canine mCA as a model for human mCA.

CONCLUSION AND OUTLOOK

Increased understanding of stromal reprogramming in tumors requires the ability to selectively analyze patient-derived CAS, ideally using untargeted methods, such as RNAseq. To date, stromal reprogramming has been mostly investigated using laser-capture microdissection of fresh-frozen tissue, coupled with

microarrays or sequencing approaches. However, use of fresh-frozen tissues most often necessitate establishing prospective trials to collect samples accordingly, requiring a high grade of coordination between surgical resection and analysis, and introducing temporal until all required cases are collected. More importantly, it also precludes the analysis of archival FFPE samples, which are the standard product of any pathology department and can be kept at room temperature over long periods of time. To circumvent these problems, we have developed a protocol that allows the analysis of subsections of FFPE patient samples by RNAseq, and have demonstrated its feasibility and usefulness by analysis of stromal reprogramming in several cohorts of patient samples. Importantly, the protocol can be adapted to interrogate transcriptional reprogramming of any area of interest in any type of tissue of any organism, provided that the area is sufficiently large to be isolated and contains sufficient RNA. Hence, we hope our approach to enable a wide range of projects to understand transcriptional reprogramming within distinct compartments of entire tissues. Over the last few years, technological advances have made it possible to analyze tumor (and other) tissues on the cellular level by single-cell RNAseq. This presents a tremendous advance in analytic power and novel insights. However, single-cell RNAseq can only be performed on fresh tissue, which again precludes its applicability for analysis of archival FFPE tissue. Also, the currently available methodology is no accurate enough yet to be routinely used in clinics. Furthermore, it requires sometimes lengthy digestion steps to dissociate tissues into single cells that quite possibly also introduce a fair amount of gene expression changes during the preparation. Finally, the cost of single-cell RNAseq experiments still is substantially higher than that of "canonical" RNAseq analyses. Due to all these aspects, analysis of tissue subsections of FFPE tissues using our LCM-RNAseq approach presents a complementary approach of great value to further understand transcriptional changes in defined locations of clinical specimen.

Despite the advances in analysis of stromal reprogramming that occurs in human tumor samples, the study of CAS in canine tumors has strongly lagged behind. Although a subset of molecular and cellular aspects have been relatively well studied in CMTs, the field is still far from having a more wide-angled overview of stromal reprogramming that occurs in these tumors. For the first time, our studies have started to shed light into stromal reprogramming in canine simple mCA and canine simple mammary adenoma, and begun to analyze the extent to which CAS in canine mCA and human mCA compare. Our data show wide-ranging stromal reprogramming in both canine mCA and adenoma, and also reveal strong molecular homologies between stromal reprogramming in human and canine tumors. Further in-depth analysis of these data have the potential to significantly increase our understanding of stromal reprogramming in canine mCA, and also to identify the conserved aspects between species that are likely drivers of the disease. Better understanding of the molecular underpinnings of canine and human CAS holds enormous potential for further interesting findings.

AUTHOR CONTRIBUTIONS

The author designed and wrote the entire manuscript without external help.

FUNDING

Work in the author's laboratory was financially supported by the A.+S. Huggenberger Stiftung, the Heuberger Stiftung, the Forschungskredit of the University of Zurich, the Promedica Stiftung Chur, the Kurt und Senta Herrmann Stiftung, and the Sassella Stiftung.

ACKNOWLEDGMENTS

The author thanks Dr. Parisa Amini (University of Zurich, Switzerland), Dr. Sina Nassiri (SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland), Dr. Alexandra Malbon [University of Zurich, Switzerland, and The Royal (Dick) School of Veterinary Studies and The Roslin Institute Easter Bush Campus, Midlothian, United Kingdom], and Dr. Julia Ettlin (University of Zurich, Switzerland), as well as Dr. Maria Domenica Moccia and Lennart Opitz (Functional Genomics Center Zurich, Switzerland) for their invaluable contributions toward the analysis of CAS from canine mammary carcinomas. Further, the author thanks the Histology Laboratory of the Institute of Veterinary Pathology (University of Zurich, Switzerland) for slide preparation and technical assistance.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

PERSPECTIVE
published: 20 December 2019
doi: 10.3389/fcell.2019.00335

Therapeutic and Mechanistic Perspectives of Protein Complexes in Breast Cancer

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Breast cancer affects one in eight women making it the most common cancer in the United Kingdom, accounting for 15% of all new cancer cases. One of the main challenges in treating breast cancer is the heterogeneous nature of the disease. At present, targeted therapies are available for hormone receptor- and HER2-positive tumors. However, no targeted therapies are currently available for patients with triple negative breast cancer (TNBC). This likely contributes to the poor prognostic outcome for TNBC patients. Consequently, there is a clear clinical need for the development of novel drugs that efficiently target TNBC. Extensive genomic and transcriptomic characterization of TNBC has in recent years identified a plethora of putative oncogenes. However, these driver oncogenes are often critical in other cell types and/or transcription factors making them very difficult to target directly. Therefore, other approaches may be required for developing novel therapeutics that fully exploit the specific functions of TNBC oncogenes in tumor cells. Here, we will argue that more research is needed to identify the protein-protein interactions of TNBC oncogenes as a means for (a) mechanistically understanding the biological function of these oncogenes in TNBC and (b) providing novel therapeutic targets that can be exploited for selectively inhibiting the oncogenic roles of TNBC oncogenes in cancer cells, whilst sparing normal healthy cells.

Keywords: transcription factor, breast cancer, TNBC, protein-protein interaction, protein complexes, cancer therapy, PROTAC, post-translational modification

OPEN ACCESS

Edited by:
Vida Vafaizadeh,
University of Basel, Switzerland

Reviewed by:
Masaru Miyano,
Lawrence Berkeley National Laboratory, United States
Yoo-Ah Kim,
National Institutes of Health (NIH), United States

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Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: *27 September 2019*
Accepted: *27 November 2019*
Published: *20 December 2019*

Citation:
Waterhouse MP, Ugur R and Khaled WT (2019) Therapeutic and Mechanistic Perspectives of Protein Complexes in Breast Cancer. Front. Cell Dev. Biol. 7:335. doi: 10.3389/fcell.2019.00335

BREAST CANCER SUBTYPES AND ASSOCIATED THERAPIES

Breast cancer is the most common cancer in the United Kingdom, accounting for 15% of all new cancer cases, and is the leading cause of cancer death in women worldwide (Bray et al., 2018). Historically, breast cancers have been classified based on the expression of several cell-surface receptors, namely the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Onitilo et al., 2009). Based on the presence or absence of these markers, breast cancers can be broadly stratified into luminal A/B, HER2+, or basal-like (triple-negative) subtypes. Specifically, luminal A/B breast cancers are characterized as hormone receptor positive (high expression of ER and/or PR); HER2+ breast cancers are characterized by amplification of HER2 (and can be ER+ and/or PR+); and triple negative breast cancers (TNBCs) are characterized as hormone receptor negative and HER2 negative (ER−/PR−/HER2−) (Onitilo et al., 2009). Receptor status continues to act as a critical assessment for all breast cancers, likely due to the quick, easy and cost-effective stratification of patients to determine suitability for

targeted treatments. These include tamoxifen, an ER modulator, and trastuzumab (Herceptin), a monoclonal antibody targeting the HER2 receptor, which are first-line therapies for ER+ tumors in pre-menopausal women and HER2+ tumors, respectively. Due to the availability of effective treatment options, hormone receptor positive breast cancers generally have a better prognosis (Fallahpour et al., 2017). Prior to the advent of modern therapies, HER2+ patients had a worse prognosis. However, since the introduction of HER2-targeted therapies, such as trastuzumab, there has been a significant improvement in prognosis (Slamon et al., 2001). Conversely, TNBCs (i.e., negative for all three hormone receptors) still lack targeted treatments and continue to have a comparatively poor prognosis (Dent et al., 2007; Onitilo et al., 2009).

For tumors that are susceptible to targeted therapies various therapies are available (Lumachi et al., 2013; Fallahpour et al., 2017). For hormone receptor-positive cancers, these include selective ER modulators, inhibitors of the aromatase enzyme and antigonadotropic therapies. Patients with HER2+ tumors also benefit from the availability of the monoclonal antibodies trastuzumab and pertuzumab, which function by preventing HER2 from functioning by inhibiting HER2-associated signaling (Molina et al., 2001; Agus et al., 2002; Franklin et al., 2004; Junttila et al., 2009). For tumors that are unsuitable for targeted therapies (i.e., TNBC), treatment involves chemotherapy in combination with radiotherapy and/or surgery (Foulkes et al., 2010; Wahba and El-Hadaad, 2015). Chemotherapeutic agents that are currently approved for use in breast cancer therapy typically target DNA synthesis and repair pathways and tend to have more serious side effects. Mechanistically, these therapies comprise alkylating agents that irreversibly crosslink DNA and lead to apoptosis (Hall and Tilby, 1992) [cyclophosphamide, mitomycin C (Tomasz, 1995)]; inhibitors of DNA biosynthesis enzymes such as dihydrofolate reductase [methotrexate (Goodsell, 1999)], thymidine synthase [fluorouracil (Longley et al., 2003)] and type II topoisomerase [mitoxantrone (Fox, 2004)]; or DNA intercalators that inhibit DNA and RNA synthesis [epirubicin, doxorubicin (Gewirtz, 1999)] (**Figure 1A**). Cytoskeletal drugs are also approved for use in breast cancer therapy (e.g., paclitaxel) and block cell cycle progression by stabilizing microtubule polymers (Horwitz, 1994; **Figure 1B**).

In hormone receptor and/or HER2+ positive tumors, it appears that much efficacy is derived from the availability of therapies targeting protein-protein interactions (PPIs) that drive disease. These therapies, which include tamoxifen, anastrozole and trastuzumab, either directly or indirectly block interactions between growth factors and their receptors. However, due to the lack of actionable receptors in TNBC, chemotherapy remains the first-line standard of care in combination with radiotherapy and/or surgery. It is apparent that these non-targeted chemotherapeutic agents represent generic therapeutic strategies that broadly target cancerous tissues, as they preferentially target rapidly dividing cells such as those found in tumors. However, normal cells that divide rapidly such as those in the digestive tract, hair follicles, and bone marrow are also highly susceptible to cytotoxicity, which leads to common chemotherapeutic side effects such as mucositis, alopecia, and myelosuppression with subsequent immunosuppression (Partridge et al., 2001). As a result, there is a clear clinical need for the identification of actionable targets in TNBC that can be used as the basis for the production of new and more targeted therapies. It is likely that, to ensure specificity in targeting, the identification of PPIs or protein networks that drive disease will be necessary for this purpose.

A promising example of this concept is the inhibition of interactions that induce immune tolerance such as the PD-1/PD-L1 axis (Gatalica et al., 2014), inhibition of which promotes T-cell proliferation, survival and cytotoxicity (**Figure 1A**). Most recently this has included the approval of atezolizumab, an anti-PD-L1 antibody, in combination with chemotherapy for the treatment of PD-L1+ metastatic TNBC by the US FDA in March 2019 (Schmid et al., 2018; Dolgin, 2019). Another example is the recent use of poly ADP ribose polymerase (PARP) inhibitors for the treatment of *BRCA1*-mutated tumors. Although there continues to be a lack of targeted treatment options for TNBC patients, ∼15% of TNBC tumors are driven by germline mutations within the *BRCA1* and *BRCA2* genes (Engel et al., 2018). These mutations result in defective double-strand DNA repair machinery and lead to the accumulation of DNA damage. PARP is another DNA repair protein that is crucial for the repair of single-strand DNA breaks (Audebert et al., 2004; Heale et al., 2006), which can develop into double-strand breaks (DSBs) if not repaired before the initiation of DNA replication (Farmer et al., 2005). In this context, *BRCA1/BRCA2* mutated tumors cannot repair these DSBs, ultimately resulting in cell death, whereas normal cells can compensate for the loss of PARP function (Farmer et al., 2005). As a result, patients with mutated *BRCA1/BRCA2* are suitable candidates for additional treatment with PARP inhibitors, such as the recently approved drug olaparib which was approved in 2019 in Europe for germline *BRCA1/2*-mutated HER2− breast cancer (Griguolo et al., 2018; Le and Gelmon, 2018). However, this therapy class is only suitable for patients with *BRCA*-mutated tumors and there is still intense interest in the identification of the molecular drivers of TNBC.

MOLECULAR PROFILING OF BREAST CANCERS FOR TARGET IDENTIFICATION

Much effort has been invested into the molecular profiling of breast cancers for the identification of novel drivers in TNBC pathogenesis and to better define breast cancer subtypes. The first of these classification models, proposed by Sørlie et al. (2003), was based on the transcriptomic profiling of 115 malignant breast tumors and identified five intrinsic subtypes of breast cancers (Sørlie et al., 2001, 2003). Although the identification of these intrinsic subtypes has provided much insight into breast cancer biology, attempts to define possible somatic drivers of breast cancer subtypes has remained difficult due to the heterogeneity of the disease as well as a lack of clear driver mutations. More recent work has aimed to tackle this issue by integrating genomic and transcriptomic breast cancer data in much larger patient sizes, a prime example of which is the recent METABRIC

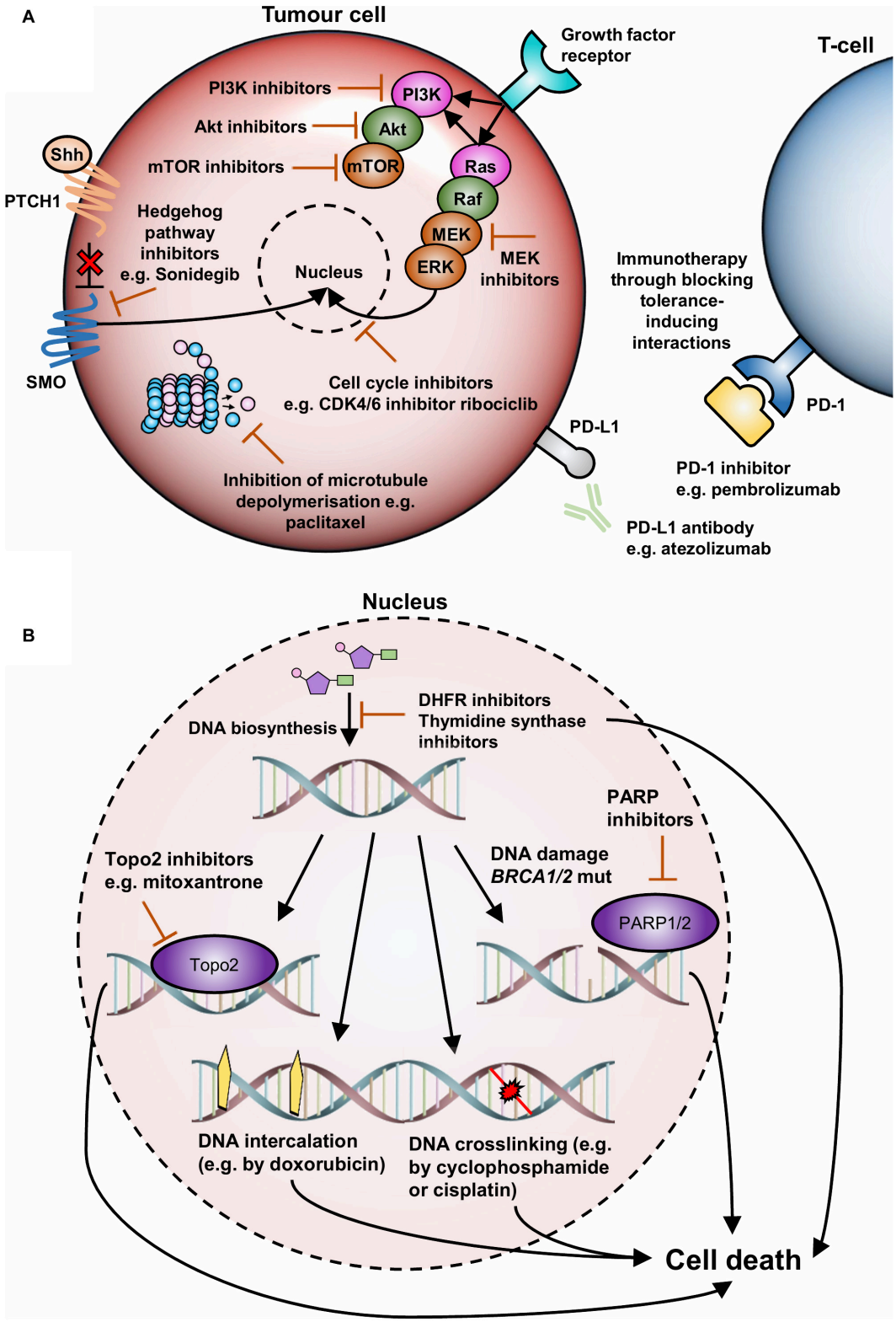

FIGURE 1 | An overview of current and emerging agents for TNBC therapy. **(A)** TNBC therapies targeting the cell surface and cytoplasm. Cell surface therapies include inhibitors of immune tolerance inducing proteins such as PD1 and PD-L1. Cytoplasmic therapies include inhibitors of the Ras/MAPK pathway, especially MEK inhibitors, inhibitors of the PI3K/AKT/mTOR pathway, inhibitors of the Hedgehog signaling pathway, and cell cycle inhibitors such as paclitaxel and CDK inhibitors. **(B)** TNBC therapies targeting the nucleus. These therapies tend to target DNA synthesis and repair pathways or affect DNA viability to induce cell-cycle arrest and cell death.

dataset. This work characterized the genomic and transcriptomic architecture of 2000 breast tumors (Curtis et al., 2012) and resulted in the identification of 10 novel molecular subgroups, known as integrative clusters, which are clustered according to copy number alterations and gene expression data (Dawson et al., 2013). Crucially, each integrative cluster is associated with distinct clinical features and outcomes (Dawson et al., 2013). In addition, the clusters have identified heterogeneity within tumors classified according to receptor status and divided all previously identified intrinsic subtypes into separate groups. Additional transcriptomic studies have further highlighted the heterogeneity of TNBC, which include studies by Lehmann et al. (2011, 2016) and Burstein et al. (2015), both of which identified four molecular subtypes of TNBC. As a result, breast cancer classification is now evolving to describe a number of distinct molecular subgroups based on multiple genomic factors, which has produced more robust patient classifiers and is leading to a new stratification and treatment paradigm for breast cancer patients. However, despite this progress in the molecular characterization of TNBC, these tumors remain to be mostly characterized by *TP53* alterations and copy number alterations involving 5q loss and gains at 8q, 10p and 12p (Dawson et al., 2013).

A limited number of studies have therefore attempted to investigate the mutational landscape in TNBC, which has mostly identified that TNBC is characterized by a low rate of activating point mutations in common oncogenes, as well as extensive individually rare mutations in other genes (Shi et al., 2018). However, TNBCs appear to be particularly enriched for alterations in tumor suppressor proteins, such as *TP53*, *RB1*, and *PTEN*, as well as oncogenic alterations in the PI3K/AKT pathway (Curtis et al., 2012; Koboldt et al., 2012). Regardless, common TNBC "oncogenes" such as *PIK3CA* and other actionable targets, such as the Ras/MAPK (Balko et al., 2013), JAK/STAT (Marotta et al., 2011), Wnt (DiMeo et al., 2009), TGF-β (Bhola et al., 2013), Hedgehog (Liu et al., 2006), and Notch (Harrison et al., 2010) pathways, are all critical genes/signaling pathways in a wide range cell types and contexts. As a result, any therapies designed against these pathways are highly likely to result in off-target cytotoxicity. Overall therefore, genome-wide studies have failed to identify driving mutations distinct from those affecting TP53, PIK3CA, and PTEN (Peluffo et al., 2019), and new therapeutic angles are required to define better and more specific targets for the production of TNBC therapies. One such angle to consider is that alterations in epigenetics and transcriptional machinery may be largely contributing to the transcriptional dysregulation seen in TNBC malignancies.

TRANSCRIPTION FACTOR TARGETING FOR POTENTIAL ENHANCED THERAPEUTIC SPECIFICITY

Downstream effectors of traditionally targeted pathways, namely transcription factors (TFs) involved in normal cellular function, are often those subjected to dysregulation resulting in cancer (Bass et al., 2009). Indeed, many cancer-related events either directly involve TFs or indirectly modulate TF activity. This highlights targeting TFs as a promising anticancer strategy and as potentially superior therapeutic targets compared to upstream signaling proteins and kinases (Konstantinopoulos and Papavassiliou, 2011). Our progression in understanding of the mechanistic properties of TFs and their associated networks, in both diseased and normal cells, has created huge potential for precision medicine in cancer. For example, targeting oncogenic TFs may lead to preferential cancer cell death in tumors that display TF dependency, whereas normal cells may be more likely to tolerate a loss of TF function due to redundancies in normal signaling pathways. One such case is the *TRPS1* TF, which demonstrates breast lineage-specific transcriptional dependency, likely due to lineage-restricted expression (Witwicki et al., 2018). As a result, breast cancer cells lines display sensitivity to *TRPS1* shRNA targeting compared to cell lines derived from colon, neuroblastoma, leukemia, prostate, and rhabdoid tumors (Witwicki et al., 2018). TFs in this context are therefore likely to have a high therapeutic potential, owing to their critical role in tumor pathogenesis along with their dispensability for physiologic cell function. Accordingly, many studies have tried to capture the transcriptional landscape of TNBC, thus identifying highly expressed genes and TFs that may be liable to therapeutic targeting. However, TFs have long been considered "undruggable" targets, which may result from the large interaction surface areas used by TFs for protein-DNA and PPIs as well as their predominant nuclear localization, which makes them less accessible to therapeutic agents (Yan and Higgins, 2013).

Despite these challenges, there are various opportunities available for targeting TFs at different functional levels. For example, TFs may be directly or indirectly targeted through inhibition (or activation) at the expression level, at the PPI level, at the post-translational modification level, at the protein/DNA binding level, through the binding of a small molecule in an inhibition/activation pocket or through physical degradation (**Figure 2**). In addition, post-translational modifications, which may result in context-specific PPIs and/or differential assembly of epigenetic remodeling complexes, must also be considered. To date, over 450 unique protein modifications have been described, including phosphorylation, acetylation, ubiquitination, methylation, and SUMOylation, which can alter target protein activity, intracellular distribution, protein interactions and protein longevity (Venne et al., 2014). For phosphorylation alone, there are over 500 different kinases in mammals (Woolfrey and Dell'Acqua, 2015), some of which could conceivably be expressed in a tissue-specific manner and may therefore give rise to differing versions of the same proteins in various tissues.

Like all other cellular proteins, TF expression is controlled by transcriptional activators and repressors (such as other TFs or itself in a feedback loop) as well as by epigenetic machinery. Aberrant activity of these processes may therefore result in oncogenic transcriptional programs. For example, oncogenic gene translocation and consequent juxtaposition of the c-MYC gene with enhancer elements has been reported in multiple myeloma, which may enhance c-MYC expression (Shou et al., 2000), and aberrant expression of the HOXA cluster of TFs

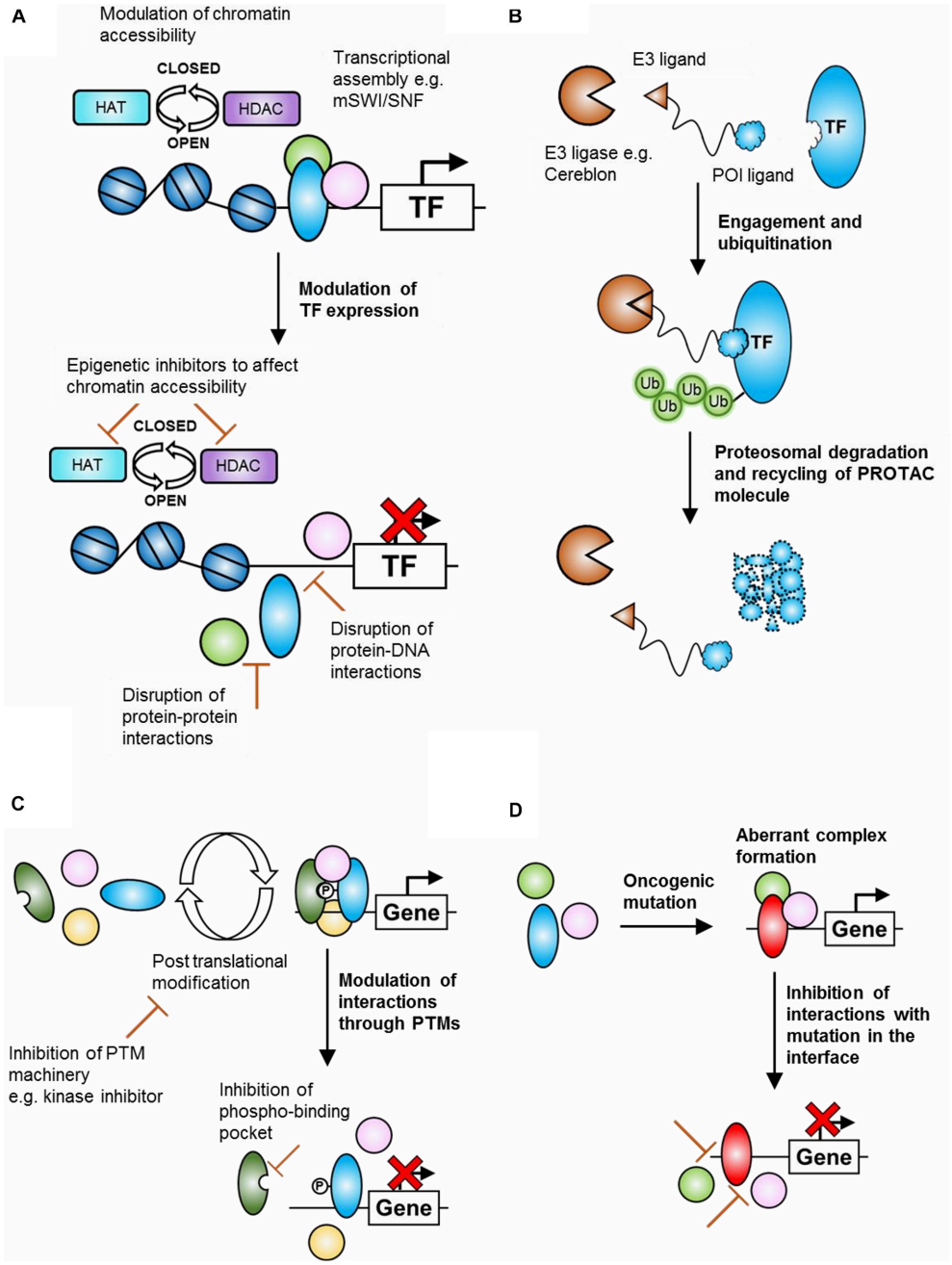

FIGURE 2 | Potential mechanisms of transcription factor (TF) targeting for cancer therapy. **(A)** Inhibition of oncogenic TF expression. This may take the form of altering chromatin accessibility, through inhibitors of epigenetic machinery, or by disrupting the assembly of transcriptional machinery at the protein-protein or protein-DNA binding level. **(B)** Depletion of oncogenic proteins by PROTAC-mediated proteasomal degradation. A bi-functional molecule containing a protein of interest-binding region and an E3-ligase binding region links the protein of interest to an E3-ligase, leading to ubiquitination and proteasomal degradation. **(C)** Inhibition of TF function through modulation of post-translational modifications. Assembly of oncogenic transcriptional (or epigenetic) assemblies may rely on post-translational modifications. Inhibiting the enzymes responsible for these modifications or inhibiting the binding pocket of the specific modification may represent feasible options for preventing the assembly of oncogenic transcriptional assemblies. **(D)** Inhibition of mutation-dependent transcriptional assemblies. Structural information regarding the binding interfaces of mutated transcriptional or epigenetic proteins may allow for the design of therapies that inhibit mutation-dependent interactions and prevent the assembly of mutation-dependent transcriptional machinery.

has been reported in several aggressive acute leukemias as a result of oncogenic rearrangements of the *MLL1* gene, a histone methyltransferase (Kawagoe et al., 1999; Guenther et al., 2005). More specifically, rearrangements of the *MLL1* gene can lead to the production of over 70 in-frame oncogenic fusion proteins, which can add functionality by enabling interactions with histone

methyltrasferases such as DOT1L (Krivtsov et al., 2007) or may direct the MLL complex to unintended genomic areas, resulting in an aberrant transcriptional program. Therapies have been designed for both of these contexts, typically through regulation at the epigenetic level. For example, HDAC and histone methyltransferase inhibitors (e.g., against DOT1L), both of which associate with the MLL complex, have entered clinical trials for acute myeloid leukemia (Daigle et al., 2011; Chen et al., 2013; Fredly et al., 2013; Morabito et al., 2016), whereas negative regulation of oncogenic c-MYC has been achieved, for example, by inhibitors of BRD4 (such as JQ1) which displace BRD4 from the c-MYC promoter (Fowler et al., 2014). Control of HOXA expression has also been attempted through disruption of the MLL complex, for example by inhibiting the incorporation of WDR5 into the MLL complex which is required for the enzymatic activity of MLL1 (Li et al., 2016; Karatas et al., 2017). These examples represent indirect TF targeting at the epigenetic and PPI levels.

As well as indirect inhibition of TF function, direct inhibition of TF interactions may be an attractive therapeutic approach. Targets in this case may include single TF homodimers, a specific heterodimeric TF pair or a multimeric transcriptional complex. Indeed, TFs have the potential to form a large number of dimeric structures with distinct biological properties (over 500 dimers in human and up to 2500 dimers when considering alternate splicing) that can allow for elaborate fine-tuning of responses (Amoutzias et al., 2008). The concentration of each monomer in the cell, its post-translational modifications and its binding affinity for other monomers all play a role in determining dimer formation and, consequently, will determine which signaling process will dominate. These regulatory mechanisms therefore offer multiple levels of complexity and likely represent an underexploited therapeutic opportunity, as targeting specific TF dimers or specifically modified TFs (e.g., phosphorylated at a specific position) may offer exquisite therapeutic specificity. This may become a viable approach through the identification of TF states that contribute toward disease pathogenesis, especially as TFs have traditionally been considered undruggable.

A good example of this concept is the Myc-Max and Mad-Max heterodimerization system, whereby Max is a ubiquitous protein that can heterodimerize with either Myc or Mad (Grandori et al., 2000; Lüscher, 2001). Similarly, Myc and Mad can only heterodimerize with Max, but not each other. Upon formation of the Myc-Max heterodimer, recruitment of the mSWI/SNF nucleosome remodeling complex or histone acetyltransferases (HATs) occurs at the promoters of target genes, resulting in transcriptional activation. Conversely, formation of the Max-Mad heterodimer leads to recruitment of HDACs, which results in silencing of target genes and is antagonistic to the Myc-Max heterodimer. As a result, variations in the concentrations or affinity of these complexes can lead to a transcriptional bias and potentially alter the oncogenic capacity of the cell. As previously discussed, post-translational modifications may offer an additional level of complexity and can determine the transition to a functional dimeric TF pair. This has been observed through the phosphorylation of STAT proteins (Levy and Darnell, 2002) as well as ReIB, which leads to the formation of p100-ReIB dimers (Maier et al., 2003). Another example is the phosphorylation of the bHLH protein E47, which blocks formation of the homodimer and favors formation of a heterodimer with MyoD, leading to the activation of muscle-specific transcriptional activity (Lluís et al., 2005). It is therefore feasible to suggest that phosphorylated versions of TFs that participate in oncogenic interactions or transcriptional programs may represent attractive therapeutic targets in the future, especially if the phosphorylated protein does not exist or is very rare in healthy tissues.

TARGETING PROTEIN NETWORKS AND CHROMATIN RE-MODELERS

In addition to TFs, it is important to consider the role played by chromatin modulators in driving transformation. Mutated protein members "hijack" remodeling machinery to localize in different areas of the genome, leading to aberrant gene expression. Although TFs in cancer are undoubtedly important, open chromatin is more likely to facilitate gene expression. Therefore, the targeting of PPIs specific to oncogenically activated chromatin modulators may offer a more viable method to silence dysregulated transcription in cancer. One clear example is the BAF or mSWI/SNF complex, where genes encoding subunits or associated proteins are mutated in over 20% of cancers (Pierre and Kadoch, 2017). Such a high frequency of mutations correlated with specific oncogenic phenotypes can be attributed to a high degree of genetic non-redundancy within the complex. An example is SMARCB1 inactivation in early pediatric rhabdoid tumors, which is considered the sole genetic driving event in an otherwise genomically stable malignancy (Wang et al., 2017). Such stability is indicative of epigenetic changes caused by an oncogenically activated BAF complex. The loss of SMARCB1 reduces levels of the BAF complex, impairing normal function and transcriptional homeostasis. Subsequent alteration in genome-wide targeting reduces BAF binding to typical enhancers required for transcription of cell differentiation genes. Instead, remaining SMARCB1-deficient complexes maintain binding at super enhancers, causing preferential transcription of genes required for current cell identity maintenance, which may be due to higher affinity BAF complex binding at these sites. When specific proliferative progenitor cells are affected, cells are effectively locked into a highly proliferative and lowly differentiated state due to impaired enhancer targeting working to drive oncogenic transformation (Wang et al., 2017). Expression of another subunit of the complex, ARID1A, is lost in colon cancer in mice causing a similar reduction in levels of the BAF complex (Mathur et al., 2017). This causes its absence at thousands of enhancers and subsequent reduction/change in gene expression. ARID1B has a similar binding preference and can compensate to some extent by binding in the place of ARID1A, but the presence of this altered complex causes extensive dysregulation of gene expression. This further highlights the importance of complex composition and the non-redundant nature of PPIs within this complex. There are therefore a great many potential targets for therapy within the BAF complex. Indeed, comprehensive understanding of

the relationships between biochemistry and function must be reached in order to unlock their greatest potential.

Another emerging field for PPI targeting is the modulation of the ubiquitin pathway. For example, proteasome-mediated degradation can be biased toward the preferential break down of tumor suppressors and the apparent preservation of oncoproteins in cancer cells (Wertz and Wang, 2019). As the process is a cascade, there are several proteins which offer valuable targets for anticancer therapies. There are three classes of enzymes responsible for ubiquitination, E1, E2, and E3, which comprise 2, 40, and over 600 isozymes in humans respectively (Li et al., 2008; Deshaies and Joazeiro, 2009; Schulman and Wade Harper, 2009). Although it has been possible to modulate the E1 and E2 members of the ubiquitination pathway, E3 enzymes have higher substrate specificity and offer greater potential for specific targeting. One of the most notable E3 PPIs for targeting is the MDM2:p53 interaction, where MDM2 is the negative regulator for p53 and therefore an important oncoprotein. Another method of modulation involving target proteins and E3 enzymes are Proteolysis targeting chimeras (PROTACs) (Sakamoto et al., 2001). PROTACs contain two moieties which independently bind a relevant target protein and an E3 ubiquitin ligase (**Figure 2B**). This brings the target into close proximity for ubiquitination by the E3 enzyme and marks the protein for degradation by the proteasome. This system has the advantage of being able to target proteins such as TFs, as PROTACs require only transient drug-target binding, whilst not inhibiting substrate activity. The ubiquitin pathway therefore offers an attractive therapeutic angle to the targeting of TFs, helping to modulate proteins which are otherwise difficult to mark.

It is clear that the wealth of existing proteomics data needs to be harnessed to address this area, looking at the specific interactions and phosphorylation states of putative TNBC oncogenes in disease contexts versus those observed in healthy tissue. This may take the form of targeted approaches using emerging techniques such as rapid immunoprecipitation mass spectrometry of endogenous proteins (RIME) (Mohammed et al., 2016) or co-immunoprecipitation coupled with mass spectrometry for the identification of PPIs and the analysis of post-translational modifications. However, unbiased and high-throughput approaches to investigate interactions and post-translational modifications in a whole-cell format are still lacking and therefore knowledge of particular TNBC oncoproteins is currently required to take this approach. The emerging field of single cell proteomics may offer the opportunity to perform unbiased screens to correlate particular protein states with cellular phenotypes in the future but, as of yet, high-throughput single-cell proteomics methods are not available for this purpose (Marx, 2019). However, the rate with which the single cell field is progressing bodes well for this technology and no doubt its development will offer unprecedented insight into PPIs driving malignancy. The combination of the above approaches may provide new therapeutic angles for the development of novel, more targeted and more effective TNBC therapies, as well as providing valuable insights into the mechanism of TNBC pathogenesis.

AUTHOR CONTRIBUTIONS

MW wrote the first draft of the manuscript and produced the figures. MW and RU wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

FUNDING

RU is funded by a NC3Rs studentship. MW is funded by a CRUK grant to WK. WK is funded by a CRUK Career Establishment Award (C47525/A17348), CRUK Small Molecule Drug Discovery Project Award (C47525/A25850), Breast Cancer Now Project Grant (2017MayPR907), and University of Cambridge and Magdalene College, Cambridge.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with one of the authors WK.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 22 January 2020
doi: 10.3389/fcell.2019.00395

'Omics Approaches to Explore the Breast Cancer Landscape

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Breast cancer incidence is increasing worldwide with more than 600,000 deaths reported in 2018 alone. In current practice treatment options for breast cancer patients consists of surgery, chemotherapy, radiotherapy or targeting of classical markers of breast cancer subtype: estrogen receptor (ER) and HER2. However, these treatments fail to prevent recurrence and metastasis. Improved understanding of breast cancer and metastasis biology will help uncover novel biomarkers and therapeutic opportunities to improve patient stratification and treatment. We will first provide an overview of current methods and models used to study breast cancer biology, focusing on 2D and 3D cell culture, including organoids, and on *in vivo* models such as the MMTV mouse model and patient-derived xenografts (PDX). Next, genomic, transcriptomic, and proteomic approaches and their integration will be considered in the context of breast cancer susceptibility, breast cancer drivers, and therapeutic response and resistance to treatment. Finally, we will discuss how 'Omics datasets in combination with traditional breast cancer models are useful for generating insights into breast cancer biology, for suggesting individual treatments in precision oncology, and for creating data repositories to undergo further meta-analysis. System biology has the potential to catalyze the next great leap forward in treatment options for breast cancer patients.

Keywords: breast cancer, system biology, proteomics, transcriptomics, genomics, organoids, PDX

OPEN ACCESS

Edited by:
Zuzana Koledova,
Masaryk University, Czechia

Reviewed by:
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University of Dhaka, Bangladesh
Kamalakannan Rajasekaran,
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *30 October 2019*
Accepted: *30 December 2019*
Published: *22 January 2020*

Citation:
Parsons J and Francavilla C (2020) 'Omics Approaches to Explore the Breast Cancer Landscape. Front. Cell Dev. Biol. 7:395. doi: 10.3389/fcell.2019.00395

BREAST CANCER – WHERE ARE WE?

Breast cancer is the leading cause of cancer-related deaths in women worldwide (Bray et al., 2018). It is a heterogeneous disease (Nik-Zainal et al., 2016), commonly separated into Luminal A (LumA), Luminal B (LumB), epidermal growth factor receptor ERBB2/HER2-overexpressing (HER2+), basal epithelial-like (BL) based on gene expression profiles (Sørlie et al., 2001). Breast cancer is currently treated with surgery, radiotherapy, cytotoxic chemotherapy and/or targeted therapies to eradicate viable cancer cells (Fisher et al., 2002).

LumA and LumB breast cancers are both estrogen receptor (ER)-positive (Sørlie et al., 2001). Deregulated ER signaling is associated with cancer hallmarks (Hanahan and Weinberg, 2011). For instance, ER target genes like cyclin-dependent kinase (CDK) 1 or the kinase Src promote

cell proliferation, invasion and epithelial–mesenchymal transition (EMT) (Stender et al., 2007; Saha Roy and Vadlamudi, 2012). LumB cancers have high expression of the proliferation marker Ki67, which correlates with increased risk of developing distant metastases (Colzani et al., 2014), and reduced expression of the progesterone receptor (PR) (Cho, 2016), which shifts gene expression toward more tumorigenic genes (Mohammed et al., 2015). LumA and LumB tumors are treated using ER antagonists (e.g., tamoxifen), aromatase inhibitors and selective estrogen receptor degraders (e.g., fulvestrant). However, therapeutic resistance may arise through loss of ER expression, mutations in ER or overexpression of alternative breast cancer-driving pathways such as ERBB1/EGFR (Garcia-Becerra et al., 2012; Clarke et al., 2015; Ma et al., 2015). To overcome resistance to traditional ER antagonists targeted therapies against phosphoinositide 3-kinases (PI3K), mammalian target of rapamycin (mTOR), and CDK4/6 have recently been proven beneficial in the clinical setting (Beaver and Park, 2012; Kornblum et al., 2018; Pernas et al., 2018).

HER2 + breast cancers overexpress ERBB2/HER2 (Iqbal and Iqbal, 2014) which promotes proliferation by regulating CDKs and Cyclins (Timms et al., 2002). Additionally, HER2 dimerization with EGFR induces activation of mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinases (JNK), and phosphoinositide phospholipase C (PLCγ) signaling pathways resulting in increased cell proliferation, migration and apoptosis resistance (Masuda et al., 2012). HER2 + breast cancers are treated with targeted agents such as trastuzumab, pertuzumab, and neratinib. Trastuzumab is an antibody which inhibits HER2 dimerization, promotes natural killer cell recruitment to tumors and stimulates ubiquitin-dependent HER2 degradation (Vu and Claret, 2012; McCann and Hurvitz, 2018; Schmid et al., 2018; Vikas et al., 2018). Therapeutic resistance to trastuzumab occurs via HER2 dimerization with other ERBB family members or constitutive HER2 activation (Vu and Claret, 2012).

BL breast cancers do not generally express ER, PR or HER2 (Milioli et al., 2017), like triple negative breast cancers (TNBCs) (Lehmann et al., 2016). BLs are highly heterogeneous and include basal-like1-2, claudin-low, and immunomodulatory subgroups (Garrido-Castro et al., 2019). BLs have a highly proliferative and invasive phenotype with high risk of relapse in early breast cancer (Fallahpour et al., 2017). BLs are typically treated by chemotherapy and radiotherapy (Wahba and El-Hadaad, 2015) although recent advances have led to novel treatment opportunities for BL cancer patients. For instance, immunomodulatory BLs can be treated with immune checkpoint programed cell death protein 1 (PD-1) and poly (ADP-ribose) polymerase (PARP) inhibitors (McCann and Hurvitz, 2018; Schmid et al., 2018; Vikas et al., 2018).

Two major challenges in breast cancer treatment are therapeutic resistance and the formation of metastasis to secondary sites (lung, bone, lymph nodes, brain, and liver) inevitably leading to patient mortality (Minn et al., 2005). As 10 year survival for metastatic breast cancer patients remains below 5% (Kontani et al., 2014) and response to targeted therapies varies from 15 to 40% for all subtypes (Bartsch et al., 2007; Haque and Desai, 2019) the need for novel therapeutic options for breast cancer patients remains a priority.

Here, we will describe several models that have contributed to knowledge of breast cancer biology and the repertoire of currently available therapeutic targets. Thereafter, we will introduce system biology-based approaches and finally discuss how their integration with traditional models is revolutionizing breast cancer translational research.

MODELS TO STUDY BREAST CANCER

Cell Lines

Breast cancer has been traditionally studied using immortalized cell lines derived from patient samples (Holliday and Speirs, 2011) which are easy and inexpensive to grow. These cell lines express biomarkers of the different molecular subtypes of breast cancer (Dai et al., 2017) and recapitulate some parent tumor characteristics including drug responses (Holliday and Speirs, 2011) and transcriptomic profiles (Neve et al., 2006). Cell lines have enabled major discoveries in breast cancer research, such as the identification of oncogenes (Elenbaas et al., 2001) and drivers of metastatic tropism (Minn et al., 2005). However, breast cancer cell lines have increased gene copy number variations compared to primary tumors (Larramendy et al., 2000), lack the *in vivo* microenvironment (Vincent et al., 2015), and do not maintain primary tumor heterogeneity (Dai et al., 2017; Liu et al., 2019) (**Figure 1A**).

Organoids

Organoids are three dimensional (3D) cell cultures which mimic healthy tissues and cancer lesions (Xu et al., 2018). Organoids are usually grown in matrices such as Matrigel^{TM}, collagen or peptide hydrogels which aim to recapitulate the breast microenvironment (Djomehri et al., 2019). The group of Mina Bissel in the '80s began to investigate how organoids were a better model for studying breast tissue compared to 2D cell culture (Weaver et al., 1995). More recently, primary and metastatic organoids have been developed which accurately recapitulate parent tumor characteristics including histopathology, genomic abnormalities and drug responses (Sachs et al., 2018). Organoids are easy to modify, can be propagated for up to 3 months (Fatehullah et al., 2016), and allow drug screening (Dutta et al., 2017). Recently, the issue of availability of primary patient samples for laboratories without access to biobanks has been solved by the creation of living biobanks of frozen organoids (Dutta et al., 2017). Organoids can be used as models to study different breast cancer subtypes and to identify potential novel therapeutic targets. Organoid are better models than 2D cultures to analyze drug response due to a more representative microenvironment and selection for stem-like cells, like those responsible for metastatic initiation (Velasco-Velazquez et al., 2011; Imamura et al., 2015). Despite these promising characteristics for breast cancer translational research, organoids lack components of the *in vivo* microenvironment and may suffer for counterselection of hyperproliferative cells (Fujii et al., 2016; Weeber et al., 2017) (**Figure 1B**).

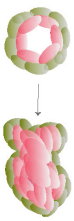
	Model	Advantages	Disadvantages	Major discoveries
A	CELL LINES 	- Cheap and easy to culture	- Increased mutation rate compared to tumours - Clonal population does not represent tumour heterogeneity - Heterogeneity within cell lines used in different labs	- Identification of the role of oncogenes - Identification of drivers of metastatic tropism to bone, brain and lung - Identification of genetic aberrations involved in resistance to targeted therapies
B	ORGANOIDS 	- Similar structures to those seen in the breast (such as milk-producing acini) - Similar histopathology, genomic abnormalities and drug responses of tumours - Suitable for medium throughput drug screens	- Availability of the initial patient samples and variability in the culturing systems among laboratories - Lack of stromal and immune components - Counterselection for hyperproliferative cells	- Creation of living biobank in which tumour tissue can be expanded whilst maintaining genomic and transcriptomic profiles of the original sample - Recapitulation of drug responses - Modelling of breast cancer tissue characteristics
C	GEMMs 	- Presence of natural micro-environment and immune system -Partial recpaitulation of human breast cancer subtypes	-Extensive breeding time and costs - Non-physiological levels of oncogenes - Genetically different from human tumours and rarely mimicking clinical metastases	- Identification of mutations - Identification of cell of origin - Identification of novel drugs combination or mechanisms of resistance
D	PDX 	-Maintainance of the genomic, transcriptomic and proteomic profiles of tumours through multiple passages - Maintainance of metastatic tropisms and heterogeneity of patient tumours - Formation of spontaneous metastasis	- Use of immunocompromised mice to prevent rejection of human cells -The mouse microenvironment may result in the clonal selection of the more aggressive cells of the patient sample	- Creation of a bank of tumour tissue which can be passaged and maintained in different laboratories - Identification of the contribution of heterogeneity to breast cancer progression - Identification of drug combinations to overcome resistance
E	-'OMICS APPROACHES 	- Unbias analysis of the DNA, RNA and protein landscape starting from any sample - Rapid and robust data generation - Creation of data repositories that can be used for other studies or for validation by other researchers	- High costs in terms of sample handling and starting amount, instrumentation and time for data analysis and integration - Poor correlation between -'Omics approaches (e.g. transcriptomics vs. proteomics) - Single cell analysis held great potential, but is still under development	- Identification of potential novel biomarkers, drivers, and therapeutic targets - Identification of specific mutations linked to drug responses - Identification of basal-like subsets

FIGURE 1 | Models and methods to study breast cancer. Summary of the advantages (left column) and disadvantages (middle column) of existing breast cancer models **(A–D)** and 'omics technologies **(E)** to study breast cancer. Right column reports a brief summary of how different methods and models have contributed to major discoveries in the field of breast cancer.

Genetically Engineered Mouse Models (GEMMs) and Syngeneic Mouse Models (SMMs)

In vivo modeling of breast cancers generally entails inducing oncogene expression (e.g., *Erbb2*) or knocking out a tumor suppressor gene (e.g., *p53*) in mice. Examples include the mouse mammary tumor virus (MMTV) promoter-driven or the 4T1-based SMMs (Holen et al., 2017). GEMMs include a natural (mouse) microenvironment and immune system, and partially mimic all human subtypes save luminal cancers (Pfefferle et al., 2013; Holen et al., 2017). However, GEMMs involve extensive costs and breeding time, often express supra-physiological levels of the transgene, and can be genetically different compared to their human counterpart (Pfefferle et al., 2013). Only 16 of the 30 most commonly mutated genes in human breast cancers were found to be mutated in a panel of metastatic GEMMs and SMMs (Yang et al., 2017). Although SMMs have higher mutational burden in metastases than in primaries like human breast cancers (Yang et al., 2017; Yates et al., 2017), GEMMs and SMMs rarely mimic clinical metastasis (Holen et al., 2017). In spite of these pitfalls, GEMMS have been instrumental in generating insights into breast cancer biology – e.g., determining that BRCA1 mutant tumors derive from luminal progenitor rather than basal cells (Molyneux et al., 2010) and in testing novel drugs combinations (Jaspers et al., 2013) (**Figure 1C**).

Patient-Derived Xenografts (PDXs)

Patient-derived xenografts (PDXs), which involve injection of human cancer cells either orthopically in the mouse mammary fat pad or subcutaneously into immunocompromised mice, provide an *in vivo* alternative to GEMMs (Hidalgo et al., 2014; Holen et al., 2017). They have helped address clinically relevant questions including the contribution of heterogeneity to, and the mechanism of, drug resistance (Byrne et al., 2017). PDXs can be passaged in different mice allowing expansion of patient tissue whilst still maintaining 'omics profiles of the patient tumor; and they spontaneously metastasize (DeRose et al., 2011; Dobrolecki et al., 2016). Drawbacks for the use of PDXs include the selection of more aggressive cells within the patient sample and the use of immunocompromised mice to prevent tumor rejection. Developing mice with humanized immune systems can help to address this problem (Hasgur et al., 2016), as recently shown for a metastasis model (Rosato et al., 2018) (**Figure 1D**).

In conclusion, choosing the correct model to study breast cancer depends on several factors including the biomedical question, sample availability, costs, etc. (**Figure 1**). We envision that future interdisciplinary research will be based on a combination of different models to identify and validate new therapeutic targets for breast cancer treatment with the advent of next generation sequencing and more robust instrumentation, 'omics approaches, like genomics and proteomics, are becoming more accessible and are increasing the information that can be obtained from breast cancer models. Thus, 'omics approaches applied to the combination of different models will provide molecular information on a global scale and will identify novel targets.

SYSTEM BIOLOGY APPROACHES TO STUDY BREAST CANCER

System biology based on 'omics approaches and network science are becoming popular in cancer research (Manem et al., 2018), despite high costs in terms of sample handling, instrumentation, and time for data analysis. Integrating 'omics approaches allows the unbiased analysis of the whole genome, transcriptome, proteome, or metabolome starting from different types of samples (**Figure 1E** and **Table 1**).

Genomics

Next generation sequencing (NGS) allows rapid and relatively inexpensive DNA sequencing covering the whole genome (Park and Kim, 2016). Genomic approaches helped redefine breast cancer subtypes (Cancer Genome Atlas Network, 2012), identify mutational landscapes (Stephens et al., 2012) or single nucleotide polymorphisms (SNPs) as a biomarker of breast cancer susceptibility (Michailidou et al., 2017) or therapeutic response (Kus et al., 2016). NGS has also facilitated the discovery of breast cancer driver mutations (Nik-Zainal et al., 2016), tumor heterogeneity (Yates et al., 2015) and novel therapeutic targets in metastatic disease (Bertucci et al., 2019). Finally, single-cell analysis allowed the study of breast cancer stem cells (Lawson et al., 2015). However, accurate genomic analysis requires large numbers of sequence reads which increases both time and cost.

These discoveries demonstrate the potential for genomics to transform breast cancer treatment (Hamdan et al., 2019). For instance, genomics helped identify patients for clinical trials (Curtis et al., 2012) or high risk individuals through mutation screening in breast cancer susceptibility (BRCA) 1–2 genes (Evans et al., 2008) and contributed to therapeutic decision making (Tsoutsou et al., 2017; Bergom et al., 2019). As an invaluable resource for researchers, the Catalogue of Somatic Mutations in Cancer (COSMIC) has compiled genomic data from breast cancer patient samples and correlated them to cellular functions and drug resistance (Forbes et al., 2017). Finally, genomic analysis for the early identification of tailored therapy for cancer patients has been made possible with the development of the Cancer Genome Atlas (TCGA)[1]. We envision that TCGA and COSMIC databases will revolutionize cancer patient diagnosis and treatment (Ashton-Prolla et al., 2015). This is already being realized in the MOSCATO trial where druggable genomic aberrations were identified and targeted in patients (Massard et al., 2017).

In addition, cell-free/circulating tumor DNA (cf/ctDNA) can be useful in monitoring clonal evolution and residual tumor presence following treatment (Buono et al., 2019). However, as ctDNA usually comprises 180–200 bp fragments from apoptotic cells, there are varying degrees of success in identifying useful biomarkers with high sensitivity (Sefrioui et al., 2015). Despite this, serial screening for mutations in ctDNA has allowed metastatic detection 8 months before clinical presentation (Garcia-Murillas et al., 2015).

[1]https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga

TABLE 1 | A selection of single- and multi-'omics-based breast cancer studies that have contributed to major discoveries in the field of breast cancer research where method strengths and weakness are reported.

Study	Topic area	'Omics approaches				Method strengths	Method weaknesses	Major discoveries
		G	T	P	M			
Nik-Zainal et al., 2016	Novel Breast Cancer Drivers	×				The whole genome sequence can be determined relatively cheaply in less than a week	Sequences must be read many times to account for inaccuracies in sequencing analyzers	Five novel cancer genes were identified. A total of 93 genes were suggested to contain breast cancer driver mutations
Playdon et al., 2017	Breast Cancer Risk				×	This technique is dependent on serum samples which are far easier to obtain than biopsies needed for other 'omics techniques	Controlling patient diet is very difficult	Three metabolites were found to be associated with increased breast cancer risk
Varešlija et al., 2018	Novel Therapeutic Targets	×	×			Combining DNA and RNA sequencing allows mutations to be connected to chromatin remodeling and gene expression	RNA integrity is compromised by the process of formalin fixing due to cross-link formation	RET and HER2 were found to be potential therapeutic targets for breast cancer brain metastases
Huang K. L. et al., 2017	Novel Therapeutic Targets	×	×	×		Proteomic isobaric labeling methods allow multiple samples to undergo relative quantification reducing variability	Large amounts of starting protein is required for phospho-proteomics. Also proteomic labeling reagents are very expensive	Novel therapeutic targets previously undiscovered at the genomic, transcriptomic or proteomic level were identified at the level of the phosphoproteome in PDX models
Massard et al., 2017	Informing Clinical Therapeutic Decisions	×	×			When tumor cell population is low in a biopsy, targeted sequencing of known cancer genes can still be used to search for actionable targets without having to purify the epithelial population	Extensive analysis is required to determine if a mutation is actionable. Also biopsies are often sent to pathologists before freezing so the molecular profile may be changed by the time the tissue is frozen	The treatment of 199 patients was based on an actionable genomic alteration which was found using DNA and RNA sequencing In 33% of patients. progression-free survival was significantly increased and in 11% there was objective response
Mertins et al., 2016	Breast Cancer Signaling	×		×		In situations where mutations produce unpredictable consequences, e.g., altering splice variants, proteogenomics can identify single amino acid variants and link these to mutations	Proteins which are missing in one or more replicates of a proteomic experiment are often excluded despite the fact the protein may have been present below the detection threshold	A number of highly phosphorylated kinases were identified that were not seen as potential therapeutic targets at the genomic level. Also the impact of mutations was traced to the signaling level to identify therapeutic targets, e.g., CETN3 loss was associated with EGFR upregulation. highlighting how this loss could be druggable
Johansson et al., 2019	Breast Cancer Subtypes	×	×	×	×	Integrating 'omics technologies allowed the mRNA- based subtypes to be expanded to a more clinically useful resource	Tumors are heterogenous and so 'omics data from one part of a biopsy may not be representative of the whole tumor	Breast cancer subtypes (Sørlie et al., 2001) were validated at a multi-omic level. Basal-like tumors were separated into two clusters that could inform therapeutic decisions

G, genomics; T, transcriptomics; P, proteomics; M, metabolomics.

Together with genomics, epigenomics (the study of DNA modifications and their impact) is also providing novel markers for breast cancer prognosis (Davalos et al., 2017) and for detection of metastasis (Legendre et al., 2015). Epigenomics has begun to illuminate the link between menopause and lifestyle factors with breast cancer risk and so may provide prognostic utility in future (Crujeiras et al., 2017).

Transcriptomics

Transcriptomics uses microarrays, which quantify a set of predetermined sequences, and RNA sequencing (RNA-Seq), which uses high-throughput sequencing to capture all sequences to determine the quantity of a transcript (Lowe et al., 2017). These approaches have been used to classify breast cancer molecular subtypes in cell lines (Neve et al., 2006) and patient-derived samples (Wu et al., 2018; Jiang et al., 2019), to compare primary breast cancers and their metastases (Varešlija et al., 2018), and to visualize phenotypic features of breast cancer cells in 3D culture (Tirier et al., 2019). In addition, transcriptomics is allowing immune cell characterization in normal breast and tumor tissue (Chung et al., 2017; Azizi et al., 2018), potentially providing a mechanism to inform immunotherapeutic decisions.

As transcriptomics does not provide information on the expression, post-translational modifications (PTMs), or activation status of proteins it is less informative than proteomics for novel therapeutic target discovery. Recent advancements in

single cell analysis may open a new era in breast cancer research to identify drivers, biomarkers, and novel therapeutic targets (Hong et al., 2019).

In the clinic, analysis of mRNA expression of gene subsets, involved in ER signaling, HER2 signaling, proliferation and invasion, is already used to predict relapse and determine whether patients would benefit from neoadjuvant chemotherapy (Vieira and Schmitt, 2018). Furthermore, as patients with elevated expression of a migratory mRNA signature had worse overall survival than those with a proliferative mRNA signature and so responded significantly better to chemotherapeutics that targeted the cytoskeleton (Nair et al., 2019) transcriptomics has the potential to inform chemotherapeutic decisions in future.

As patient tumor biopsies are typically formalin fixed and paraffin-embedded (FFPE), a preservation procedure that reduces RNA integrity (von Ahlfen et al., 2007), fresh frozen tissue collection should become the standard procedure for mRNA expression to inform clinical decisions.

Proteomics

Proteomics studies the expressed proteome and its PTMs by mass spectrometry (MS), protein microarrays, and, more recently, mass cytometry. Advances in samples handling, instrumentation, and data analysis now provide unprecedented insights into the abundance and function of the (modified) proteome (Doll et al., 2019). Proteomics can assess tissue or blood samples, thus lending itself to clinical applications (Mardamshina and Geiger, 2017). For instance, specific serum biomarkers have been discovered by proteomic studies (Li et al., 2002; Raso et al., 2012), potentially providing an early diagnosis signature (Saadatmand et al., 2015). Correlation between RNA or gene copy number with protein expression is rather low (Mertins et al., 2016; Johansson et al., 2019) thus analyzing the patient proteome holds promise for identifying novel preventative or therapeutic targets not previously identified at the genomic or transcriptomic level. This idea is supported by the fact that currently used anti-breast cancer drugs predominantly act against proteins.

MS-based proteomics has been used to characterize cell lines (Huang F. K. et al., 2017), to reveal novel layers of breast cancer classification (Tyanova et al., 2016; Yanovich et al., 2018), and to identify proteins involved in drug resistance (Liu et al., 2006). Furthermore, phosphoproteomics that identify phosphorylated proteins (von Stechow et al., 2015) has been used to connect somatic mutations to signaling (proteogenomics) (Mertins et al., 2016), to identify kinases signatures in TNBC (Zagorac et al., 2018), and to map drug targets for personalized treatments (Pierobon et al., 2018). These discoveries have diagnostic and prognostic potential which is worth further exploring and implementing in the clinic when phosphoproteomics methods will become common practice.

An alternative to MS-based proteomics is provided by mass cytometry where single cells are probed with metal ion-labeled antibodies and then samples are analyzed by time-of-flight mass spectrometry (Leelatian et al., 2017). In breast cancer research this technology has been recently used to identify cell types and immune infiltrates within a tumor (Wagner et al., 2019). However, this method remains limited by antibody availability. Similarly to transcriptomics, phosphoproteomics is also limited by the availability of fresh frozen tissue as the phosphoproteome is substantially altered by FFPE preservation (Wakabayashi et al., 2014).

In conclusion, analyzing the proteome and phosphoproteome of patients at different breast cancer stages will help identify signatures for personalized treatments, ideally starting from liquid biopsies. In future proteomics may be used to follow the response to treatment by analyzing changes in patient proteome so to adapt the therapeutic plan.

Metabolomics

Metabolomics is the system-wide identification of endogenous metabolites from bodily fluids in a targeted or unbiased manner (Silva et al., 2019). Metabolomics has been used to correlate changes in metabolism with proliferation rate in breast cancer cells (Jerby et al., 2012), to cluster tumor subtypes (Haukaas et al., 2016), to analyze the lipids content in breast cancer cells (Lisa et al., 2017), and to correlate nutrients with breast cancer risk (Playdon et al., 2017). More recently, this approach has begun paving the way for the identification of metabolic-state specific biomarkers for breast cancer diagnosis (Jasbi et al., 2019). Therefore, metabolomics will allow further insights into correlation between metabolism, epigenomic and proteomic alterations and breast cancer progression or treatment.

Data Integration

The contribution of each aforementioned 'omics technology to the understanding of breast cancer biology and to the discovery of novel targets or biomarkers has been substantial. Integrating these approaches is predicted to be even more powerful (Chakraborty et al., 2018; Manem et al., 2018) (**Table 1**) For instance, a genomic/transcriptomic/proteomic combined approach has confirmed the existence of the known molecular subtypes (LumA, LumB, HER2+, and BL) of breast cancer (Cancer Genome Atlas Network, 2012) as well as allowing identification of novel therapeutic targets in PDX models (Huang K. L. et al., 2017). Recently, a comprehensive analysis of clinical, genomics, and transcriptomics data has uncovered the TNBC landscape (Jiang et al., 2019). Proteogenomics has challenged the way in which somatic mutations contribute to signaling changes (Mertins et al., 2016), highlighting the need of both these analyses to confirm the therapeutic importance of a genetic alteration. For instance, patients lacking HER2 amplification were found to have enriched HER2 signaling (Pierobon et al., 2018), underlining the importance of analyzing changes in signaling to plan the correct therapeutic approach. With the development of single cell analysis in genomics, transcriptomics and proteomics (Linnarsson and Teichmann, 2016; Hong et al., 2019; Marx, 2019; Wagner et al., 2019) there are opportunities to better understand breast cancer heterogeneity and the role of the microenvironment. Finally, it would be fascinating to integrate 'omics approaches with radiomics (quantitative information from digital images) (Pinker et al., 2018) and with imaging-based mass spectrometry that is rapidly changing the field of spatial proteomics (Keren et al., 2018) to guide patient-specific therapy or patient stratification.

TABLE 2 | A selection of 'omics data repositories built for data sharing and to support research questions (Bamford et al., 2004; Fontaine et al., 2011; Omenn, 2014; Speake et al., 2015; Tomczak et al., 2015; Clough and Barrett, 2016; Rudnick et al., 2016; Chou et al., 2019; Tate et al., 2019).

Database	'Omics data					Additional information	References
	G	T	P	M	E		
Catalogue of Somatic Mutations in Cancer (COSMIC)	×	×			×	COSMIC contains data from over 13 million tumor samples, identifying 6 million coding mutations and over 19 million non-coding mutations. This resource collates all genes implicated in cancer through somatic mutation, of which 719 are currently listed.	Bamford et al., 2004; Tate et al., 2019
The Cancer Genome Atlas (TCGA)	×	×	×		×	TCGA contains multi omic data for 30 different tumor types. In regards to breast cancer it has enabled confirmation of the existence of the four main breast cancer subtypes, it has identified several novel breast cancer drivers and it has identified potentially druggable novel targets.	Tomczak et al., 2015
Clinical Proteomic Tumor Analysis Consortium (CPTAC)			×			CPTAC contains mass spectrometry-based proteomic analysis of tumors from TCGA. The aim of CPTAC is to create a proteogenomic resource where dysregulated proteins and phosphorylation sites can be identified and potentially connected to genomic alterations.	Rudnick et al., 2016
Proteomics Identification Database (PRIDE)			×			PRIDE aims to be a resource for open access sharing of mass spectrometry data, not just across cancer. They currently have over 9200 datasets available, including 297 breast cancer datasets.	Jones et al., 2006
GENIE	×					GENIE combines genomic and clinical data in an attempt to associate genomic alterations with phenotypic changes	Fontaine et al., 2011
GXB		×				GXB compiles immunological transcriptomic data	Speake et al., 2015
Genomic Expression Omnibus (GEO)	×	×			×	GEO is a database of transcriptomic and epigenomic data	Clough and Barrett, 2016
Human Proteome Organization (HUPO)			×			The human proteome project, run by HUPO aims to identify all the proteins in the human proteome and to begin to assess their functionalities and interactions	Omenn, 2014
Transciptome Alterations in Cancer Omnibus (TACCO)		×				TACCO is a resource for identifying differentially regulated transcripts within different cancer types and combining these with survival data to determine prognosis based ongene expression profiles	Chou et al., 2019

G, genomics; T, transcriptomics; P, proteomics; M, metabolomics; E, epigenomics.

'OMICS APPROACHES APPLIED TO EXISTING BREAST CANCER MODELS

Integrating 'omics approaches with traditional methods has already helped underline the validity of some of the models, for example, highlighting that omics profiles are maintained in PDX models through multiple passages (Zhang et al., 2013). Multiomics technologies have also facilitated novel discoveries in existing models (Chakraborty et al., 2018). A combination of genomics, transcriptomics and proteomics has elucidated drivers of mesenchymal-to-epithelial transition in 2D culture (Bhatia et al., 2019). Transcriptomics in GEMM and SMM-derived cell lines allowed identification of differentially regulated genes and their contribution to metastases (Yang et al., 2017) Transcriptomics and proteogenomics in PDXs have finally helped to profile gene/proteins expression to identify novel targets (Huang K. L. et al., 2017).

'Omics technologies have not only improved the power of traditional models in breast cancer research, but also revolutionized the analysis of patient samples, making them an indispensble tool in translational studies. Integration of 'omics approaches requires powerful computational and statistical methods to analyze and interpret the vast quantity of available data, for instance combining linear mathematical models with machine learning and network science principles (Manem et al., 2018). This requires collaboration between cancer scientists, computational biologists and medical statisticians to create robust methods to gain insights into cancer biology and to inform clinical trials and personalized therapeutic regimes.

CONCLUSION AND PERSPECTIVES

With 'omics technologies applied to patient samples becoming robust, our understanding of the mechanisms driving breast cancer and the discovery of novel biomarkers and therapeutic targets have improved significantly over the last few years (Chakraborty et al., 2018; Manem et al., 2018). For instance, the use of molecular assays, including OncotypeDx and MammaPrint in the clinic is based on advancements in genomic technologies (Gupta et al., 2015; Vieira and Schmitt, 2018). Transparent sharing of 'omics data in databases like COSMIC (Forbes et al., 2008), PRIDE (Jones et al., 2006) and others (**Table 2**) will allow unbiased analysis of available data by different groups to find previously unnoticed potential genes or proteins of interest as biomarkers or therapeutic targets.

The implementation of 'omics approaches in clinical practice will allow analysis of changes in patients at a global level by improving diagnosis and choice of therapeutic plan so far based on a few markers. We predict that 'omics technologies-guided biomarker identification will allow early tumor detection so that treatments can start earlier and that the identification of novel

targets will decrease reliance on non-targeted therapies, thus improving the quality of life for breast cancer patients.

AUTHOR CONTRIBUTIONS

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

FUNDING

Research in the lab of CF was supported by Wellcome Trust (Sir Henry Dale Fellowship grant number 107636/Z/15/Z) and by the Biotechnology and Biological Sciences Research Council (BBSRC responsive mode project grant number BB/R015864/1). JP was supported by R-UK Non-Clinical Training Award – 2018 grant number A27445.

ACKNOWLEDGMENTS

We thank all members of the Francavilla lab and Dr. Bruno Simoes, Prof. Robert Clarke (The University of Manchester) and Dr. Ciara O'Brien (The Christie Hospital NHS Foundation Trust and The University of Manchester) for helpful discussion and for reading the manuscript. We also thank members of the Bio-MS facility, The University of Manchester. We apologize to authors whose work could not be cited due to space limitations.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 31 January 2020
doi: 10.3389/fcell.2020.00025

Aberrant WNT/CTNNB1 Signaling as a Therapeutic Target in Human Breast Cancer: Weighing the Evidence

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WNT signaling is crucial for tissue morphogenesis during development in all multicellular animals. After birth, WNT/CTNNB1 responsive stem cells are responsible for tissue homeostasis in various organs and hyperactive WNT/CTNNB1 signaling is observed in many different human cancers. The first link between WNT signaling and breast cancer was established almost 40 years ago, when *Wnt1* was identified as a proto-oncogene capable of driving mammary tumor formation in mice. Since that discovery, there has been a dedicated search for aberrant WNT signaling in human breast cancer. However, much debate and controversy persist regarding the importance of WNT signaling for the initiation, progression or maintenance of different breast cancer subtypes. As the first drugs designed to block functional WNT signaling have entered clinical trials, many questions about the role of aberrant WNT signaling in human breast cancer remain. Here, we discuss three major research gaps in this area. First, we still lack a basic understanding of the function of WNT signaling in normal human breast development and physiology. Second, the overall extent and precise effect of (epi)genetic changes affecting the WNT pathway in different breast cancer subtypes are still unknown. Which underlying molecular and cell biological mechanisms are disrupted as a result also awaits further scrutiny. Third, we survey the current status of targeted therapeutics that are aimed at interfering with the WNT pathway in breast cancer patients and highlight the importance and complexity of selecting the subset of patients that may benefit from treatment.

Keywords: canonical Wnt signaling, non-canonical Wnt signaling, beta-catenin, breast cancer, mammary gland, stem cells, cancer stem cells

OPEN ACCESS

Edited by:
Vida Vafaizadeh, University of Basel, Switzerland

Reviewed by:
Jeffrey M. Rosen, Baylor College of Medicine, United States
Caroline Alexander, University of Wisconsin–Madison, United States

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Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: *30 September 2019*
Accepted: *14 January 2020*
Published: *31 January 2020*

Citation:
van Schie EH and van Amerongen R (2020) Aberrant WNT/CTNNB1 Signaling as a Therapeutic Target in Human Breast Cancer: Weighing the Evidence. Front. Cell Dev. Biol. 8:25. doi: 10.3389/fcell.2020.00025

INTRODUCTION

WNT proteins and their downstream effectors form a highly conserved signaling network that regulates tissue morphogenesis during development and adult tissue homeostasis in virtually all multicellular animals studied to date (van Amerongen and Nusse, 2009; Loh et al., 2016; Schenkelaars et al., 2017). The mammalian genome contains 19 *WNT* genes, encoding 19 different WNT proteins. These can bind and activate 10 different FZD receptors and a handful of co-receptors, thereby initiating different intracellular signaling cascades. 'Canonical' WNT signaling is defined by its use of β-catenin (CTNNB1) as main downstream effector and transcriptional co-activator of TCF/LEF target gene expression (MacDonald et al., 2009; Clevers and Nusse, 2012; Nusse and Clevers, 2017). 'Non-canonical' WNT signaling responses

do not use CTNNB1, but instead activate different signaling molecules with profound impact on the cytoskeleton and cell migration (Komiya and Habas, 2008; van Amerongen, 2012; VanderVorst et al., 2018).

For both historic and experimental reasons, the intestinal epithelium has become the benchmark against which all other tissues are weighed when it comes to WNT signaling. This has shaped both our thinking and our terminology, with the intestine frequently being referred to as the "typical" example. A large body of literature shows that stem cell self-renewal and differentiation in the intestine and other endodermal derivatives is critically dependent on WNT/CTNNB1 signaling (Sato et al., 2009; Barker et al., 2010; Huch et al., 2013a,b; Clevers et al., 2014; Clevers, 2016). Hyperactive WNT/CTNNB1 signaling is a hallmark of colorectal cancer, both in early stages of polyp formation and at later stages of invasion and metastasis (Zhang and Shay, 2017). In this context, increased WNT/CTNNB1 signaling mainly results from genetic mutations in the *APC* gene, which encodes a negative regulator of CTNNB1 (Fodde, 2002). The unambiguous genetic evidence from human tumors leaves little doubt about the relevance of aberrant WNT/CTNNB1 signaling in the initiation and progression of colorectal cancer.

The involvement of WNT signaling in breast cancer remains less well understood (Yu et al., 2016; Alexander, 2018). This is surprising, given that the link between WNT signaling and breast cancer is as old as the WNT research field itself (Nusse and Varmus, 2012). In fact, the first mammalian WNT gene (*Wnt1*, originally identified as *int-1*) was discovered as a proto-oncogene capable of driving mammary tumor formation in mice (Nusse and Varmus, 1982). Here we review the evidence, highlight current research gaps and indicate future avenues worth exploring to dissect the role of WNT signaling in human breast cancer.

HOW IMPORTANT IS WNT SIGNALING FOR DEVELOPMENT AND MAINTENANCE OF THE HUMAN BREAST?

A first major knowledge gap is our lack of a basic understanding of the role of WNT signaling in human breast development and physiology. The mammary gland largely develops after birth and undergoes dynamic tissue remodeling throughout life. The most prominent changes occur in puberty (when the breast tissue develops under the influence of rising levels of estrogen and progesterone), and during pregnancy and lactation (when it differentiates and produces milk to nurture the offspring). Given how critical this tissue has been for our survival as a mammalian species and in view of the prevalence and mortality of breast cancer across different societies in women worldwide, it remains somewhat strange that we still have an incomplete picture of the molecular, cell and tissue biology of the human breast. In fact, one of the most detailed studies of human breast development, and individual variation therein, arguably dates back to 1840[1].

[1]https://jdc.jefferson.edu/cooper/

Most of what we know about WNT signaling in mammary gland biology and breast cancer comes from studies in mice, where both CTNNB1-dependent and -independent signaling are essential for mammary gland development, branching morphogenesis and function during embryogenesis and in postnatal life (Brisken et al., 2000; Chu et al., 2004; Veltmaat et al., 2004; Badders et al., 2009; Roarty et al., 2015; Yu et al., 2016). The mouse was discovered as a useful organism for studying the link between hormones and breast cancer well over a century ago (Lathrop and Loeb, 1916), but it really came to the fore as an experimental model system with the discovery of the fat pad transplantation assay (Deome et al., 1959). This technique remains indispensable for studying the growth, differentiation and regenerative properties of different mammary epithelial cell populations (Faraldo et al., 2015; Wronski et al., 2015). Nowadays, robust protocols allow the prospective isolation of mammary stem cells (capable of forming a new epithelial network upon transplantation) via fluorescence activated cell-sorting (FACS) (Shackleton et al., 2006; Stingl et al., 2006; Prater et al., 2013; Gao et al., 2016). More recently, genetically engineered mouse models have allowed sophisticated lineage tracing approaches, which have been instrumental for studying mammary stem and progenitor cell behavior *in situ* (van Amerongen, 2015; van de Moosdijk et al., 2017).

Multiple efforts have been made to delineate the mouse mammary epithelial cell hierarchy. The cumulative lineage tracing literature suggests that postnatal mammary gland development, homeostasis and remodeling are mainly driven by unipotent basal and luminal stem cells (Van Keymeulen et al., 2011; Davis et al., 2016; Wuidart et al., 2016, 2018; Scheele et al., 2017), although a rare fraction of bipotent stem cells likely co-exists (Wang et al., 2015). At least some mammary stem cells are WNT/CTNNB1 responsive (Zeng and Nusse, 2010; De Visser et al., 2012; van Amerongen et al., 2012a; Plaks et al., 2013; Wang et al., 2015; Blaas et al., 2016). However, this does not automatically imply that homeostasis and remodeling of the mammary epithelium is as strictly controlled by WNT/CTNNB1 responsive stem cells as appears to be the case for the intestinal epithelium. Moreover, stem cell plasticity can be induced by transplantation (Van Keymeulen et al., 2011; van Amerongen et al., 2012a) or oncogenic mutations (Koren et al., 2015; Van Keymeulen et al., 2015), raising the question if mammary stem and progenitor cells should be forced into a rigid hierarchy to begin with.

How findings from the mouse translate to the human breast remains unclear. In both human and mouse, the mammary gland is comprised of a non-stereotypically branched, ductal network composed of a bilayer of basal and luminal epithelial cells. Yet neither the two tissues, nor the experimental systems available to study each of them, are directly comparable between the two species. Major differences exist in the composition of the stroma, with the mouse mammary gland containing a higher proportion of adipocytes (hence the name 'fat pad' for the stromal pocket into which cells can be transplanted) and the human breast containing considerably more collagen. This constitutes a different molecular signaling environment with very different mechanobiological properties.

Breast tissue composition changes throughout life and varies between individual women (Sun et al., 2014). Prominent differences in the expression pattern of epithelial cell markers between mouse and human also exist, although these are frequently ignored. For example, KRT14 reliably marks basal cells in the mouse mammary gland but is also expressed in a fraction of luminal cells in the human breast (Santagata et al., 2014; Dontu and Ince, 2015; McNally and Stein, 2017; Gerdur Ísberg et al., 2019).

Unlike in mice, human stem cell activity cannot be readily visualized *in vivo*. Unraveling the stem and progenitor cell hierarchy in the breast has thus proven difficult, but a recent study managed to use Cytochrome C Oxidase deficiency to identify multi-lineage differentiation in the healthy breast, presumably from stem cells in the luminal layer (Cereser et al., 2018). Experimental systems to study self-renewal and differentiation of human breast epithelial cells are limited to *in vitro* cell culture assays. Primary mammosphere cultures (in which cells are grown in suspension to enrich for cells with self-renewal properties) are frequently used to evaluate human breast stem cell activity (Shaw et al., 2012). However, this link is indirect and may not reflect the *in vivo* situation.

Access to healthy human breast tissue for experimental purposes is usually restricted to the leftover material from breast reduction surgeries. FACS protocols have been developed to isolate different cell populations from these specimens, including an ALDH + population with stem/progenitor cell properties as evaluated by multi-lineage differentiation in a 2D clonogenic colony formation assay (Ginestier et al., 2007). Transcriptional profiling of these cells revealed that they express high levels of *WNT2* and *RSPO3*, suggesting an autocrine source of ligands and agonists (Colacino et al., 2018). Mammosphere cultures are typically maintained in the absence of exogenous WNT proteins, but cells in these cultures do express *FZD2* (Shaw et al., 2012). Although primary human mammosphere cultures appear to be relatively insensitive to DKK1-mediated inhibition of WNT signaling (Lamb et al., 2013), multiple *WNT* genes can be induced in these cultures upon stimulation with estrogen or progesterone (Arendt et al., 2014). Comparative transcriptional profiling between mouse and human epithelial cells suggests that active WNT/CTNNB1 signaling in the basal cell population is conserved between the two species (Lim et al., 2010) and long-term maintenance of primary human as well as mouse mammary epithelial cells in Matrigel has been reported in the presence of WNT3A-containing media (Zeng and Nusse, 2010; Sachs et al., 2018).

Summarizing, the human breast likely also uses WNT signaling for growth and differentiation. However, the WNT-secreting and WNT-responsive cells have not been clearly demarcated. Single cell RNA sequencing studies will likely shed more light on the stem and progenitor cell hierarchy in the healthy human breast, and on the position of WNT/CTNNB1 signaling in this hierarchy, in the foreseeable future (Holliday and Speirs, 2011). If and how CTNNB1-dependent and –independent signaling functionally controls proliferation, differentiation and branching morphogenesis of primary human breast epithelial cells is something that can likely only be answered using primary 3D organotypic cultures (Linnemann et al., 2015, 2017).

IS WNT SIGNALING DEREGULATED IN HUMAN BREAST CANCERS?

A second research gap is the lack of specific markers to reliably measure WNT signaling activity in human breast cancer. CTNNB1-independent signaling responses are notorious for their lack of robust readouts in most mammalian cells and tissues. For CTNNB1-dependent signaling, such readouts are available: Reporter constructs with concatemerized TCF/LEF binding sites can be introduced into cells and patient derived xenografts to measure WNT/CTNNB1 signaling (Green et al., 2013; Many and Brown, 2014). However, this approach is unsuitable for monitoring pathway activity in histological specimens, nor does it probe multifactorial signaling in the endogenous chromatin context (Nakamura et al., 2016; Doumpas et al., 2019).

Two of the earliest described WNT/CTNNB1 target genes are *CCND1* and *MYC* (He et al., 1998; Shtutman et al., 1999). Elevated protein levels of CCND1 and MYC are detected in a high proportion of invasive ductal breast carcinomas, but this does not always correlate to CTNNB1 expression levels (Wong et al., 2002; He et al., 2014). Given their general involvement in cell proliferation, upregulation of *CCND1* and *MYC* can be achieved in myriad ways (Lindqvist et al., 2014). So far, *AXIN2* appears to be one of the few universal target genes that could be used to reliably measure relative WNT/CTNNB1 signaling activity in human breast cancer (Lustig et al., 2001; Jho et al., 2002).

In the absence of a well-defined, mammary-specific WNT/CTNNB1 target gene expression program and given the preponderance of paraffin embedded tumor specimens, immunohistochemical detection of CTNNB1 protein levels has been used as the most direct way to readout WNT/CTNNB1 signaling. From these analyses it has been known for a long time that elevated intracellular levels of CTNNB1, a hallmark of active WNT/CTNNB1 signaling, can be detected by immunohistochemistry in a significant (13–77%) proportion of all ductal and lobular breast cancer samples (Jonsson et al., 2000; Karayiannakis et al., 2001; Wong et al., 2002; Ozaki et al., 2005; Prasad et al., 2008a; He et al., 2014; Hou et al., 2018). Care should be taken when performing and interpreting these experiments: Dogma dictates that active WNT/CTNNB1 signaling results in increased nuclear CTNNB1 levels, but those with more hands on experience in the field know that changes in CTNNB1 can be quite subtle and even modest (2–5 fold) increases in the levels of intracellular CTNNB1 can be more than sufficient to robustly activate TCF/LEF target gene expression (Jacobsen et al., 2016).

Clinical evidence suggests that WNT/CTNNB1 signaling is elevated across multiple subtypes of human breast cancer. Aggressive triple negative breast carcinomas (TNBC) were found to be enriched for elevated CTNNB1 levels compared to luminal A, luminal B or HER2+ tumors (Khramtsov et al., 2010). Higher levels of intracellular CTNNB1 are associated with a higher tumor grade (Sormunen et al., 1999) and poor prognosis (Lin et al., 2000; Khramtsov et al., 2010). The highest levels of CTNNB1 are found

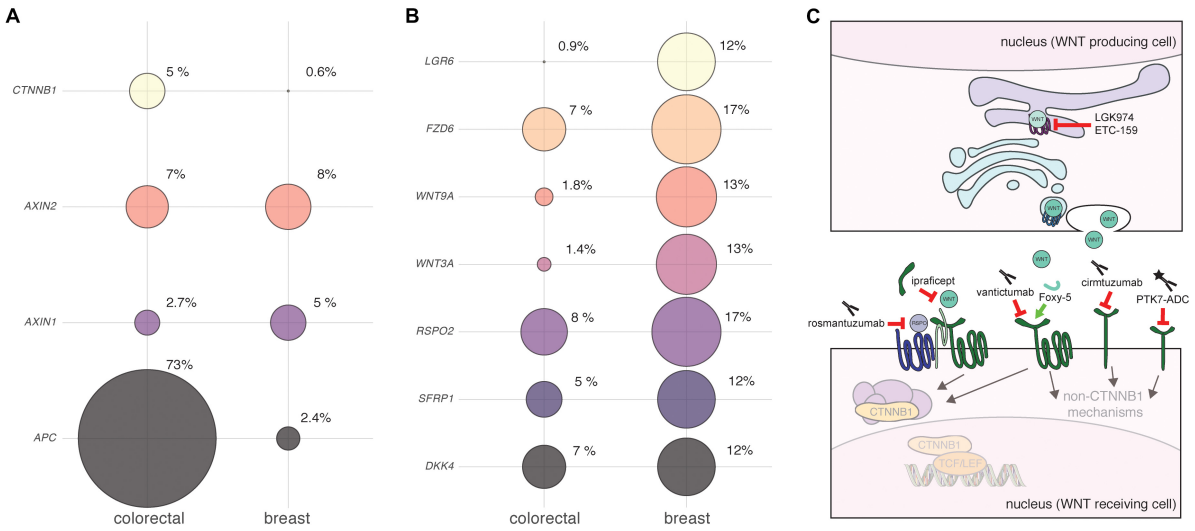

FIGURE 1 | Detecting and targeting aberrant WNT signaling in human breast cancer. **(A,B)** Bubble plots illustrating the alteration of different WNT pathway components in breast versus colorectal cancer. Plots were generated using data from http://cbioportal.org (accessed on 20 September 2019), using the following datasets: Colorectal Adenocarcinoma (TCGA, Provisional), samples with mutation and copy number alteration data (220 patients/samples). Breast Invasive Carcinoma (TCGA, Provisional), samples with mutation and copy number alteration data (963 patients/samples). Circle sizes reflect the proportion of samples with alterations in each of the genes depicted, with the actual percentages shown. Note that copy number alterations (amplifications + deletions) and mutations (truncations + substitutions) were combined into a single score. No distinction was made between breast cancer subtypes. Data were not corrected for overall differences in mutation rates or genome instability between the different tumor types. No inference can be made about RNA and protein expression level changes based solely on these analyses. **(A)** *APC* is the most prominently mutated gene in colorectal cancer. Other endodermal cancers, including hepatocellular carcinoma, also show frequent genetic mutations in WNT/CTNNB1 signaling components (White et al., 2012). Depending on the tissue of origin and tumor subtype, activating mutations in *CTNNB1* itself or inactivating mutations in negative regulators like *APC* or *AXIN1* are more or less prevalent (Yanagisawa et al., 1992; Morin, 1997; Ishizaki et al., 2004). In breast cancer, genetic mutations in *APC* are rare. However, epigenetic changes such as *APC* promoter hypermethylation have been reported in the literature, with the highest incidence observed in inflammatory breast cancer (Jin et al., 2001; Van Der Auwera et al., 2008; Lindqvist et al., 2014). **(B)** The top genes that show genetic alterations in breast cancer are implicated to a lesser extent in colorectal cancer. Note that all of these components function at the level of ligand and receptor binding. The top two hits, *RSPO2* and *FZD6*, have both been linked to reduced metastasis free survival, but likely operate via different WNT signaling mechanisms (Corda et al., 2017; Coussy et al., 2017). It should be stressed that in this respect, breast cancer is not unique. As more and more genome-wide expression profiling studies are becoming available, evidence is accumulating that many different cancers likely display changes in WNT/CTNNB1 signaling in the absence of mutations in *APC* or *CTNNB1* (Wiese et al., 2018; Flanagan et al., 2019b). In addition, it was recently demonstrated that FZD7, which functions upstream of APC and CTNNB1, is required for WNT/CTNNB1 signaling in gastric tumors irrespective of their *APC* status (Flanagan et al., 2019a). This is reminiscent of earlier studies hinting toward a similar phenomenon for other upstream components (Suzuki et al., 2004). Even in colorectal cancer, the situation may thus be far more complex than envisioned, and the local niche may continue to affect signaling levels even when the WNT/CTNNB1 pathway is intrinsically activated through genetic mutations in *APC* (van Neerven and Vermeulen, 2019). **(C)** Cartoon showing the points of interception for WNT-pathway targeting drugs that are currently in clinical trials. See text for details.

in metaplastic carcinomas and non-metastasizing fibromatosis – two rare subsets of breast cancer (Lacroix-Triki et al., 2010). Here, up to 90% of tumors show increased levels of CTNNB1 and a proportion of these may contain activating genetic mutations in the *CTNNB1* gene (Abraham et al., 2002; Hayes et al., 2008; Hennessy et al., 2009). For the most part however, and unlike the situation encountered in colorectal cancer, genetic mutations in *APC*, *AXIN* or *CTNNB1* are virtually non-existent in human breast tumors (**Figure 1A**). As first proposed many years ago, this discrepancy can likely be explained by tissue-specific differences in sensitivity to WNT/CTNNB1 signaling (Gaspar and Fodde, 2004; Gaspar et al., 2009).

In the absence of any apparent genetic mutations, what then is the cause of elevated CTNNB1 levels in human breast cancer? In the normal human breast, CTNNB1 is mainly detected in the cell membrane as part of adherens junctions (Hashizume et al., 1996). It cannot be excluded that the increase in CTNNB1 could therefore, at least partially, be due to its release from these junctions upon loss of CDH1, given that this is a frequent event in more advanced and invasive tumors (Prasad et al., 2008b; Zeljko et al., 2011). However, another possibility is that CTNNB1 levels are increased as a direct result of enhanced WNT/CTNNB1 signaling due to changes in the expression levels of upstream WNT pathway components. In large public breast cancer datasets, changes at the level of ligands, (ant)agonists and receptors are readily apparent (**Figure 1B**). Moreover, the cumulative literature provides ample evidence of changes in the levels of ligands and receptors in primary or metastatic human breast cancer (**Table 1**). In interpreting these findings, some caution is warranted. First, few of the RNA expression level changes have been shown to affect protein levels. Second, where such follow up is performed, antibody specificity has not always been properly validated.

Since absolutely no inference about cell biological mechanisms can be made solely based on expression level changes, functional follow up is crucial to determine the implications of these

TABLE 1 | Comprehensive overview of ligand (*WNT1-16)* and receptor (*FZD1-10, LRP5-6, ROR1-2, RYK, PTK)* genes and their implication in human breast cancer based on a survey of the primary literature.

Gene	Mechanism*		Drug**	Gene expression changes detected at the level of		Reference
	CTNNB1	*other*		*RNA*	*protein*	
WNT1	X	?	PORCN$_i$	0		Corda et al., 2017
				0		Milovanovic et al., 2004
				0		Watanabe et al., 2004
				+		Ayyanan et al., 2006
				+		Ain et al., 2011
					+	Wong et al., 2002
WNT2	X	?	PORCN$_i$	+		Dale et al., 1996
				+		Ellsworth et al., 2009
				+		Huguet et al., 1994
				+		Katoh, 2001
				+		Watanabe et al., 2004
WNT2B	X	?	PORCN$_i$	n.a.	n.a.	n.a.
WNT3	X	?	PORCN$_i$	0		Huguet et al., 1994
WNT3A	X	?	PORCN$_i$	n.d.		Huguet et al., 1994
				0		Corda et al., 2017
WNT4	X	X	PORCN$_i$	+		Ayyanan et al., 2006
				+		Huguet et al., 1994
				+		Tsai et al., 2015
WNT5A	X	X	PORCN$_i$ Foxy-5	–	–	Borcherding et al., 2015
					–	Dejmek et al., 2005
					–	Jönsson et al., 2002
				–		Martin et al., 2005
				–		Trifa et al., 2013
					–	Zhong et al., 2016
				+		Iozzo et al., 1995
				+		Lejeune et al., 1995
WNT5B	X	?	PORCN$_i$	+		Corda et al., 2017
				+		Klemm et al., 2011
WNT6	X	?	PORCN$_i$	0		Milovanovic et al., 2004
				+		Ain et al., 2011
WNT7A	X	?	PORCN$_i$	n.d.		Huguet et al., 1994
				+		Avgustinova et al., 2016
				–		Yi et al., 2017
WNT7B	X	?	PORCN$_i$	–		Milovanovic et al., 2004
				+		Huguet et al., 1994
				+		Yeo et al., 2014
WNT8A	?	?	PORCN$_i$	n.a.	n.a.	n.a.
WNT8B	?	?	PORCN$_i$	n.a.	n.a.	n.a.
WNT9A	?	?	PORCN$_i$	n.a.	n.a.	n.a.
WNT9B	?	?	PORCN$_i$	n.a.	n.a.	n.a.
WNT10A	X	?	PORCN$_i$	–		Ain et al., 2011
WNT10B	X	?	PORCN$_i$	+		Bui et al., 1997
					+	Wend et al., 2013
WNT11	?	X	PORCN$_i$	+		Corda et al., 2017
WNT16	?	?	PORCN$_i$	n.a.	n.a.	n.a.
FZD1	?	?	OMP18R5 (vantictumab)	+		Milovanovic et al., 2004

(Continued)

TABLE 1 | Continued

Gene	Mechanism*		Drug**	Gene expression changes detected at the level of		Reference
	CTNNB1	*other*		*RNA*	*protein*	
FZD2	?	?	OMP18R5 (vantictumab)	+		Gujral et al., 2014
				+		Milovanovic et al., 2004
FZD3	?	?		+		Bell et al., 2017
FZD4	X	?		n.a.	n.a.	n.a.
FZD5	X	?	OMP18R5 (vantictumab)	n.a.	n.a.	n.a.
FZD6	?	X		+	+	Corda et al., 2017
FZD7	X	?	OMP18R5 (vantictumab)	+		Chakrabarti et al., 2014
				+		Dey et al., 2013
				+		Jia et al., 2018
				+		Yang et al., 2011
FZD8	X	?	OMP18R5 (vantictumab)		+	Jiang et al., 2016
			OMP-54F28 (ipafricept)	–		Wang et al., 2012
FZD9	?	?		$C^{M}pG$		Conway et al., 2014
FZD10	?	?		0		de Groot et al., 2014
LRP5	X	–		n.a.	n.a.	n.a.
LRP6	X	–		+		Lindvall et al., 2009
				+		Liu et al., 2010
				–		Ma et al., 2017
ROR1	?	X	Cirmtuzumab		+	Balakrishnan et al., 2017
					+	Cao et al., 2018
					+	Chien et al., 2016
					+	Cui et al., 2013
					+	Zhang et al., 2012
ROR2	?	X			–	Li et al., 2014
					+	Henry et al., 2015
RYK	?	?		–		Borcherding et al., 2015
PTK7	?	?	PTK7-ADC	+		Ataseven et al., 2013
				+		Damelin et al., 2017
				+		Gärtner et al., 2014

*Only data collected from freshly isolated tumors (e.g., microarrays, qRT-PCR, Western blotting) or fixed tumor samples (e.g., immunohistochemistry) were used. Data obtained from experiments on established human breast cancer cell lines or patient-derived xenografts were not included. Subtype-specific differences have been incompletely investigated, partially due to small cohort sizes. As an example, when all breast cancer subtypes were grouped together, 75% scored negative for WNT10B protein expression (Wend et al., 2013), corresponding to an earlier finding at the RNA level (Bui et al., 1997). However, 90% of TNBC samples scored positive (Wend et al., 2013). Similarly, FZD9 shows more frequent hypermethylation in hormone-receptor positive invasive breast cancers compared to those that are scored as hormone-receptor negative, as well is in those tumors that have a wildtype as opposed to a mutant TP53 status (Conway et al., 2014). *Potential signaling mechanism based on evidence from the cumulative Wnt literature supporting involvement of the gene product in WNT/CTNNB1 signaling and/or non-canonical (other) signaling events. **Potential target for the indicated drugs based on substrate specificity of the listed therapeutics described in the literature. –, Lower RNA or protein expression detected in primary breast cancer tissue compared to normal tissue and/or lower expression is associated with worse prognosis. 0, similar expression in breast cancer tissue and normal tissue. +, Higher RNA or protein expression detected in primary in breast cancer tissue compared to normal tissue and/or higher expression is associated with worse prognosis. n.a., no data available. n.d., tested, but not detectable. $PORCN_i$, PORCN inhibitors. $C^{M}pG$, DNA methylation detected.*

alterations. For example, only FZD7 is consistently found to signal through CTNNB1/TCF in human breast cancer cells, thereby affecting cell proliferation (Yang et al., 2011; Chakrabarti et al., 2014; Riley and Day, 2017). In contrast, copy number gain of the *FZD6* gene, which can be readily detected in human breast cancer cohorts (**Figure 1B**) and most predominantly in TNBC, most likely exerts its effects on cell motility and invasion via alternative, non-canonical WNT signaling mechanisms (Corda et al., 2017). For other components, such as *RSPO2*, *RSPO4* and to a lesser extent *LGR5* and *LGR6*, the overexpression of which is enriched in TNBC, the mechanism is more likely to involve amplification of the WNT/CTNNB1 signaling response (Coussy et al., 2017). Importantly, the separation between canonical and non-canonical WNT signaling is not black and white.

For instance, WNT5A, still frequently regarded as the "typical" non-canonical WNT ligand, can both repress and activate CTNNB1-dependent signaling, *in vitro* as well as *in vivo* (Mikels and Nusse, 2006; van Amerongen et al., 2012b). Especially in the context of cancer, where cellular signaling pathways are invariably deregulated, unexpected signaling activities are likely to be encountered (Grossmann et al., 2013).

Summarizing, more extensive transcriptional and epigenetic profiling of tumor and adjacent normal tissue is needed to reveal the true extent of aberrant WNT signaling in human breast cancer. Early studies reported hypermethylation, and presumably silencing, of genes encoding secreted WNT-pathway inhibitors as a potential mechanism for disrupting the balance in WNT signaling in breast cancer. Examples are widespread and include *WIF1* (Wissman et al., 2003; Ai et al., 2006; Veeck et al., 2009), *SFRP1* (Ugolini et al., 2001; Veeck et al., 2006; Suzuki et al., 2008), *SFRP2* (Suzuki et al., 2008; Lindqvist et al., 2014), *SFRP5* (Suzuki et al., 2008; Veeck et al., 2008a; Lindqvist et al., 2014), *DKK1* (Forget et al., 2007; Suzuki et al., 2008) and *DKK3* (Veeck et al., 2009; Lindqvist et al., 2014; Yamaguchi et al., 2015). Epigenetic analyses, such as those measuring DNA methylation levels, are now becoming part of the standard work flow for large consortia. The first of such analyses indeed revealed extensive changes in WNT signaling components across breast tumors (Koval and Katanaev, 2018). The main challenge still lies ahead as we face the daunting task of properly interpreting these experimental findings. For instance, *DKK3* and *WIF1* methylation was detected in a similar proportion of breast cancer patients, but only *DKK3* methylation was a prognostic marker of survival (Veeck et al., 2009). And while one study reported *SFRP2* promoter hypermethylation in more than 80% of breast cancer patients (Veeck et al., 2008b), a recent report suggests that, in contrast, elevated serum levels of SFRP2 may serve as an independent marker for poor prognosis (Huang et al., 2019). Future studies will also have to focus on subtype-specific differences.

WILL BREAST CANCER PATIENTS BENEFIT FROM DRUGS TARGETING THE WNT PATHWAY?

Our current lack of understanding which patients are most likely to benefit from treatment with WNT inhibitors is a third major knowledge gap. Several drugs that interfere with the WNT signaling pathway are currently being tested in clinical trials (for recent reviews see Krishnamurthy and Kurzrock, 2018; Ghosh et al., 2019). After decades of ill-fated attempts to block WNT signaling downstream of CTNNB1, the current developmental pipeline is fueled by two different rationales (**Figure 1C**). The first is the conceptual notion that, even in the absence of apparent mutations, WNT/CTNNB1 plays a central role in the maintenance of multiple adult tissue stem cell populations and, by analogy and extension, in cancer stem cells. This line of reasoning forms the basis for the development of drugs that inhibit WNT protein secretion, such as the PORCN inhibitors LGK974 and ETC-159 (Liu et al., 2013; Madan et al., 2016). The main adverse effects reported for PORCN inhibitors in Phase I clinical trials are related to loss of bone density (Ng et al., 2017; Tan et al., 2018). Somewhat surprisingly, the systemic toxicity of PORCN inhibitors appears to be relatively limited. One potential explanation for this observation comes from experiments conducted in mice. Here, the WNT-secreting intestinal myofibroblasts, which constitute the intestinal stem cell niche, were shown to be intrinsically resistant to xenobiotics, including PORCN inhibitors, because they express a subset of multidrug efflux pumps (Chee et al., 2018). While this opens a therapeutic window, it also leads to the sobering conclusion that tumor cells may likely evolve similar resistance mechanisms upon prolonged treatment. In fact, these same ATP-binding cassette (ABC) transporters have long been implicated in acquired multidrug resistance in cancer, albeit in the context of classical chemotherapeutic agents rather than targeted therapeutics (Robey et al., 2018). In addition, although it is generally assumed that all WNT ligands require PORCN for their secretion, exceptions to this rule may exist (Rao et al., 2018).

The second rationale for designing drugs that interfere with WNT signaling are more focused and evidence based. These efforts are directed toward specific WNT-pathway components that show altered expression in human tumors. Examples include the anti-RSPO3 antibody OMP-131R10/rosmantuzumab and the decoy receptor FZD8-CRD OMP-54F28/ipafricept (Cattaruzza et al., 2015; Le et al., 2015). So far, the most promising results for breast cancer have been obtained with the broad-spectrum anti-FZD antibody OMP-18R5/vantictumab, which blocks FZD1, 2, 5, 7, and 8 (Gurney et al., 2012). In pre-clinical trials, OMP-18R5 was shown to inhibit the outgrowth of patient derived breast cancer xenografts, thus demonstrating potential efficacy against breast cancer (Gurney et al., 2012; Fischer et al., 2017). A phase Ib clinical trial in HER2$^-$ breast cancer patients identified a four-gene signature (*FBXW2*, *CCND2*, *CTBP2,* and *WIF1*) as a potential predictive biomarker for the response to combined treatment with paclitaxel and vantictumab (Zhang et al., 2018). Structure guided design will likely help in generating more specific antibodies that target individual FZD receptors (Raman et al., 2019). Based on the available data, FZD6 and FZD7 seem obvious candidates for therapeutic intervention (**Figure 1** and **Table 1**).

Few WNT-pathway targeting drugs that are currently in clinical trials were explicitly developed with breast cancer in mind. A notable exception is Foxy-5, a peptide mimetic of WNT5A that was designed with the goal of blocking breast cancer metastasis by reconstituting a – presumably non-CTNNB1 driven –WNT5A signaling response in cancers that had lost *WNT5A* expression (Säfholm et al., 2008). While WNT5A protein expression was found to be low in 75% of TNBC tumors, medium to high expression was detected in 75% of ER+ breast cancer samples (Borcherding et al., 2015). Furthermore, expression levels may change upon treatment, as WNT5A protein levels were significantly higher in 79% of patients after relapse and elevated WNT5A levels were also associated with the induction of multidrug resistance (Hung et al., 2014).

In many cancers, including breast cancer, only a small population of tumor cells, the so-called 'cancer stem cells,' may be responsible for driving tumor growth. Human breast

cancer stem cells were first identified as tumor initiating cells following transplantation into immunocompromised mice (Al-Hajj et al., 2003) and have been connected to metastasis formation and resistance to therapy. Given the presumed importance of WNT/CTNNB1 signaling in breast cancer stem cell maintenance (Lamb et al., 2013; Jang et al., 2015; Hou et al., 2018), it is somewhat counterintuitive that the non-canonical co-receptor ROR1 is emerging as a potential key mediator of chemoresistance in breast cancer stem cells (Zhang et al., 2019). Overexpression of ROR1 is a prognostic marker in TNBC (Chien et al., 2016) and the anti-ROR1 antibody cirmtuzumab, originally developed for treating chronic lymphocytic B-cell leukemia (Zhang et al., 2013), is therefore also in clinical trials for human breast cancer. Initial interest in ROR1 as a potential therapeutic target arose because of its low expression in healthy adult tissues, although a new antibody against ROR1, specifically designed for immunohistochemistry on FFPE samples, shows higher endogenous ROR1 expression than previously suspected (Shabani et al., 2015; Balakrishnan et al., 2017). Another unexpected candidate for targeting breast cancer stem cells surfaced in the form of PTK7, a WNT receptor whose function is not yet completely elucidated (Damelin et al., 2017). PTK7-ADC, a PTK7-targeting antibody that is conjugated to a cytotoxic drug, has also entered phase I clinical trials for metastatic TNBC (Radovich et al., 2019).

Summarizing, it is still too early to conclude anything about the impact of these drugs on breast cancer patient survival. If these therapeutics continue on to more advanced stages of clinical testing, the main challenge will still be to demonstrate true clinical efficacy by rationally selecting those patients that are most likely to benefit from treatment.

DISCUSSION

The absence of well-defined genetic mutations complicates our assessment of the functional importance of aberrant WNT signaling in human breast cancer. No definitive or generalized conclusions can be drawn about the role of either WNT/CTNNB1 or CTNNB1-independent WNT signaling at this point. Given their pleiotropic effects, we need a lot more insight into how these different signal transduction routes affect breast cancer initiation and progression. For this, we need to unravel the basic biological mechanisms through which the complex WNT signaling network controls normal human breast development and physiology. These studies will do more than just satisfy scientific curiosity: They will ultimately be critical to determine which breast cancer subtypes or individual patients are most likely to benefit from targeted therapeutics designed to interfere with WNT signaling activity, taking into account the growth promoting and inhibitory activities of individual ligand/receptor pairings in different cellular contexts.

Both patient selection and monitoring of their clinical response will require new assays and biomarkers. Our drug intervention strategies, in turn, need to be fine-tuned in such a way that individual WNT/receptor interactions or downstream signaling responses can be blocked or activated with great precision. For instance, whereas downregulation of DKK1 has been linked to lung metastases, patients with high levels of DKK1 more frequently present with bone metastases (Zhuang et al., 2017). And while the former has been suggested to occur via a non-canonical signaling mechanism, the latter likely occurs through DKK1-mediated inhibition of WNT/CTNNB1 signaling. In either case, the use of a PORCN inhibitor or a pan-FZD antibody would seem ill advised in both of these cases. Moreover, the adverse effects of these pan-WNT inhibitors on bone density will need to be overcome to advance their clinical use (Madan et al., 2018).

Finally, breast cancer is a systemic disease and the involvement of WNT signaling should be considered from this perspective as well. Both in mice and humans, loss of *TP53* has recently been associated with the induction of WNT protein production, which may in turn stimulate the immune system to promote metastasis (Kim et al., 2019; Liu et al., 2019; Wellenstein et al., 2019). Likewise, cytokine signaling from the local bone microenvironment may promote metastatic colonization by initiating an autocrine WNT signaling loop in human breast cancer stem cells (Eyre et al., 2019). At present, functional studies almost invariably fall back on the use of established human breast cancer cell lines. It is unlikely that these suffice to unravel the contribution of WNT signaling to human breast cancer. Comparing the results obtained in breast cancer cell lines to those obtained in studies with primary human breast cancer organoids and the analysis of patient-derived xenografts is warranted. Given the (epi)genetic diversity of the human breast cancer landscape, patient-to-patient heterogeneity and the interplay between breast cancer cells and their local and systemic environment, the inclusion of stromal and immune components in these experimental model systems will be essential (Holliday and Speirs, 2011; Stephens et al., 2012; Pereira et al., 2016).

AUTHOR CONTRIBUTIONS

RA contributed to the conception and design of the study and wrote the first draft of the manuscript. ES performed the literature survey that is summarized in **Table 1** and wrote sections of the manuscript. ES and RA contributed to acquisition, analysis and interpretation of the literature. All authors contributed to the manuscript revision, read and approved the submitted version.

FUNDING

RA acknowledges funding from the following sources: KWF Kankerbestrijding (Dutch Cancer Society, career development award ANW 2013-6057, project grant 11082/2017-1), NWO (Netherlands Science Foundation, VIDI 864.13.002) and a MacGillavry fellowship from the University of Amsterdam.

ACKNOWLEDGMENTS

The authors thank Yorick van de Grift for critical reading and feedback on the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 03 March 2020
doi: 10.3389/fcell.2020.00037

In vitro Models of Breast Cancer Metastatic Dormancy

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Delayed relapses at distant sites are a common clinical observation for certain types of cancers after removal of primary tumor, such as breast and prostate cancer. This evidence has been explained by postulating a long period during which disseminated cancer cells (DCCs) survive in a foreign environment without developing into overt metastasis. Because of the asymptomatic nature of this phenomenon, isolation, and analysis of disseminated dormant cancer cells from clinically disease-free patients is ethically and technically highly problematic and currently these data are largely limited to the bone marrow. That said, detecting, profiling and treating indolent metastatic lesions before the onset of relapse is the imperative. To overcome this major limitation many laboratories developed *in vitro* models of the metastatic niche for different organs and different types of cancers. In this review we focus specifically on *in vitro* models designed to study metastatic dormancy of breast cancer cells (BCCs). We provide an overview of the BCCs employed in the different organotypic systems and address the components of the metastatic microenvironment that have been shown to impact on the dormant phenotype: tissue architecture, stromal cells, biochemical environment, oxygen levels, cell density. A brief description of the organ-specific *in vitro* models for bone, liver, and lung is provided. Finally, we discuss the strategies employed so far for the validation of the different systems.

Keywords: cancer dormancy, metastatic dormancy, *in vitro* models cancer, cancer metastasis, breast cancer, metastasis biology

OPEN ACCESS

Edited by:
Emilia Peuhu,
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Reviewed by:
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *31 October 2019*
Accepted: *15 January 2020*
Published: *03 March 2020*

Citation:
Montagner M and Sahai E (2020) In vitro Models of Breast Cancer Metastatic Dormancy. Front. Cell Dev. Biol. 8:37. doi: 10.3389/fcell.2020.00037

METASTATIC DORMANCY

Dormancy is an old concept that describes a clinical phenomenon (Klein, 2011; Uhr and Pantel, 2011), i.e., the relapse of a cancer after surgical removal of the primary tumor in a patient considered clinically disease-free for a long time. This implies that cancer cells disseminated prior to surgery and persisted as Minimal Residual Disease (MRD) for a prolonged time (arbitrarily defined, but usually longer than 5 years) before switching to aggressive growth and overt metastasis. The recurrence can be at the primary site (primary tumor dormancy) or at a secondary site (metastatic dormancy). The mechanisms underlying the two types of dormancy are likely to be partially overlapping if involving cell intrinsic genetic/epigenetic mechanisms, or distinct, if dependent on the tissue microenvironment. Clinical dormancy is common for breast, prostate, melanoma, renal, and thyroid cancers, while it is rarely observed in lung and colon cancers (Uhr and Pantel, 2011). In breast cancers, estrogen receptor (ER) status seems to profoundly influence the rate of relapse: ER− patients tend to recur within the first 5 years following primary tumor diagnosis, while ER+ patients have increased risk between 5 and 20 years (Pan et al., 2017; Pantel and Hayes, 2018). While anti-estrogen therapy significantly improved patient outcomes, a significant fraction of them still

develops distant relapses and extending the duration of the treatment beyond 5 years yields little benefit (Pan et al., 2017; Bense et al., 2018; Pantel and Hayes, 2018).

In this review we specifically focus on *in vitro* models developed to study metastatic dormancy. Upon dissemination in a secondary organ, metastatic breast cancer cells (BCCs) can undergo three fates: death, dormancy, or growth. Dormancy does not have a clear biological definition, it has been proposed a classification of dormant phenotypes into cellular dormancy (entering into reversible quiescence in G0) and tumor mass dormancy (a small cluster of cells where proliferation is counterbalanced by apoptosis due to lack of nutrients, blood supply or because of immune surveillance) (Linde et al., 2016; Goddard et al., 2018; Weidenfeld and Barkan, 2018; Lan et al., 2019). However, these states are likely to coexist within the same patients and probably the same cells can dynamically fluctuate between these different states.

Growth arrest mechanisms generally fall into three main categories: quiescence, terminal differentiation and senescence (Pack et al., 2019). While the former is reversible upon withdrawal of restrictive signals, the latter is associated with permanent exit from cell cycle and persistent activation of stress signals. Cyclin-dependent kinases (CDKs) coupled with cyclins promote cellular proliferation by inhibiting pocket protein family (Rb, p107, p130), conversely CDK-cyclin couples are inhibited by CIP/KIP inhibitors (p21, p27, p57) and INK4 inhibitors (like p16). Intrinsic and extrinsic factors are integrated into the regulation of this core machinery, for example, serum starvation triggers upregulation of p27 and exit from proliferation, while CDKs are induced by mitogenic signals. DNA damage is the strongest internal signal regulating proliferation and mediates growth arrest via stabilization of p53 and its target p21. Apart from the prominent role of p27 (Bragado et al., 2013; Touny et al., 2014), little is known about the role of cell-cycle machinery in the different stages of metastatic dormant phenotype and whether dormant cells lie closer to quiescence or senescence in the growth arrest spectrum.

Several strategies have been implemented to visualize dormant disseminated cells *in vivo*. The easiest methods are staining of fixed tissues for the proliferation-associated protein Ki67, growth arrest marker p27 or for DNA-incorporated synthetic nucleosides (such as BrdU or EdU) (Ghajar et al., 2013; Fluegen et al., 2017; Carlson et al., 2019; Montagner et al., 2020). The main limit of these methods is that they are not compatible with tissue viability and don't allow isolation of non-proliferating cells. To circumvent this problem, De Cock et al. (2016) utilized an intracellular fluorescent vital dye to label cells prior to injection into mice. The dye is diluted at each cell division, allowing for isolation of cells that didn't proliferate (Cock et al., 2016). Similarly, Fluegen et al. (2017) generated metastatic cells stably expressing a photoconvertible fluorescent protein, Histone 2B-Dendra2. This is photoconverted from green to red before injecting cells in mice, and nuclear red fluorescent signal decreases at each cell division, similar to a vital dye (Fluegen et al., 2017). The fluorescence ubiquitination–based cell cycle indicator (FUCCI) system has also been applied (Albrengues et al., 2018) which allows dynamic visualization of each phase of the cell cycle during *in vivo* imaging.

Whether dormant cells are quiescent or undergo a balanced proliferation (where proliferation rate is compensated by apoptosis) has a profound impact on the design of new therapies (Wells et al., 2013), because it is assumed that dormant cells are inherently resistant to conventional chemotherapy as they are not cycling (Wells et al., 2013; Linde et al., 2016). This is not entirely correct as recent data show that chemoresistance is in part actively supported by the metastatic niche and is not just a consequence of cell-cycle arrest (Carlson et al., 2019). Moreover, it has been recently shown that several patients with bone marrow-disseminated cancer cells (DCCs) that resisted treatment with FEC (fluorouracil, epirubicin, and cyclophosphamide), benefited from additional treatment with docetaxel; as this drug induces microtubule stabilization, cell-cycle arrest in the G(2)M phase and apoptosis, this suggests that a considerable fraction of dormant cells still has proliferative activity (Naume et al., 2014; Goddard et al., 2018). Notably, patients with dormant DCCs that persisted after the second therapy had worst prognosis, further supporting the idea that metastatic lesions develop from pre-existing dormant DCCs (Braun et al., 2005; Naume et al., 2014).

DATA FROM PATIENTS

Despite the fact that metastatic lesions account for the vast majority of cancer-related deaths, metastatic colonization is an extremely inefficient process (Massagué and Obenauf, 2016). Each step of the hematogenous metastatic cascade of epithelial cancers (loss of polarity, detachment from primary tumor, migration through basal membrane and stromal layers, intravasation, survival in the blood stream, extravasation) represents a significant hurdle that contributes to the selection of aggressive cancer cell clones. Even focusing on the steps that follow intravasation, less than 0.01% of cells will eventually develop metastatic lesions and not even in all patients (Naumov et al., 2002; Braun et al., 2005). These numbers are confirmed by experimental models of metastatic dissemination (Valastyan and Weinberg, 2011), with the switch from micrometastasis to macrometastasis estimated to happen with a frequency lower than 0.02% for liver metastases from melanoma cells (Luzzi et al., 1998; Cameron et al., 2000). From these numbers the expectation might be that persistence in secondary organs is a feature restricted to few highly aggressive cells (seed) and/or to target organs with a peculiar permissive environment (soil). Yet, clinical and experimental evidence of early dissemination of breast cancers have been reported (Goddard et al., 2018), indicating that even cells from early stage disease can disseminate and persist. Moreover, several registries reported people who have developed cancers following organ transplants (Buell et al., 2005; Klein, 2011), indicating that disseminated cells survived in a quiescent state in different organs of donors with prior undiagnosed or cured cancers. Of note, transplanted organs were not the most common sites of metastasis, such as kidney or heart. This evidence supports the idea that survival after metastatic spreading might not be limited, *per se*, to highly aggressive

cells or few target organs, and that indolent disease can seed additional sites.

CHALLENGES FOR THE DEVELOPMENT OF *IN VITRO* MODELS

Transgenic mouse models of dormant/indolent metastatic mammary cancers have been described over the years (Li et al., 2000; Hüsemann et al., 2008) and have been recently used to discover the roles of progesterone receptor, Her2 and partial-EMT into early dissemination (Harper et al., 2016; Hosseini et al., 2016). However, these models also have significant limitations, such as the hurdles associated with tracking asynchronous disseminated metastatic cells. Moreover, dormancy is often the result of the crosstalk between the cancer cells and the metastatic stroma; thus, parameters should be modulated at single cell resolution, which is often impossible *in vivo*. Lastly, removing single stromal populations *in vivo* to prove their requirement into control of dormancy is incompatible with animal viability; the design of *in vitro* models is a valuable strategy to bypass these limitations.

The development of reliable *in vitro* models to investigate dormancy is hampered by the limited data from patients (Chéry et al., 2014; Vishnoi et al., 2015). Scattered dormant DCCs lie far below the radar of current diagnostic tools and significant advancements in that direction will be challenging and will run the risk of detecting lesions that would never progress (Srivastava et al., 2019). Thus, together with new tools for detection of metastatic clusters at single-cell resolution, development of markers for dangerous vs. harmless disseminated cells are highly desirable. Over the last decade, in parallel with advances in microfluidic technologies, biomaterials and biofabrication techniques, many groups developed and optimized *in vitro* tools to explore the issue of metastatic dormancy with different objectives, from discovery of basic mechanisms of survival to platforms for high-throughput drug discovery (Pradhan et al., 2018; Rao et al., 2019). Even though these *in vitro* models are increasing in number and complexity, their descriptive and/or predictive power is unknown, given the paucity of markers, metrics and expression data from patients. Nevertheless, there are common themes emerging from different models that led to the approval of clinical trials (Goddard et al., 2018) and to the development of tools to predict likelihood of relapse (Borgen et al., 2018). Moreover, recent publications provided explanations for epidemiological data linking inflammation with higher risk of breast cancer relapse (Cock et al., 2016; Albrengues et al., 2018). Recent reviews have covered in depth the history, evolution and recent advances in the dormancy field (Giancotti, 2013; Ghajar, 2015; Linde et al., 2016; Aguirre-Ghiso and Sosa, 2018; Goddard et al., 2018), this review focuses instead on *in vitro* models for breast cancer metastatic dormancy that have been more extensively validated and that, regardless of their complexity, led to discoveries supported by independent *in vitro* systems, animal models or by data from patients. Moreover, we provide a framework for the development of further *in vitro* models, by critically discussing metrics and parameters that should ideally be integrated to tightly anchor new and old models with data from animal models or breast cancer patients with the hope of circumventing the limitations discussed above (**Figure 1**).

CELLS

To establish *in vitro* models that reflect the *in vivo* situation, it is first necessary to have cells that exhibit dormant behavior *in vivo*, several BCCs lines with different dormant proclivity and tropism have been generated.

The first option is the use of cell lines derived from *in vivo* selection of dormant clones from an aggressive parental cell line (comparison between parental and selected subclones). A list of those cellular variants is provided in **Supplementary Table S1**, alongside with the selection strategy. The fact that subclones with stable dormant phenotype can be isolated from the aggressive parental cell line is something more than a technical opportunity, but might reveal something more profound about the biology of the dormant phenotype, i.e., that heritable characteristics of single cells, most likely epigenetically specified, are as important as the dormant microenvironment to dictate the choice between quiescence and proliferation.

The second option is the use of cell line series generated from a common precursor, but then selected independently from different animals (**Supplementary Table S1**). A notable example of these cell lines is the D2 series (D2.A1, D2.1, D2.0R) established by Fred Miller lab and characterized by Ann Chambers lab in her pioneering works on cancer dormancy (Mahoney et al., 1985; Aslakson and Miller, 1992; Rak et al., 1992; Morris et al., 1993, 1994). These cells have been cloned from spontaneously growing tumor in different BALB/cfC_3H mice transplanted with a D2 hyperplastic alveolar nodule (HAN) line (Medina, 1976). D2.0R and D2.A1 cells grow with comparable rate on plastic, but with extremely different dynamics in 3D systems, coculture models and *in vivo*: A1 form overt metastases in lung and liver, OR lie dormant in the same organs for several months (Naumov et al., 2002; Barkan et al., 2008; Shibue and Weinberg, 2009; Touny et al., 2014; Montagner et al., 2020). Notably, another breast cancer cell series of great interest has been developed by the same laboratory in BALB/c mice. These cells show progressive acquisition of aggressive traits, from primary tumor growth, local invasion, intravasation, lung homing, overt metastasis (67NR > 168FARN > 4T07 > 4T1) (Aslakson and Miller, 1992). Often used in studies about dormancy, the cell line 4T07 was generated by sequential intravenous injection and isolation from lungs of a thioguanine- and ouabain-resistant cell line (Dexter et al., 1978; Blazar et al., 1980; Miller et al., 1987). The comparison between the two cell lines has led to the discovery of important molecules involved in the dormant state of lung, bone and brain disseminated cells (Gao et al., 2012, 2016).

A third option is the comparison among cell lines from completely different origin. Examples of these classes are the widely used triple negative cell line MDA-MB-231 (on the aggressive side of the spectrum) and the ER+ cells MCF7, T47D, ZR-75-1 that form quiescent metastatic lesions upon intravenous

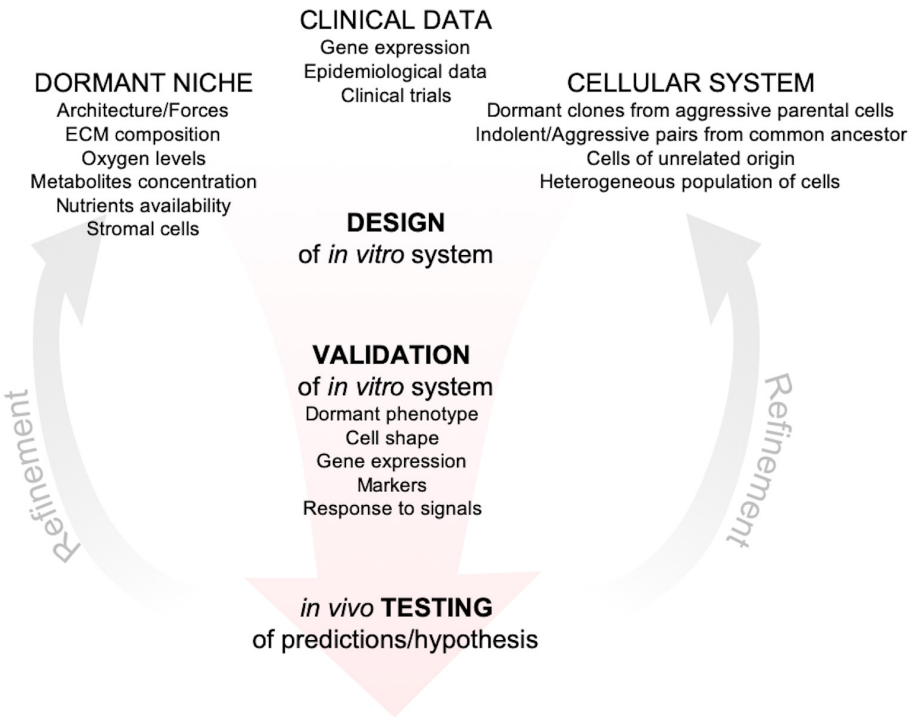

FIGURE 1 | Framework for the development of *in vitro* models of metastatic dormancy. *In vitro* models should include one or more features of the dormant niche starting from measurements/observations of the target metastatic tissue *in vivo*. Then cellular systems have to be chosen based on clinical evidences that support their specific use, i.e., correct metastatic tropism, correct gene expression (when available), correct response to signals (such as inflammation). After the initial setup, the *in vitro* system must be validated and the cell lines, as well as the microenvironment, should exhibit one or more features specific of the dormant phenotype (obtained from animal models or clinical data). The *in vitro* system should then be exploited to generate hypothesis and prediction that could be tested experimentally in animal models or from clinical data. Finally, according to the feedbacks from the *in vivo* validation, the system can be refined for more accurate predictions and hypothesis.

injection (Harrell et al., 2006; Holen et al., 2016; Wright et al., 2016; Gawrzak et al., 2018). Recently, bone metastatic versions of MCF7 cell line have been developed (Pavlovic et al., 2015; Clements and Johnson, 2019).

The last option is the comparison within the same cell line. This approach is a valuable alternative whenever the question is related to the drivers of cellular heterogeneity within the same population *in vitro* (Ghajar et al., 2013).

DORMANT NICHE COMPONENTS

During the last two decades, the role of the microenvironment has been gaining importance in understanding several steps of the malignant transformation. For metastatic dormancy, the context where cells disseminate is key, as these cells are likely not to gain further mutations once they have entered quiescence. Components of the dormant niche include, but are not limited to: tissue architecture (geometry and stiffness, adhesion, cell density, ECM), biophysical (shear stress, tissue stiffness) and biochemical (oxygen levels, ROS concentration, nutrients, metabolites) environment, stromal populations. Examples and details of *in vitro* systems including tissue architecture and stromal cells is provided in **Figure 2** and **Supplementary Table S2**.

Tissue Architecture

The rapid development of bioengineering and a better understanding of the principles behind mechanotransduction (Iskratsch et al., 2014; Piccolo et al., 2014; Dupont, 2016; Montagner and Dupont, 2020) has led to several *in vitro* approaches to study metastatic dormancy of BCC. Models involving scaffolds of natural or artificial biomaterials, microfabrication, microfluidics, bioreactors, implantable niches have been developed (extensively reviewed in Pradhan et al., 2018; Rao et al., 2019). 3D spheroids can be generated simply as clusters of cells floating into medium (Wenzel et al., 2014; Cavnar et al., 2015; Imamura et al., 2015) or by employing natural (collagen-I, hyaluronic acid, and Cultrex) (Barkan et al., 2008; Fang et al., 2016; Kassim et al., 2017) or synthetic biomaterials (hydrogels of silica-polyethylene, polycaprolactone scaffolds) (Guiro et al., 2015; Preciado et al., 2017). Cells within these structures showed different degrees of quiescence, apoptosis, hypoxia and have been tested for their sensitivity to drugs. However, same caveats apply and, although informative, these models require more validation to address if their findings translate in a dormant phenotype. A notable exception is the well-known 3D system developed in Green laboratory. In this *in vitro* model, D2 cells lie on top of a stiff layer of basement membrane matrix and are embedded in a second layer of diluted

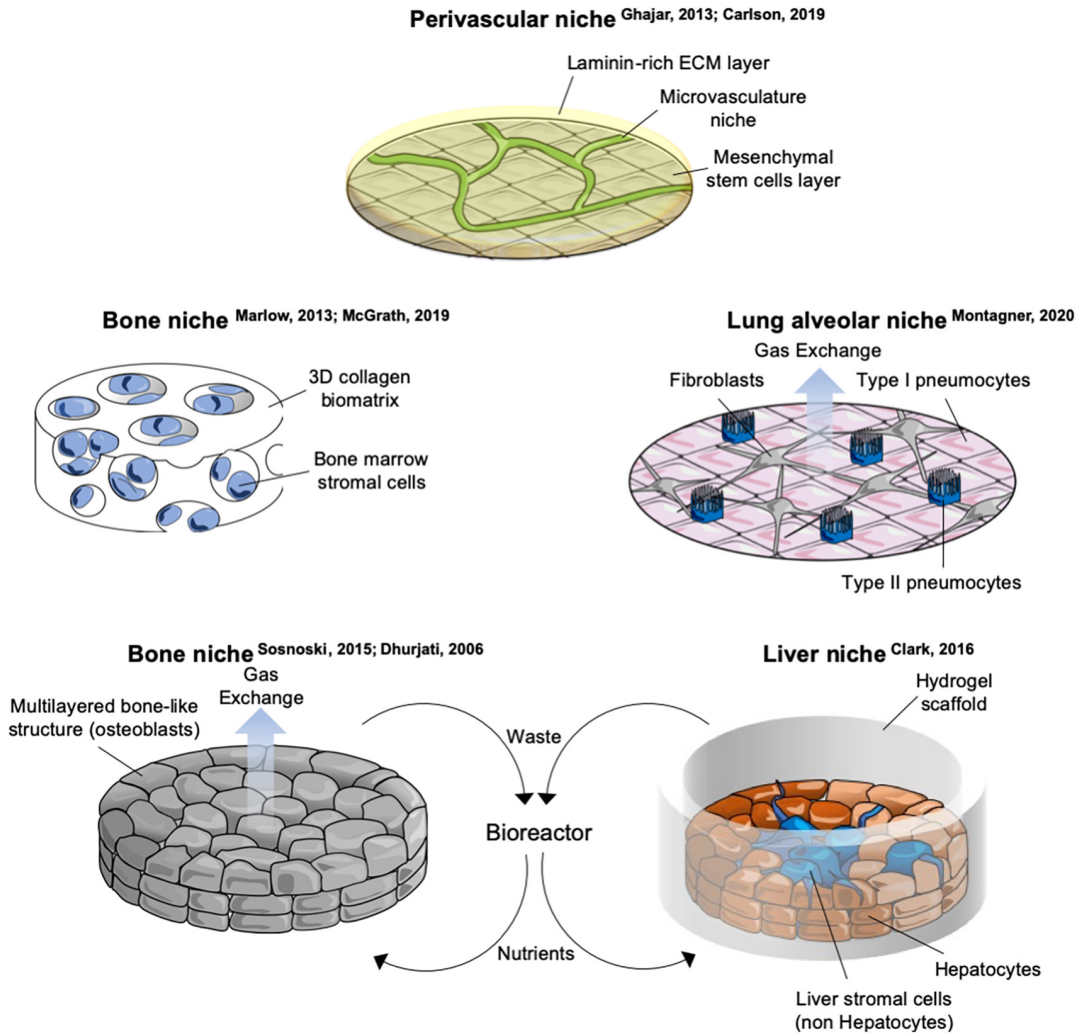

FIGURE 2 | Schematics of selected *in vitro* organotypic systems designed for studying breast cancer cells (BCCs) metastatic dormancy. Schemes of *in vitro* systems developed to mimic microenvironments of specific metastatic organs. Details and references of each system are provided in **Supplementary Table S2**.

basal matrix. Under these conditions D2.0R cells remain dormant in round structures, while D2.A1 continuously grow and invade surrounding territories. This conformation can then be further functionalized by adding other ECM proteins, such as collagen-I that drives the proliferative switch of otherwise dormant cells (Barkan et al., 2008, 2010). This system has been extensively validated *in vivo* and by other laboratories as well (Shibue and Weinberg, 2009; Shibue et al., 2012), and led to the discovery of the integrin-Src-ERK axis in the dormant-to-proliferation switch (Barkan et al., 2008, 2010). The use of ECM proteins can be combined with other niche components such as stromal cells to increase the complexity of the system (Ghajar et al., 2013).

Stromal Cells

Resident organ parenchymal cells are an essential component of the dormant niche contributing to each step of quiescence-to-proliferative switch. Difficulties in the coculture of BCCs together with stromal cells are primarily two: (i) availability of organ-specific stromal cells and (ii) finding the culturing protocol that allows survival of all the cellular components. Moreover, the cellular composition of a tissue is often dynamic and heterogeneous, including different lineages of the same cell type as well as specific resident and transient immune populations. This complexity is often not captured by current *in vitro* models. Cocultures developed so far involving BCCs include osteoclasts (Lu et al., 2011), osteoblasts (Sosnoski et al., 2015), lung alveolar cells (Montagner et al., 2020), endothelial cells (Ghajar et al., 2013; Carlson et al., 2019), hepatocytes and non-hepatocytes liver stromal cells (Wheeler et al., 2014), bone marrow stromal cells (Ghajar et al., 2013; Marlow et al., 2013; Carpenter et al., 2018), neutrophils (Albrengues et al., 2018), peripheral blood mononuclear cells (Carpenter et al., 2018; **Figure 2** and **Supplementary Table S2**). While for some populations primary cells are available [lung and bone marrow stromal cells (Ghajar et al., 2013; Marlow et al., 2013; Carpenter et al., 2018), hepatocytes and non-hepatocytes (Clark et al., 2016),

NK cells (Malladi et al., 2016), neutrophils (Albrengues et al., 2018), human osteoblasts (Sosnoski et al., 2015), mouse SNO osteoblast-like cells (Capulli et al., 2019)], other cells require immortalization [endothelial cells (Ghajar et al., 2013), fibroblasts and type1-like pneumocytes (Montagner et al., 2020), human fetal osteoblasts and mesenchymal cells of bone marrow origin (Marlow et al., 2013), spontaneously immortalized mouse calvaria osteoblasts (Sosnoski et al., 2015)] or transformation to be cultivated [murine preosteoclasts (Lu et al., 2011), type2-like pneumocytes (Montagner et al., 2020)] and this might influence the correct crosstalk with the dormant BCC. Moreover, it has been shown that fibroblasts and endothelial cells have organ-specific gene expression (Chang et al., 2002; Nolan et al., 2013) and thus using unmatched stromal cells might overlook organ specific signaling. On the other side, the use of immortalized, homogeneous stromal cells allows a precise and repeatable experimental setup compared to deriving primary cells. An important detail in the *in vitro* models cited above is the use of a very low number of BCCs relative to stromal cells (Ghajar et al., 2013; Wheeler et al., 2014; Montagner et al., 2020).

Biochemical Environment

Mitogens and nutrients directly impact on cell-cycle machinery (Pack et al., 2019), it is not surprising that decreasing their concentration in culture medium to a more physiological level already has an effect on proliferation. In a recently developed lung organotypic system, we used a Mitogen Low Nutrient Low medium (MLNL) that didn't have a different effect on D2 cells *per se*, but that allowed to pinpoint some factors of the signaling network after stromal cells were added (Montagner et al., 2020). Mitogen Low Medium (MLM) alone had a remarkable effect on HCC1954-LCC1 (Latency Competent Cells) cells instead (Malladi et al., 2016). Cultivating LCC1 subclones in MLM medium drove expression of quiescence genes, such as Sox9, downregulation of several mediators of anti-tumor responses from NK cells and downregulation of Wnt, myc, NF-kB pathways, higher TGFβ response and lower P-ERK/P-p38 ratio (Malladi et al., 2016).

Hypoxia

Oxygen concentration for most of the tissues oscillates between 5 and 7%, compared to the 20% in air at normal atmospheric pressure (McKeown, 2014). Bone marrow is a particularly hypoxic environment (Spencer et al., 2014) and a favorable metastatic site for BCCs. The use of physiological oxygen levels decreases proliferation for most of cells (Hubbi and Semenza, 2015) and, as with low serum, it might not be specific to dormant cells (de Prati et al., 2017; Lee et al., 2018). However, hypoxia has been implicated in dormancy in two studies where it has been shown to repress LIFR-STAT3 pathway leading to metastatic outgrowth (Johnson et al., 2016) and to preset primary tumor cells with a dormant program, then manifested after dissemination (Fluegen et al., 2017).

Cell Density

Plating cells at a clonogenic density *in vitro* is already sufficient to induce heterogeneous growth arrest in BCCs. The Wieder laboratory developed an *in vitro* system of bone marrow dormancy that, despite its simplicity, has been shown to recall several aspects of quiescence validated in other laboratories. BCCs that are plated onto fibronectin-coated plates undergo quiescence in presence of FGF2 and activation of integrin α5β1, PI3K and ERK pathways. These cells express partial EMT markers and can re-enter proliferation upon treatment with IL6/8 and TGFβ) (Korah et al., 2004; Najmi et al., 2005; Barrios and Wieder, 2009; Tivari et al., 2015).

VALIDATION OF *IN VITRO* MODELS OF DORMANCY

What are we really modeling? This is the first question when designing any model and although this is an issue not unique to the topic of this review, the limited availability of clinical data makes it harder to unambiguously describe a dormant cell *in vitro*. Because an unequivocal list of dormant cells' features is unavailable, several groups have validated their models by looking at a number of aspects that justified the parallel between the proposed *in vitro* model and the *in vivo* evidences, although a single model encompassing all of them has not yet been developed (**Figure 1**).

Reversible Quiescence

The most important behavior underlying the dormant phenotype is growth arrest, and most of the *in vitro* models discussed in this review successfully achieve cell-cycle arrest of cells that can be reversed upon changing experimental conditions, such as serum levels, oxygen tension or with specific signals, such as inflammation. However, this does not demonstrate the relevance of the model. For example, it has been shown that adjusting the mechanical properties of the cell culture surface (using ECM-conjugated polyacrylamide gels) alone has a dramatic impact on cellular proliferation *in vitro* (Tilghman et al., 2012), but this does not imply that changes in local tissue mechanics are cause of entry and exit from dormancy. Ideally, the model conditions should be based on appropriate measurements of the *in vivo* environment in which dormant cells are found in terms of biophysical, biochemical and cellular composition of the niche (ECM composition and architecture, nutrients and metabolites concentration, ligands concentration, communication with stromal cells). However, this information is hard to determine at single-cell resolution in murine models and even harder to measure in clinical material. To distinguish between quiescence and senescence (or even apoptosis), cells must re-enter the proliferative state upon withdrawal of the factors used to trigger dormancy or upon treatment with signals able to drive exit from dormancy. Examples of such signals for BCCs are inflammation (LPS, smoke) (Cock et al., 2016; Albrengues et al., 2018), POSTN (Montagner et al., 2020), TGFβ1 (Ghajar et al., 2013), RTKs (Tivari et al., 2015; Montagner et al., 2020), IL6, Collagen I (Barkan et al., 2010), Src (Barkan et al., 2010; Montagner et al., 2020), SFRP2 (Montagner et al., 2020), IKKβ (Lamiaa et al., 2017), integrins activation (as discussed below); while examples

of inhibitors are: TSP1 (Ghajar et al., 2013), p38 (Marlow et al., 2013), Alk5 (Marlow et al., 2013), BMP2 (Gao et al., 2012), TGFβ2 (Bragado et al., 2013), MSK1 (Gawrzak et al., 2018), IFN-β (Lan et al., 2019).

Markers of Dormancy

Together with a reversible growth arrest, expression of gene/protein marker of dormancy should be addressed. Not many well-established markers are available for BCCs, those that have been widely validated *in vitro* and *in vivo* so far include DEC2/SHARP1, p27, NR2F1 and the ratio between P-ERK/P-p38 proteins (Touny et al., 2014; Johnson et al., 2016; Linde et al., 2016; Malladi et al., 2016; Borgen et al., 2018). We recently reported an RNA-seq analysis of lung-disseminated dormant BCCs that will hopefully provide new markers for the characterization of these cells *in vitro* (Montagner et al., 2020).

Regardless of the metrics adopted, the predictive power of an *in vitro* system represents its best validation and testing the predictions generated in mice or patients is the ultimately goal (**Figure 1**).

ORGAN-SHARED MECHANISMS OF DORMANCY

Whether the same mechanisms for quiescence or reawakening are shared among different organs *in vivo* is unknown. The observations that dormant subclones isolated from one organ show quiescence in other organs suggests that there might be some overlap and thus either intrinsic genetic/epigenetic mechanisms dominate over microenvironmental cues or there are common traits in very different niches. For example, D2.0R cells are dormant in liver and lung, HCC1954-LCC1 are derived from brain disseminated cells, but are found latent in lungs as well (Malladi et al., 2016), T47D-DBM have been isolated as bone dormant variant (Gawrzak et al., 2018), but they survive in quiescent state in lungs as well (Montagner et al., 2020). Mechanistic similarities between dormancy in different organs will aid the development of universal clinical strategies with the ability to eliminate dormant cells regardless of their anatomical site.

Activation of ERK and p38, associated with metastatic outgrowth and quiescence, respectively, have been consistently observed in bone and lungs (Barkan et al., 2010; Touny et al., 2014; Linde et al., 2016; Malladi et al., 2016; Gawrzak et al., 2018). THBS1 from PVN (perivascular niche) was shown to induce dormancy in lung and bone marrow (Ghajar et al., 2013). Src has been validated as BCCs survival signal in bone and lungs (Barkan et al., 2010; Zhang et al., 2013; Montagner et al., 2020). Similarly, Akt was found as a factor supporting survival or outgrowth of BCCs in bone *in vivo* (Zhang et al., 2013) and *in vitro* (Korah et al., 2004) and in an *in vitro* model of lung (Montagner et al., 2020). The proinflammatory cytokine LPS induces outgrowth of quiescent cells in lung (Cock et al., 2016; Albrengues et al., 2018) and in an *in vitro* model of dormancy in the liver (Clark et al., 2018).

A recurring theme in several models of BCCs dormancy is the importance of integrins into survival or chemoresistance. Many groups independently reported the key role of different integrin dimers. Integrinβ_1-dependent activation of Src and ERK downstream of collagen-I has been found to drive exit from quiescence *in vitro* and *in vivo* (Barkan et al., 2010; Touny et al., 2014), to sustain reawakening of dormant cells following NET proteolysis of laminin *in vivo* [in the $\alpha_3\beta_1$ form (Albrengues et al., 2018)] and to support survival after engagement of fibronectin in *in vitro* bone dormancy models [in the $\alpha_5\beta_1$ form (Barrios and Wieder, 2009; Barney et al., 2019)]. Perivascular-driven chemoresistance of dormant BCCs also relies on $\alpha_5\beta_3$ and $\alpha_4\beta_1$ activation by von Willebrand Factor and VCAM-1 in endothelium, respectively. By using blocking antibodies against those isoforms in combination with doxorubicin and cyclophosphamide Carlson et al. (2019) were able to circumvent chemoresistance and decrease tumor burden in bone marrow. Finally, we recently found that acute treatment of mice with cilengitide (inhibitor of $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ integrins) effectively reduced lung-disseminated dormant BCCs (Montagner et al., 2020). In sum, quiescent BCCs seem to rely on integrins in many ways and might prove more sensitive to integrin inhibitors then established or actively growing cancer cells.

CONCLUDING REMARKS

So far, the battle to defeat metastatic breast cancer has achieved only limited advances since the advent of hormone target therapies. For some types of cancer, the period metastatic dormancy offers an opportunity to eliminate disease before it resumes aggressive growth, but the inherent lack of data from patients slows down the development of new therapies. The development of *in vitro* models to bypass this limitation has been the goal for several laboratories during the last decade, and common themes in the survival and growth of disseminated BCCs in different organs are starting to emerge. Here we present cellular models and microenvironmental factors implemented so far, together with a critical discussion on validation strategies. The discovery of new markers from patients and validation of same mechanisms among different systems will give confidence to translate these findings into clinical trials and hope to finally impact on the origin and development of metastatic breast cancer.

AUTHOR CONTRIBUTIONS

MM and ES equally contributed to conceiving and writing the review.

FUNDING

ES and MM were funded by the Francis Crick Institute, which receives its core funding from Cancer Research UK (FC001144),

the UK Medical Research Council (FC001144), and the Wellcome Trust (FC001144). MM also received funding from Marie Curie Actions-Intra-European Fellowships no. 625496 and BIRD Seed grant from Department of Molecular Medicine (University of Padua).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00037/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 17 March 2020
doi: 10.3389/fcell.2020.00162

Immuno-Oncology Biomarkers for Personalized Immunotherapy in Breast Cancer

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The immune checkpoint blockade therapy has drastically advanced treatment of different types of cancer over the past few years. Female breast cancer is the second leading cause of death in the overall burden of cancers worldwide that is encouraging healthcare professionals to improve cancer care management. The checkpoint blockade therapies combined with novel agents become the recent focus of various clinical trials in breast cancer. However, identification of the patients who are responsive to these therapeutic strategies remained as a major issue for enhancing the efficacy of these treatments. This highlights the unmet need in discovery and development of novel biomarkers to add predictive values for prosperous personalized medicine. In this review we summarize the advances done in the era of biomarker studies and highlight their link in supporting breast cancer immunotherapy.

Keywords: immunotherapy, biomarker, breast cancer, immune checkpoint blockade, anti-PD-1 and anti-PD-L1

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: *04 December 2019*
Accepted: *28 February 2020*
Published: *17 March 2020*

Citation:
Vafaizadeh V and Barekati Z (2020) Immuno-Oncology Biomarkers for Personalized Immunotherapy in Breast Cancer. Front. Cell Dev. Biol. 8:162. doi: 10.3389/fcell.2020.00162

INTRODUCTION

Recent advances in cancer therapy present immunotherapy as a prospect change in treating various cancers. The immune-checkpoint blockade (ICB), designated as a cutting edge therapy, is used in increasing number of advance cancer diseases with durable responses compared to most chemotherapy and targeted therapies (Ansell et al., 2015; Gettinger et al., 2015; Larkin et al., 2015; Spencer et al., 2016; Tray et al., 2018). Breast cancer is the most common malignancies among women worldwide and many breast cancers have been recently determined immunogenic and enriched in tumor-infiltrating lymphocytes (TILs) (Cimino-Mathews et al., 2016).

The ICB monotherapy, anti-programmed death-1/programmed death-ligand-1 (anti-PD1/PD-L1), has demonstrated promising outcomes in metastatic triple negative breast cancer (Nanda et al., 2016; Schmid et al., 2018; Adams et al., 2019a,b; Emens et al., 2019). There is a considerable attention for developing immunotherapy-based strategies to escalate anti-cancer responses and to reduce the side effects, such as trials on combination therapy of anti-PD-1/PD-L1 with chemotherapy agents or combination with targeted therapies in metastatic patients (Nolan et al., 2017; Domchek et al., 2018; Nicolas et al., 2018). Moreover, developing strategies on combining different ICBs are appealing in breast cancer treatment, such as combining anti-PD-1/PD-L1 with anti-cytotoxic T-lymphocyte associated protein-4 (anti-CTLA-4) (Wolchok et al., 2017) or other co-inhibitory molecules (Chester et al., 2018; Harding et al., 2019). One of the major challenges in this regard is to establish predictive biomarkers for the stratification of breast cancer patients benefiting from these ICBs therapeutic strategies.

In March 2019, the U.S. Food and Drug Administration (FDA) approved the first ICB drug, a PD-L1 antibody called Atezolizumab (Tecentriq) in combination with a chemotherapy agent, for the treatment of triple-negative metastatic breast cancer patients (TNBC) (NCT02425891)

(Schmid et al., 2018; Emens et al., 2019). The TNBC is a subtype of the disease with frequency of 15% and lacks hormone receptors, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) (Anders et al., 2016). The Atezolizumab authorized to be applied only on metastatic TNBC patients whose tumors express the PD-L1 protein that is characterized by an FDA-approved test, VENTANA PD-L1 (SP142), as a predictive biomarker. This was a significant fundamental step in predicting clinical benefit of only one ICBs combination strategies in breast cancer treatments. However, different ICBs agents and strategies are going to bring new treatment modalities for this disease. Therefore, there is an unmet need in developing novel predictive biomarkers for proper selection of patients who are benefiting from ICBs treatments and for avoiding unnecessary toxicity in unresponsive patients. Furthermore, identifying predictive biomarkers are necessary for better management of the expensive health care costs, especially for those patients that are unlikely to be responsive to the ICBs therapies. Here we summarize the attempts that have been done on the discovery of major predictive biomarkers in liquid biopsies, tumor tissues and tumor microenvironment that might contribute into advancing prediction of therapeutic decisions as well as the future challenges in this era.

LIQUID BIOPSIES BIOMARKERS

Over the past few years, considerable effort has been done in discovery and development of liquid biopsy-based biomarkers, as it is minimally invasive, cost effective and can be replicated during patients' follow-up (Wan et al., 2017; Pantel and Alix-Panabieres, 2019; Rothwell et al., 2019). These biomarkers can be detected in blood, cerebrospinal fluid and urine of cancer patients but not in healthy individuals. Today, liquid biopsy-based biomarkers are defined as soluble proteins, exosomes or other vesicles transmitting proteins or nucleic acids driven from a tumor, circulating tumor cells (CTCs) and circulating tumor DNAs (ctDNAs) (Kohler et al., 2011; Siravegna et al., 2017). All these properties make liquid biopsy-based biomarkers attractive in immunotherapy for the assessment of predictive biomarkers at the baseline or in monitoring therapy response.

The plasma proteins such as soluble PD-L1, cytokines and exosomes' bound proteins are considered as important source of information in biomarker discovery and development. A high level of soluble PD-L1 has been demonstrated a poor prognosis in ICB response (Okuma et al., 2018). Nonetheless, the plasma level of soluble PD-L1 can be increased due to the different physiological conditions and diseases e.g., pregnancy or autoimmune diseases (Yanaba et al., 2016; Jovanovic et al., 2018). Therefore, the utility of soluble PD-L1 as a cancer biomarker remained as a controversial issue that need more in depth studies to define proper cut-off to differentiate between cancer therapeutic response and other diseases or physiological conditions. The cytokine and chemokine signature in cancer indicated potential predictive value in ICB therapy (Arrieta et al., 2017). Development of a cytokine panel to evaluate ICB response for patient classification in breast cancer seems to be encouraging, for instance, it has been shown that IL-27 up-regulated PD-L1 and promoted breast cancer growth (Yan et al., 2019), yet more studies need to be done to develop a proper panel of cytokines with predictive values in breast cancer. Another plasma protein candidate biomarkers are exosomes, a detective level of RNA molecules and proteins including PDL-1 are packed in exosome and secreted from cancer cells into the blood and lymphatic systems. Exosomes' transmitting PD-L1 can bind to PD-1 on T-lymphocytes and consequently inactivate immune system from attacking cancer cells (Yang et al., 2018; Poggio et al., 2019). Increased level of circulating exosomal PD-L1 is a predictive marker for patient clinical response, e.g., indicating poor prognosis in melanoma patients (Chen et al., 2018). Moreover, as it was mentioned, exosomes and other vesicles contain detective levels of different classes of RNA molecules including, protein-coding RNAs (mRNA) and non-coding RNAs (e.g., miRNA) (Umu et al., 2018). Recent studies demonstrated that miRNAs directly or indirectly regulate the expression of different immune checkpoints on T-cells and on Tumor cells or APCs (Zhang et al., 2019). Hence, miRNAs that specifically control one-target checkpoints are favored in biomarker development. Various miRNAs such as miR-34a, miR-17-5p, miR-15b, miR-193a-3p, miR-197miR-200c showed correlation with expression of PD-L1 in tumor tissues as well as in sera or plasmas, and purposed to have predictive values in ICBs therapy of different cancers (Chen et al., 2014; Cortez et al., 2016; Ahn et al., 2017; Audrito et al., 2017; Kao et al., 2017; Fan et al., 2019). In breast cancer, a panel of thirteen miRNAs has been identified that directly target and down-regulate B7-H3. Among these thirteen miRNAs, expression of miR-29 is associated with higher survival rate of breast cancer patients (Nygren et al., 2014). Therefore, circulating exosomal biomarkers are considerably perceived as a robust source of information – both proteins and nucleic acids e.g., miRNA – to be investigated in breast cancer (Meng et al., 2019).

Circulating tumor cells are considered as important source of liquid biopsy-based biomarkers because they are driven from different sites of a tumor and could provide with more information about overall tumor characteristic. In breast cancer CTC clusters are recognized as a valuable prognostic biomarker and are associated with increasing metastatic potential (Aceto et al., 2014). Importantly, CTCs can interact with neutrophils and form CTC-neutrophil clusters that are proliferative and highly efficient metastatic precursors in breast cancer (Szczerba et al., 2019). The first study on expression of PD-L1 on CTCs was reported in patients with metastatic breast carcinoma (Mazel et al., 2015), and later it was investigated in other types of cancer. Further study on CTC/PD-L1 indicated that the frequency of PD-L1 positive CTCs are significantly higher in metastatic breast cancer patients compared to non-metastatic patients (Schott et al., 2017). These findings suggested CTC/PD-L1 assay as a potential non-invasive marker for stratification of patients benefiting from anti-PD-L1 therapies in clinical trials (Mazel et al., 2015; Schott et al., 2017). The expression of another important immune checkpoint member, B7-H3, on CTCs of breast cancer patients has been also reported. The B7-H3 positive CTCs showed pronounced correlation with Ki-67 expression, a tumor proliferation marker, and were proposed to be a potential biomarker or target for immune checkpoint

blockade therapies in breast cancer (Pizon et al., 2018). CTCs are valuable source of tumor information to develop new biomarkers, however, there are some challenges on the reproducibility of the results which are linked to the applying various techniques for isolation and enriching of these cells. The techniques should be standardized among health service laboratories with the aim to provide qualified analysis of both the epithelial CTCs and the cells undergoing epithelial to mesenchymal transition (EMT), and at the same time to minimize contaminations with other cells such as circulating macrophages with the same surface marker expression. This will enable investigators to establish the CTC biomarkers with predictive or prognostic value in immune therapy.

The ctDNAs have been for decades center of attentions as a crucial source of liquid biopsy-based biomarkers. Today advances in the ctDNAs extraction and enrichments combined with next-generation sequencing (NGS) have contributed to development of valuable biomarkers such as genome instability number (GIN) (Jensen et al., 2019), tumor mutational burden (TMB) and aberrant DNA methylation (Sina et al., 2018) for cancer immunotherapy response stratification of patients.

Different attempts on identification of ctDNA GIN have indicated some merit in therapeutic decision-making (Ahlborn et al., 2019) and in monitoring of breast cancer recurrence (Yang et al., 2019). Nevertheless, the genome-wide analysis of GIN using ctDNA demonstrated GIN dynamic changes upon ICB therapy that enabled monitoring treatment outcome in cancer patients (Jensen et al., 2019). On the contrary, analysis of GIN using ctDNA marked no predictive value in response to ICB therapy for breast cancer (Jensen et al., 2019). Nevertheless, this study was done on a small breast cancer cohort; therefore, to have a meaningful closure about the GIN clinical values, the larger clinical trials should be conducted in breast cancer patients. The TMB assessment in tumor tissues is considered as a promising biomarker, solo or in combination with PD-L1 immunohistochemistry (IHC), with predictive value in immunotherapy efficacy in various types of cancer (Danilova et al., 2016; Ready et al., 2019; Samstein et al., 2019). The utility of ctDNA for assessment of TMBs delivers support for limited or inaccessible tissue samples to improve therapeutic decisions for some cancers (Gandara et al., 2018); however, this is highly dependent on the selection of the gene-targeted panel to evaluate TMBs, and most probably needs to be customized for each cancer type. In addition, the efficiency and precision of the assay using ctDNA to depict TMB is dependent on high coverage of the assay (Gandara et al., 2018; Georgiadis et al., 2019; Pasini and Ulivi, 2019). For breast cancer a NGS panel of mutations associated with 76 target genes, MammaSeq^{TM}, have been recently developed with applicable use of ctDNA. MammaSeq^{TM} showed encouraging result in detection of somatic mutations and monitoring disease burden. However, the assay has limitation in capturing all known mutations associated with cancer and is not specific for rare events in ctDNA (Smith et al., 2019).

DNA methylation changes are key to the development and progression of certain cancers (Chatterjee et al., 2018). Aberrant DNA methylation signature demonstrated great potential to be used as a ctDNA biomarker in cancer (Barekati et al., 2010; Radpour et al., 2011; Sina et al., 2018). It has been shown that DNA hypermethylation of promoter or distal enhancer regions play role in low expression of PD-L1 (Y. Zhang et al., 2018) and demethylation of PDCD1 promoter activates PD-1 expression (Mishra and Verma, 2018). Treating cancer cells with the DNA methyltransferase (DNMT) inhibitors resulted in a better response of anti-CTLA-4 immunotherapy (Chiappinelli et al., 2015). Moreover, other studies indicated that the expression of PD-L1 on cancer cells is associated with global hypomethylation that could play a role in the regulation of PD-L1 expression. This information is emphasizing on potential indication of DNA methylation signatures as biomarkers, which might suggest additional treatments or combination therapies to modulate responsiveness to PD-1 inhibitor treatment (Emran et al., 2019). Furthermore, the genome-wide technology and their corresponding data analysis illustrated a signature that might enable guiding the prediction of ICB immunotherapy response in cancer (Duruisseaux et al., 2018; Guo et al., 2019). However, relatively little attention has been given to develop a customized panel of genomic regions with aberrant methylation patterns for breast cancer.

TUMOR TISSUE BIOMARKERS

A limited number of tumor biomarkers were already assessed in clinical trials of the ICBs approved by the FDA (**Table 1**). In tumor cells both genomic and non-genomic factors are studied as potential biomarkers to predict the response or resistance to ICB therapies. Genomic factors include tumor immunogenicity, mutation/neoantigen-load (Snyder et al., 2014), increased TMB (Keenan et al., 2019), increased PD-L1 level (Havel et al., 2019), interferon gamma (FNγ) response (Ayers et al., 2017), human leukocyte antigen (HLA) diversity, deficient DNA mismatch repair (dMMR) (Zhao et al., 2019), high microsatellite instability (MSI-hi), copy-number alterations, checkpoint regulators e.g., CMTM4/6 (Mezzadra et al., 2017), up-regulation of checkpoint receptors and oncogenic signaling (Havel et al., 2019; Keenan et al., 2019). Non-genomic factors such as gut microbiome (Gopalakrishnan et al., 2018; Routy et al., 2018), metabolic pathway and the activity of lactate dehydrogenase (LDH) (Wein et al., 2018) have been shown to strongly modulate immune responses and success in ICB therapy. Some biomarkers from **Table 1** and other relevant tumor tissue biomarkers for ICB therapies in breast cancer are explained below.

Mutational landscape of the tumor and the neoantigen load are associated with increased immunogenicity which are recognized by T cells. Several studies show that high TMB correlates with enhanced ICB response rates (Keenan et al., 2019). A whole-genome sequencing (WGS) analysis of 442 patients' tumor tissue biopsies from metastatic breast cancer revealed two fold higher TMB compared to primary breast cancer (Angus et al., 2019). They could identify 11% of patient (threshold of $\geq$10 mutations per Mbp) with a high TMB as a potential biomarker to identify clinically relevant subgroups for immunotherapy. Interestingly, high TMB was only associated

TABLE 1 | A list of FDA-approved and validated ICBs targeting PD-1, PD-L1 or CTLA-4 axis with investigational biomarkers to predict an efficient patient response to the immunotherapy.

Targets mAB	ICB name (Trade name)	ICBs in cancer therapy (FDA approved) Combination with chemotherapy (#)	Biomarkers	Breast cancer (FDA unapproved)
PD-1	**Nivolumab** (Opdivo)	Metastatic melanoma (04/07/2014 Japan) and (13/11/2014 United States)	BRAF-V600E CD274 (PD-L1)	**Nivolumab** + short therapy of doxorubicin and cisplatin in TNBC (Voorwerk et al., 2019) + Epigenetic agent RRx-001 in TNBC (NCT02518958)
		Non-small cell lung cancer (09/10/2015) Metastatic non-small cell lung cancer (13/11/2015)		
		Metastatic renal cell carcinoma (23/11/2015)		
		Classical Hodgkin lymphoma (16/05/2016)		
		Metastatic head and neck squamous cell carcinoma (09/11/2016)		
		Advanced urolthelial carcinoma (01/02/2017)		
		Relapsed colorectal cancer (01/08/2017)	MSI-hi, dMMR	
		Advanced liver cancer (22/09/2017)		
		Metastatic small cell lung cancer (17/08/2018)		
	Pembrolizumab (Keytruda)	Advanced or unresectable melanoma (04/09/2014) Adjuvant treatment of stage III melanoma (15/02/2019)	BRAF-V600E	**Pembrolizumab** + a JAK2 inhibitor Ruxolitinib in TNBC (NCT03012230) + a CDK4/6 inhibitor Abemaciclib in HR + /HER2- BC (NCT02779751) + Trastuzumab in HER2 + BC (NCT02318901) + PARP inhibitor Niraparib in TNBC (NCT02657889)
		Squamous and non-squamous non-small cell lung cancer (02/10/2015) Metastatic non-small cell lung cancer (23/10/2016), Stage III non-small cell lung cancer (11/04/2019)	CD274 (PD-L1) EGFR ALK	
		Metastatic head and neck squamous cell carcinoma (04/08/2016) and first-line treatment of this cancer type (10/06/2019)		
		Adult and pediatric patients with refractory or relapsed classical Hodgkin lymphoma (14/03/2017)		
		Advanced non-small cell lung cancer and bladder cancer (09/05/2017) # and advanced bladder cancer (17/05/2017)		
		All metastatic solid tumor types (22/05/2017)	MSI-hi or dMMR	
		Stomach and gastroesophageal cancer (22/09/2017)		
		Advanced cervical cancer (12/06/2018)	CD274 (PD-L1)	
		Adult and pediatric primary mediastinal large B-cell lymphoma (13/06/2018)		
		Advanced hepatocellular carcinoma (09/11/2018)		
		Skin cancer merkel cell carcinoma (19/12/2018)		
		Metastatic small cell lung cancer (17/06/2019)		
		Advanced esophageal squamous cell cancer (30/07/2019)		
		Advanced endometrial carcinoma (27/09/2019)		
		High-risk non-muscle invasive bladder cancer (08/01/2020)		
	Durvalumab (Imfinzi)	Advanced bladder cancer (30/04/2017)		**Durvalumab** + Chemotherapy Taxane-anthracycline TNBC NCT02685059
		Stage III non-small cell lung cancer (16/02/2018)	CD274 (PD-L1)	
	Cemiplimab (Libtayo)	Advanced cutaneous squamous cell carcinoma (09/09/2018)		
PD-L1	**Atezolizumab** (Tecentriq)	Common type of bladder cancer (17/05/2016)		**Atezolizumab** + Trastuzumab in HER2 + BC (NCT02605915)
		Metastatic and resistant non-small cell lung cancer (17/10/2016)	Gene Signature (T-effector), ALK	
		Metastatic triple-negative breast cancer (08/03/2019) # + Nab-paclitaxel (NCT02425891)	CD274 (PD-L1)	
		Extensive-stage small cell lung cancer (18/03/2019)#		
		Metastatic non-small cell lung cancer (03/12/2019)	No EGFR or ALK aberrations	
	Avelumab (Bavencio)	Skin cancer merkel cell carcinoma (22/03/2017)		**Avelumab** ± CDK4/6 inhibitor Palbociclib + Tamoxifen in ER + BC (NCT03573648) + Fulvestrant in ER + /HER2- BC (NCT03147287)
		Advanced bladder cancer (08/05/2017)		
		Advanced renal cell carcinoma (14/05/2019)#	CD274 (PD-L1)	

(Continued)

TABLE 1 | Continued

Targets mAB	ICB name (Trade name)	ICBs in cancer therapy (FDA approved) Combination with chemotherapy (#)	Biomarkers	Breast cancer (FDA unapproved)
CTLA-4	**Ipilimumab** (Yervoy)	Metastatic melanoma (13/11/2011)	HLA-A	
	Tremelimumab (Ticilimumab)	Orphan drug status for the treatment of malignant mesothelioma (20/04/2015)		
PD-1 and **CTLA-4**	**Nivolumab** (Opdivo) and **Ipilimumab** (Yervoy)	Advanced melanoma (01/10/2015)	BRAF-V600E HLA-A	
		Advanced renal cell carcinoma (16/04/2018)		
		Relapsed or refractory colorectal cancer (10/07/2018)	MSI-hi or dMMR	

ICB, Immune-checkpoint blockade; mAB, monoclonal antibody; BC, breast cancer; NCT, NIH clinical trial research study; BRAF-V600E, BRAF mutations of valine 600 to glutamic acid; MSI-hi, high microsatellite instability; dMMR, deficient DNA mismatch repair; HLA-A, a group of human leukocyte antigens; ALK, anaplastic lymphoma kinase and #, in combination with chemotherapy.

with metastatic tissue and it was equal between breast cancer subtypes and biopsy sites.

The DNA mismatch repair (MMR) system is crucial for genomic integrity and stability and it prevents microsatellite instability (MSI). Tumors with dMMR and MSI-hi are more sensitive to anti-PD-1/PD-L1 therapies (Zhao et al., 2019). This occurs due to mutation or loss of function of DNA repair proteins. Some data show that dMMR is more frequent in early stage cancers than in metastatic cancers, which is important for the selection of the best time point for ICB therapy. Although dMMR and MSI-hi are used as predictive biomarkers for Pembrolizumab therapy of all metastatic solid tumor types, both biomarkers are rarely present in most breast tumors (Mills et al., 2018), except BRCA-deficient TNBC. BRCA1- and BRCA2-deficient breast cancers are characterized by vast genomic instability and T cell-inflamed signature. BRCA1-deficient tumors indicated high expression of PD1 and PD-L1 (Wen and Leong, 2019) and similar to TNBC, seems to have best response to ICBs, especially in combination with cytotoxic agents.

PD-L1 expression in tumor cells and TILs using immunostaining-scoring methods have been associated with response to blockade of the PD-1/PD-L1 axis (Dolled-Filhart et al., 2016; Torlakovic et al., 2020). However, there is no clear-cut between separation of responders and non-responders patients. A portion of PD-L1^{-} tumors still responds to ICB therapy and on the other hand not all PD-L1^{+} tumors are responsive to ICB therapy. PD-L1 overexpression is correlated with copy-number alteration of 9p24.1 locus containing PD-L1, PD-L2 and JAK2 (Green et al., 2010). In addition, PD-L1 protein levels and stability in tumors can be increased using inhibitors of cyclin-dependent kinases 4 and 6 (CDK4/6). It has been shown that PD-L1 protein abundance is regulated by CDK4 and cullin 3-SPOP E3 ligase via proteasome-mediated degradation (Zhang et al., 2018). To increase the ICBs efficiency in breast cancer, some clinical studies used a combination of JAK2 or CDK4/6 inhibitors with anti-PD1 therapy and evaluated the safety and efficacy of these combinational therapies in patients (NCT03012230, NCT02779751, **Table 1**).

Oncogenes such as mutated BRAF, EGFR and KRAS and amplified HER2 and loss of tumor suppressor genes e.g., PTEN often regulate inflammatory and immune suppressive cytokines like IL-6 and affect ICB response rates (Keenan et al., 2019). IFNγ is released by activated T cells upon recognition of tumor neoantigens and activates IFNγ-JAK-STAT-IRF1 axis in tumor cells. Alteration of this pathway affects the response to ICBs via different mechanisms such as increasing the expression levels of HLA and induction of PD-1 and PD-L1 gene expression by direct binding of IRF1 and STAT3 to their promoters (Garcia-Diaz et al., 2017; Keenan et al., 2019).

Besides TMB and the expression levels of IFNγ and PD-L1 as dynamic biomarkers for ICB therapy in breast cancer, multi-gene based assays to develop combinational ICB biomarkers are required. Similar assays e.g., Oncotype DX (Paik et al., 2004), MammaPrint (van't Veer et al., 2002) and Prosigna (Parker et al., 2009) have been previously used for the prediction of chemo and targeted therapy benefit. The current assays such as Enzyme-Linked Immunosorbent Spot (ELISpot) for the detection of cytokine and IFNγ secreting cells show limited sensitivity in assessing tumor-specific T-cell responses. However, a new assay "Mutation-Associated Neoantigen Functional Expansion of Specific T cells" (MANAFEST) allows a sensitive measurement of antigen-specific TCR clonotypic amplifications following treatment in blood, tumor, and normal tissue of patients receiving immunotherapy (Danilova et al., 2018).

TUMOR MICROENVIRONMENT BIOMARKERS

Extracellular matrix (ECM) changes are predicted as prognosis factors that are correlated with immunological activity. ECM dysregulation is often linked to the presence of cancer-associated fibroblasts (CAFs) expressing activated TGFβ signaling (Chakravarthy et al., 2018). The cytokine TGFβ is the major mediator of immune suppression in the tumor microenvironment and has a central role in inhibition of the both adaptive and innate immune responses during tumor progression (Batlle and Massague, 2019). Reprogramming of CAFs and anti-TGFβ therapies can enhance checkpoint blockade. A recent preclinical study has shown that the lack of response to Atezolizumab therapy was associated with TGFβ signature in fibroblasts and exclusion of CD8^{+} T-effector cells from tumor parenchyma in metastatic urothelial tumors (Mariathasan et al., 2018). Several studies revealed that combinational therapy of

both TGFβ and PD-L1 resulted in synergistic anti-tumor effect in both breast and colorectal cancers (Knudson et al., 2018; Tauriello et al., 2018). A novel bifunctional anti-PD-L1/TGFβ Trap fusion protein (M7824) was tested in EMT6 and 4T1 syngeneic mouse breast cancer models. M7824 decreased TMB and promoted $CD8^+$ T cell and NK cell activation (Knudson et al., 2018). In a neoadjuvant setting, M7824 is used in treating patients with stage II-III $HER2^+$ Breast Cancer (NCT03620201). Besides T-cell exclusion based on TGFβ-activated stroma, Wnt-β-catenin signaling plays a role in T-cell activation and $CD8^+$ T-effector cells migration by decreasing $CD103^+$ dendritic cell (DC) recruitment (Spranger et al., 2015). In a melanoma model, this migration depends on the presence of DC producing CXCL10 (Spranger et al., 2017). Importantly, anti-PD-1 efficacy depends on CXCR3 activity, which is a receptor for CXCL9, CXCL10, and CXCL11 chemokines. These CXCR3 ligands are identified as positive predictive biomarkers and their induction in non-responsive mouse tumors could restore the sensitivity to anti-PD-1 (Chow et al., 2019).

Vascular endothelial growth factor A (VEGFA) and angiopoietin-2 (ANG-2) produced by endothelial cells affect antitumor immunity. In a metastatic mouse mammary tumor model (MMTV-PyMT), Schmittnaegel et al. (2017) could show that a dual inhibition of both angiogenic factors resulted in an increase of tumor antigen presentation, activation of tumor-infiltrating CD8 + T cells, and induction of endothelial PD-L1 expression through IFNγ. Simultaneous blocking of PD-L1/PD-1 signaling in this tumor model improved antitumoral activity and increased survival rate by 30% in mice (Schmittnaegel et al., 2017).

Infiltrating and tumor-associated immune cells are the major component of tumor-associated stroma with both protumor and antitumor activities. An increase in peripheral $ICOS^+CD4^+$ T cells has been also shown as a good clinical ICB responses in patients with hormone-responsive advanced breast cancer, which were treated with the anti-CTLA4 tremelimumab in combination with exemestane (Vonderheide et al., 2010).

The efficiency of ICBs is highly based on TILs and we need a better understanding of molecular determinants of TILs phenotype in tumor and tumor microenvironment. Pre-existing of immune response in tumors and localization and density of TILs are strong prognostic indicators for selection of ICB-responsive patients in different cancer types including breast cancer (Wein et al., 2018). In addition to immunohistochemistry methods to study TILs density, single cell RNA sequencing provides high-resolution to study the immune cell diversity and tumor heterogeneity related to ICB responses. Recently, Jiang et al. (2018) have developed a computational tool for dysfunctional T-cell signature, called "Tumor Immune Dysfunction and Exclusion" (TIDE). They could show that TIDE signatures predict ICB immunotherapy response in melanoma patients treated with first-line anti-PD1 or anti-CTLA4. The TIDE tool predicts only intrinsic ICB resistance and models two distinct mechanisms of tumor immune evasion: (i) T cell dysfunction in tumors with high infiltration of cytotoxic T lymphocytes (CTL), and (ii) T cell exclusion and prevention of T cell infiltration in tumors with low CTL level.

The crosstalk between cancer cells and immune cells at the primary tumor site, in the circulation and in the metastatic niche has a strong influence on cancer progression that affects patients' response to ICBs (Saini et al., 2019). In a recent study, Wagner et al. (2019) performed large-scale mass cytometry profiling of 144 human breast tumor and 50 non-tumor tissue samples and characterized features of cancer ecosystems, inter-patient variations in tumor-associated immune cells and their associations with clinical data. They could show that $PD\text{-}L1^+$ tumor-associated macrophages (TAMs) and exhausted T cells are abundant in high-grade ER^- and ER^+ tumors. This single-cell mass spectrometric approach called mass cytometry ($CyTOF^{TM}$) can be combined with immunohistochemical methods, which were used for multiplexed imaging of tumor tissues and with subcellular resolution (Giesen et al., 2014).

Reprogramming of tumor immune microenvironment presents a powerful strategy to enhance the response to anti-PD1/PD-L1 in different type of breast cancer. Recently, Voorwerk et al. (2019) showed that a short-term treatment with doxorubicin and cisplatin was able to reprogram tumor microenvironment. This caused up-regulation of inflammatory JAK-STAT and TNF-α signaling and increased the sensitivity to the Nivolumab in metastatic TNBC (Voorwerk et al., 2019).

FUTURE BIOMARKER CHALLENGES

Scientist and healthcare professionals have gained and explained a vast knowledge about potential predictive biomarkers for ICB patient's classification. The most promising biomarkers have been presented as proteomic and transcriptomic signatures of exosomes and CTCs, genomic analysis of ctDNA (genome instability number, specific tumor mutations, and aberrant DNA methylation signature). The next crucial step is the clinical verification of these candidate biomarkers that requires a consensus on methodological standardization of the assays and in parallel to investigate these biomarkers in large patient populations.

The interaction between tumor cells and immune cells in TME leads to the dynamic change of immunotherapy targets. This is a challenging factor for the identification of appropriate biomarkers for the selection of drug responsive patients. Biological understanding of multigene-based biomarkers and combinational strategies for ICB biomarkers will give healthcare providers the opportunity to increase the effectiveness of immune therapy in breast cancer. Advanced technologies such as single cell RNA sequencing and CyTOF-based immune profiling provide high-resolution of tumor immune microenvironment. Enhancing the drug response by remodeling of dynamic tumor ecosystem is fundamental for a successful personalized cancer therapy.

AUTHOR CONTRIBUTIONS

Both authors wrote the manuscript without external help. VV prepared the table.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 17 March 2020
doi: 10.3389/fcell.2020.00146

The STRIPAK Complex Regulates Response to Chemotherapy Through p21 and p27

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The STRIPAK complex has been linked to a variety of biological processes taking place during embryogenesis and development, but its role in cancer has only just started to be defined. Here, we expand on previous work indicating a role for the scaffolding protein STRIP1 in cancer cell migration and metastasis. We show that cell cycle arrest and decreased proliferation are seen upon loss of STRIP1 in MDA-MB-231 cells due to the induction of cyclin dependent kinase inhibitors, including p21 and p27. We demonstrate that p21 and p27 induction is observed in a subpopulation of cells having low DNA damage response and that the $p21^{high}/\gamma H2AX^{low}$ ratio within single cells can be rescued by depleting MST3&4 kinases. While the loss of STRIP1 decreases cell proliferation and tumor growth, cells treated with low dosage of chemotherapeutics *in vitro* paradoxically escape therapy-induced senescence and begin to proliferate after recovery. This corroborates with already known research on the dual role of p21 and indicates that STRIP1 also plays a contradictory role in breast cancer, suppressing tumor growth, but once treated with chemotherapeutics, allowing for possible recurrence and decreased patient survival.

Keywords: breast cancer, STRIPAK, cell cycle, p21, p27, DNA damage response, chemotherapy

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *15 November 2019*
Accepted: *21 February 2020*
Published: *17 March 2020*

Citation:
Rodriguez-Cupello C, Dam M, Serini L, Wang S, Lindgren D, Englund E, Kjellman P, Axelson H, García-Mariscal A and Madsen CD (2020) The STRIPAK Complex Regulates Response to Chemotherapy Through p21 and p27. Front. Cell Dev. Biol. 8:146. doi: 10.3389/fcell.2020.00146

INTRODUCTION

Over the past few years, extensive functional and mechanistic research has been conducted to resolve the framework of the Striatin Interacting Phosphatase and Kinase (STRIPAK) complex. The accumulated findings have linked specific components of the complex to various biological functions including vesicular trafficking (Zhang et al., 2013; Lant et al., 2015), Golgi assembly (Kean et al., 2011), Hippo signaling (Ribeiro et al., 2010; Zheng et al., 2017), autophagy (Huang et al., 2017), cell migration (Madsen et al., 2015; Bazzi et al., 2017), and cell cycle control (Cornils et al., 2011; Frost et al., 2012; Kazmierczak-Baranska et al., 2015; Pandey et al., 2017). Substantiated by these findings, the STRIPAK complex is supervising embryogenesis and development (Lant et al., 2015; Madsen et al., 2015; Sakuma et al., 2015, 2016; Bazzi et al., 2017; Pal et al., 2017; Zheng et al., 2017), circadian rhythms (Andreazza et al., 2015), type 2 diabetes (Chursa et al., 2017), and progression of cancer (Wong et al., 2014; Zhang et al., 2014; Madsen et al., 2015; Huang et al., 2017).

The STRIPAK complex is an evolutionarily conserved supramolecular complex; holding the PP2A phosphatase in complex with its striatin-family of regulatory subunits (STRN, STRN3, STRN4), the two hippo kinases (MST1/MST2), the three GCKIII kinases (MST3, MST4, SOK1)

and various scaffolding proteins (Glatter et al., 2009; Goudreault et al., 2009; Ribeiro et al., 2010; Couzens et al., 2013). It is believed that the scaffolding proteins, including SLMAP, SIKE, STRIP1 (FAM40A), STRIP2 (FAM40B), direct and uphold PP2A/Striatin phosphatase specificity, and loss of these proteins consequently disassemble the STRIPAK complex; leading to hyper-phosphorylation of PP2A/Striatin target proteins. This is, for example, observed upon loss of SLMAP, which induces hyper-phosphorylation of MST1/2 kinases (Bae et al., 2017; Zheng et al., 2017; Tang et al., 2019), while loss of STRIP1 induces hyper-phosphorylation of MST3/4 kinases (Madsen et al., 2015).

The *in vivo* function of STRIP1 has been described in multiple eukaryotic organisms. In the filamentous fungus *Neurospora crassa*, the *Strip1* homolog is important for hyphal fusion (Xiang et al., 2002) and required for normal recovery from pheromone arrest in G1 of the cell cycle (Kemp and Sprague, 2003). In yeast, the *Strip1* homolog connects the Golgi, the centrosome, and the nuclear envelope to organize mitotic progression (Frost et al., 2012). The yeast homolog also antagonizes mTORC2 signaling by promoting dephosphorylation of TORC2 substrates (Pracheil et al., 2012). In *Drosophila melanogaster*, the *Strip1* homolog regulates border cell migration (Madsen et al., 2015), serves as a molecular linker for early endosome organization in axon elongation (Sakuma et al., 2014), and regulates the circadian clock by dephosphorylating the circadian oscillator CLOCK during daytime (Andreazza et al., 2015). The *Strip1* homolog in the fruit fly has also been linked to cell proliferation by antagonizing Hippo signaling and by supporting RAS/MAPK signaling (Ashton-Beaucage et al., 2014). In the mouse embryo, loss of *Strip1* arrests mesoderm migration after the gastrulation epithelial-to-mesenchymal transition (Bazzi et al., 2017). Indeed, STRIP1 has been shown to regulate cytoskeleton dynamics and cell migration on several occasions (Bai et al., 2011; Sakuma et al., 2015, 2016; Suryavanshi et al., 2018). We discovered that the STRIPAK complex is an important and ancient regulator of plasticity of cell migration during both developmental processes and cancer metastasis (Madsen et al., 2015). We demonstrated that loss of STRIP1 induces strong activation of the two MST3&4 kinases, consequently inducing breast cancer cells to metastasize using actomyosin-driven amoeboid migration. These data were the first to demonstrate that perturbation of STRIP1 could affect tumorigenesis in breast cancer (Madsen et al., 2015). In this paper, we continue to elaborate on the molecular and biological functions of STRIP1 and MST3&4 in breast cancer. We show that loss of STRIP1 induces the expression of cyclin dependent kinase inhibitors (CKI) including CDKN1A (p21), which leads to cell cycle arrest and reduced tumor growth. Surprisingly the strong induction of p21 also has an inconvenient effect if cells are treated with chemotherapeutic, as it promotes a proliferative cell fate rather than inducing a senescent phenotype when treated with sub-lethal doses of chemotherapeutics.

MATERIALS AND METHODS

Cell Culturing and Transfections

Human MDA-MB-231 breast cancer cells (ATCC) were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin under 5% CO2 and 37°C. siRNA transfections were performed using Lipofectamine 2000 (ThermoScientific). In brief, cells were subjected to transfection in serum-free OptiMEM using 25 nM siRNA. After 24 h of transfection, the cells were re-plated for subsequent analyses. Seventy-two hours post-transfection, cells were collected for flow cytometry, immunoblotting, or fixed for immunofluorescence. The following siRNAs were used in the study: Hs_FAM40A_2 FlexiTube siRNA (SI00383796, Qiagen), Hs_FAM40A_5 FlexiTube siRNA (SI04198789, Qiagen), Hs_FAM40A_7 FlexiTube siRNA (SI04295949, Qiagen), STRIP1_35 (s39935, ThermoFisher), STRIP1_36 (s39936, ThermoFisher), Hs_FAM40B_7 FlexiTube siRNA (S104300618, Qiagen), siGENOME Human STK24 (MST3) siRNA (D-004872-23, Horizon Discovery), siGENOME Human STK26 (MST4) siRNA (D-003753-04, Horizon Discovery), siGENOME Human STK25 siRNA (D-004873-02, Horizon Discovery), siGENOME Human PDCD10 (CCM3) siRNA (D-004436-01, Horizon Discovery), CDKN1A_01 (s417, ThermoFisher), CDKN1A_02 (s415, ThermoFisher), CDKN1B_01 (s2837, ThermoFisher), and CDKN1B_02 (s2838, ThermoFisher). Treatment with Doxorubicin (Sigma) and Cisplatin (Merck) for high dosage were supplemented into culture media at 1 μM for 6 h, beginning 72 h post transfection. For senescence and recovery with low dosage, doxorubicin and cisplatin were supplemented at 50 nM and 250 nM, respectively, for 24 h, beginning 48 h post-transfection, and allowed to recover in normal media for another 96 h.

RNA-Sequencing

Total RNA was prepared 72 h post-transfection using RNeasy (Qiagen), according to the manufacturer's instructions. RNA was treated with DNase I on the columns before eluting the RNA. RNA-sequencing was conducted on samples from 3 independent experiments. Quality control of the RNA and RNA-sequencing was performed by The Eukaryotic Single Cell Genomics facility, Lund University. Bioinformatic validation and quantifications were performed in house. GSEA analysis was performed using Broad Institute analysis software and publicly available gene sets. All analyses were run using 1,000 permutations.

The RNA-sequencing data generated in this study have been deposited in the GEO database under accession GSE145618.

Proliferation Analysis

Proliferation curves of cells were based on cell count analysis after siRNA-transfection, beginning 2 days post-transfection where the gene and protein knockdown was at its maximum. Proliferation with drug usage was performed using ethynyl-20-deoxyuridine (EdU) Proliferation Kit (Abcam) according to manufacturer's protocol. For immunofluorescence staining, a final concentration of 40 μM EdU was supplemented to cells in culture medium for 2 h prior to fixation. In total hundreds to thousands of cells were quantified per siRNA transfection, by assessing 50–100 cells per image, with 5 images per condition, and at least 3 independent repeats. For flow cytometry analysis, a final concentration of 20 μM EdU was supplemented to

cell in culture medium for 2 h prior to harvesting. Gating protocol for EdU proliferation analysis was performed according to manufacturer's protocol using approximately 10,000 cells per repeat.

Immunoblotting

Western blotting was performed according to standard procedures. Cells were washed with ice-cold Dulbecco's phosphate-buffered saline (PBS) and then lysed using 1 × Laemmli buffer with 50 mM DTT and further processed through sonication using Biorupture (Diagenode). The samples were resolved in 4–20% Tris-Glycine gels (Invitrogen) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham) using wet transfer. PVDF membranes were blocked with 5% milk in Tris-buffered saline with 0.02% Tween20 (TBS-T) for 1 h and then probed with primary antibodies diluted in 3% BSA in TBS-T overnight, and subsequently with secondary antibody conjugated to horseradish peroxidase diluted in 5% milk, TBS-T for 1–2 h. The specific proteins were detected with Amersham Imager 600 (GE Healthcare) after incubation with Luminata Crescendo/Forte Western HRP substrate (EMD Millipore). The following antibodies were used for western blotting: FAM40A/STRIP1 (ab199851, Abcam, 1:250), p21 (sc6246, Santa Cruz Biotechnologies, 1:500), p27 (sc1641, Santa Cruz, 1:500), cyclin A (sc271682, Santa Cruz, 1:1000), phospho-RB (8516, Cell Signaling, 1:1000), phospho-LATS1(Ser909) (9157, Cell Signaling, 1:1000), pMST1/2 (3681, Cell Signaling, 1:1000), pGCKIII [Anti-MST4 + MST3 + STK25 (phospho T174 + T178 + T190)] (ab76579, Abcam, 1:1000), phospho-AKT (Ser473) (4060, Cell Signaling, 1:1000), AKT (4691, Cell Signaling, 1:1000), phospho-GSK-3β (5558, Cell Signaling, 1:1000), γH2AX (2577, Cell Signaling, 1:400), tubulin (5335, Cell Signaling, 1:50000), anti-Rabbit HRP (7074, Cell Signaling, 1:2000), and anti-Mouse HRP (7076, Cell Signaling, 1:2000).

Flow Cytometry for Cell Cycle

Cells were collected by trypsinization and fixed with ice-cold 70% ethanol. After washing, the cell pellet was resuspended in a staining solution of 50 μg/ml propidium iodide (PI) (Sigma) and 100 μg/ ml RNase in PBS. The cell cycle phase of approximately 10,000 cells was determined by FACSverse (BD Biosciences) and further analyses of collected data points was performed using FlowJo. The whole cell population is first gated (R1) according to forward and side scatter. Further gating is performed by measuring the area (PI-A) and the width of the collected PI signal (PI-W) for removal of apoptotic cells and doublets (R2). Single cells are then sorted into subpopulations G0/G1, S, and G2/M, represented in a histogram with PI-A on the x-axis. For nocodazole treatment of cells, 200 nM of nocodazole (Sigma) was added to culture medium 18 h prior to collection.

Serum Starvation for Inducing Hippo Signaling

Glass bottom culture plates (Mattek) were coated with collagen/matrigel and allowed to polymerize for 1 h prior to addition of cells. Collagen/matrigels were made with 10% FBS, 40% Rat-tail collagen I (Corning), 20% Matrigel Basement Membrane Matrix (Corning), a 5X collagen buffer, and culture media. Cells were plated 24 h post-transfection and incubated for a further 48 h. Prior to fixation, cells were placed in serum-free media for either 30 or 60 min. At least 5 images were taken of each condition.

Immunofluorescence and Confocal Microscopy

Cells were plated on glass bottom culture plates (Mattek) for confocal microscopy. The cells were PFA-fixed 72 h post-transfection. The cells were permeabilized in 0.2% Triton X-100, PBS and then blocked with 3% BSA, PBS prior to overnight staining with primary antibodies diluted in 1% BSA, PBS. A cocktail of AlexaFluor-conjugated secondary antibodies (ThermoFisher, 1:400) with DAPI (Sigma, 1:500) and Phalloidin-TRITC (Sigma, 1:500) were then added to the samples. All fluorescent images were acquired using a Leica SP8 or a Zeiss LSM710 confocal microscope. Five images per condition were taken, containing 50–100 cells each, for each repeat, with at least 3 independent repeats. The following antibodies were used: YAP (sc101199, Santa Cruz Biotechnologies, 1:100), p21 (sc6246, Santa Cruz Biotechnologies, 1:50), p27 (3686, Cell Signaling, 1:800), and γH2AX (2577, Cell Signaling, 1:800).

Generation of Stable Crispr/Cas9 Knockout Cell Lines

The lentiCRISPRv2 (Gecko, Addgene) was used as described previously (Garcia-Mariscal et al., 2018). Single-guide RNAs (sgRNAs) targeting genes of interest coding regions were designed and cloned into the lentiviral vector lentiCRISPRv2 (Gecko, Addgene) according to the manufacturer's instructions. All sgRNAs used were selected for low off-target efficiency using algorithms at crispor.tefor.net. The oligonucleotide sequences corresponding to the sgRNAs were: STRIP1 sgRNA#1: F: 5′-CACCGCTGGTTGCGGTTGAACTCGC-3′, R: 5′-AAACGCGAGTTCAACCGCAACCAGC-3′: STRIP1 sgRNA#2: F: 5′-CACCGTGTTTGTTGTTCACGATCAG-3′, R: 5′- AAACCTGATCGTGAACAACAAACAC-3′; STRIP1 sgRNA#3: F: 5′- CACCGAGCCGCACAGCCACCACCCG-3′, R: 5′-AAACCGGGTGGTGGCTGTGCGGCTC-3′; STRIP1 sgRNA#4: F: 5′-CACCGCTATTCGGAGTCACCAGACC-3′, R: 5′-AAACGGTCTGGTGACTCCGAATAGC-3′. HEK293T cells were used for the lentivirus production by transfection of lentiCRISPRv2 vector together with pCMV-VSV-G and psPAX2 using Lipofectamine 2000 according to the manufacturer's instructions. Next day, HEK medium was exchanged and after 24 h, the supernatant containing the viral particles were collected, mixed (1:1) with fresh medium containing 8 μg/ml polybrene (Sigma Aldrich), and added to the MDA-MB-231 cells. The MDA-MB-231 cells were infected with lentivirus for 24 h, before exchanging with fresh medium containing 1 μg/ml puromycin for 5 days. The gene-modified cells were not purified further, and therefore used as a pool for the subsequent experiments. All four sgRNAs were validated and sgSTRIP1#3 was chosen for the animal experiments.

Animal Experiments

All experiments were carried out according to institutional guidelines and approved by the local ethics committee in Lund, permit number 12562/2018. Female NSG mice were purchased from Jackson. Mice (8 weeks old) were orthotopically injected into the 4th inguinal mammary fat pad with 1×10^6 MDA-MB-231-CRISPR^{CONTROL} cells on the left side and 1×10^6 MDA-MB-231-CRISPR^{STRIP1} cells on the right side. Tumors were removed 17 days post-injection. The tumor volume was calculated accordingly: volume = width^2 × length × 0.52.

Image Analysis

Quantification of western blots and immunofluorescence images was performed using ImageJ/Fiji software (imagej.net/Fiji). At least 5 images were taken for each condition for at least 3 repeats. Cell number was obtained through DAPI staining and software calculation by creation of regions of interest (ROIs) for each nucleus, with a 50-pixel exclusion. Nuclear ROIs were used to obtain mean nuclear staining of p21 and γH2AX. In house macros for Fiji software were created to unbiasedly extract intensity values of staining for images.

Statistical Analysis

All graphs and statistical tests were created using GraphPad Prism. All graphs are depicted as mean ± SD. Statistical tests were performed using one-way or two-way ANOVA or unpaired student's *t*-test (two-tailed). All tests were performed at least three independent times. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

RESULTS

Loss of STRIP1 Arrests Cells in the G1-Phase of the Cell Cycle

We previously demonstrated that the STRIPAK complex is an important regulator of breast cancer cell migration and metastasis in mouse models (Madsen et al., 2015). Interestingly, previous findings also suggest that the STRIPAK complex may, as well, perturb the cell cycle. We therefore decided to investigate if loss of individual STRIPAK components would interfere with the cell cycle. A flow cytometry cell cycle analysis of MDA-MB-231 breast cancer cells was conducted 72 h after siRNA-depletion of individual STRIPAK genes known to regulate cancer cell migration and metastasis. The siRNA-mediated gene-depletion was conducted with already validated siRNAs (Madsen et al., 2015) and demonstrated that loss of STRIP1 significantly increased the numbers of cells arrested in the G0/G1 phase of the cell cycle, while lowering the numbers of cells in S and G2/M, as compared to the siRNA control (siAllstar) cells (**Figures 1A,B**). The siRNA-depletion of STRIP2 and CCM3 induced minor differences, while loss of MST3 did not affect the cell cycle, probably due to redundancy from MST4 (Madsen et al., 2015). We decided to focus on the role of STRIP1 due to its strong impact. As the flow analysis was conducted using a smart pool of STRIP1 siRNAs, we confirmed our findings using four individual siRNAs, obtained from different companies (**Figure 1C**). Immunoblotting analysis validated that all siRNAs successfully depleted the STRIP1 protein (**Figure 1D**).

We next set out to investigate whether the cells were able to progress through the cell cycle by treating them with nocodazole, an agent that interferes with the polymerization of microtubules. Thus, adding nocodazole to proliferating cells will arrest them in G2/M phase due to the spindle assembly checkpoint. siRNA-depleted MDA-MB-231 cells were treated with 200 nM nocodazole for 18 h before collecting them for flow cytometry. Approximately 70% of control cells were found to be in G2/M phase after nocodazole treatment (**Figure 1E**). On the contrary, close to 60% of STRIP1-depleted cells were still found in the G0/G1 phase, emphasizing the role of STRIP1 in the G1-exit of the cell cycle (**Figure 1E**). We then tested if loss of STRIP1 affected overall cell proliferation. Our data shows that loss of STRIP1 slightly reduced proliferation rate (**Figure 1F**), while the numbers of apoptotic cells were unchanged, according to the flow cytometry analysis (**Figure 1A**, inset). These data demonstrate that loss of STRIP1 maintain or prolong cells in the G1-phase and, as a consequence, lower the net proliferation of MDA-MB-231 cells. To substantiate these findings, we genetically manipulated MDA-MB-231 cell using CRISPR/Cas9 technology. The knockdown efficiency was validated, and MDA-MB-231-CRISPR^{CONTROL} and -CRISPR^{STRIP1} cells were implanted orthotopically into the mammary fat pad of NSG mice and the tumor size quantified (**Figure 1G**). These findings demonstrate that loss of STRIP1 reduces cell proliferation and tumor growth.

Loss of STRIP1 Induces Expression of CDK Inhibitors p21 and p27

Cells in the G1 phase are preparing to enter the S-phase, but must ensure that the genome is undamaged and that there are enough resources to replicate the DNA (Bertoli et al., 2013). The G1 checkpoint is regulated by cyclin-dependent kinase (CDK) inhibitors that physically interact and inhibit the activity of CDKs, thus preventing the cells from entering the cell cycle prematurely (Bertoli et al., 2013). Cyclins are proteins that control cell cycle progression by activating CDKs. In early G1 phase, CDK4/6 interacts with cyclin D to mono-phosphorylate Retinoblastoma (RB). The phosphorylation of RB is further enhanced by the cyclin E/CDK2 complex, and as RB gradually becomes more phosphorylated throughout the G1 phase, it dissociates from the transcription factor E2F, allowing E2F to drive the expression of genes needed to enter the S phase and for the initiation of DNA replication (Bracken et al., 2004; Bertoli et al., 2013).

We performed RNA sequencing of siRNA-depleted MDA-MB-231 cells to look for cell cycle regulated changes. Gene set enrichment analysis (GSEA) of STRIP1-depleted cells demonstrated a general decrease in E2F-target genes responsible for the G1/S transition (**Figure 2A**). The analysis identified CCND2 (cyclin D2), CCNE1 (cyclin E1), as well as CDK2 and CDK4 to be downregulated after loss of STRIP1 (**Figure 2B**). On the contrary, the expression of all members of the CIP/Kip family of CDK inhibitors; CDKN1A (p21), CDKN1B (p27), and CDKN1C (p57), were all increased, while the INK4 family of

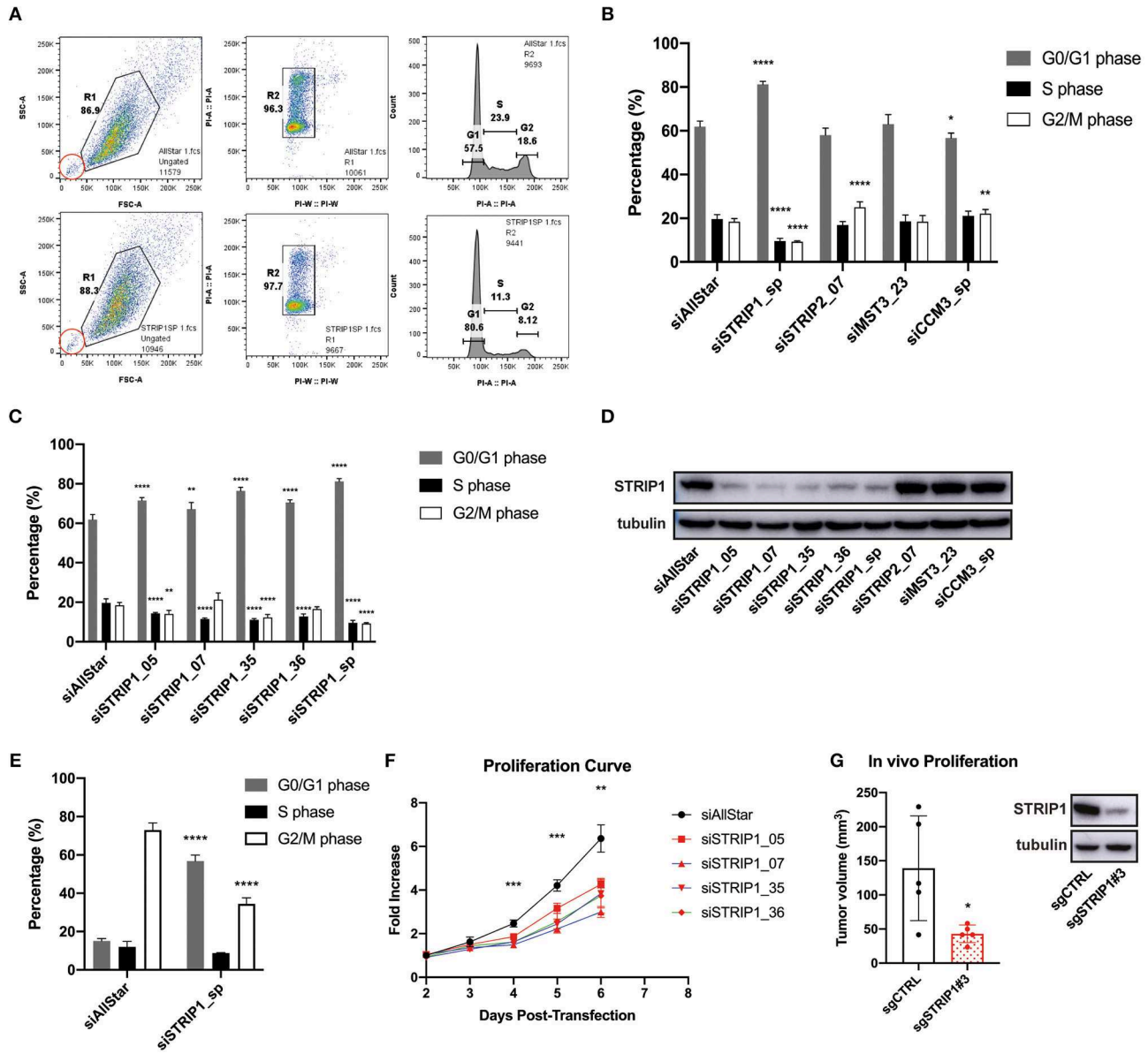

FIGURE 1 | Loss of STRIP1 arrests cells in the G1-phase of the cell cycle. **(A)** Gating strategy of cell cycle analysis using flow cytometry of control/AllStar (top) and STRIP1 depleted (bottom) cells. MDA-MB-231 cells were stained with propidium iodide (PI) to analyze the DNA content. Side scatter area (SSC-A) describes the granularity of each passing cell, forward scatter area (FSC-A) measures the cell size. A population of cells (R1) is gated (left) and the cells are plotted according to the area and width of detected PI (middle). Three subpopulations (R2) describe the amount of DNA content (G0/G1, S, G2/M) and can be better visualized in a histogram (right). Inset, in red, indicates unchanged quantity of apoptotic cells between control and STRIP1 depleted cells. **(B)** Cell cycle analysis of MDA-MB-231 cells siRNA-depleted for core components of the STRIPAK complex incl. STRIP1, STRIP2, MST3, and CCM3. Depletion of STRIP1 leads to a greater percentage of cells in the G0/G1 phase compared to control. A smart pool (sp) of differently targeted siRNAs were used for STRIP1 and CCM3. **(C)** Cell cycle analysis of multiple STRIP1 siRNAs and combined smart pool (sp). The use of individual siRNAs for STRIP1 similarly result in a higher percentage of cells in G0/G1 phase compared to control. **(D)** STRIP1 protein knockdown efficiency of siRNA-depleted MDA-MB-231 cells was demonstrated by immunoblotting. **(E)** Cell cycle analysis of control and STRIP1 depleted MDA-MB-231 cells after treatment with nocodazole for 18 h to synchronize cells in G2/M phase. Loss of STRIP1 maintains the cells at a G0/G1 arrest. All cell cycle analyses were conducted at least three independent times. **(F)** Cell proliferation assay of STRIP1 siRNA-depleted MDA-MB-231 cells. Proliferation analysis was performed at least five independent times. Statistical analysis using two-way ANOVA was performed on siSTRIP1_05 compared to siAllStar, $^{**}P < 0.0021$ and $^{***}P < 0.0002$. **(G)** MDA-MB231 cells were genetically modified using CRISPR/Cas9 for removal of STRIP1 and orthotopically injected into the mammary fat pad of NSG mice. Tumor volume quantification shows decreased size with loss of STRIP1. Results obtained are from five individual animals carrying one tumor of each. Knockout efficiency of CRISPR cells was demonstrated by immunoblotting. All statistical tests of the cell cycle analyses were performed using one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ to siAllStar.

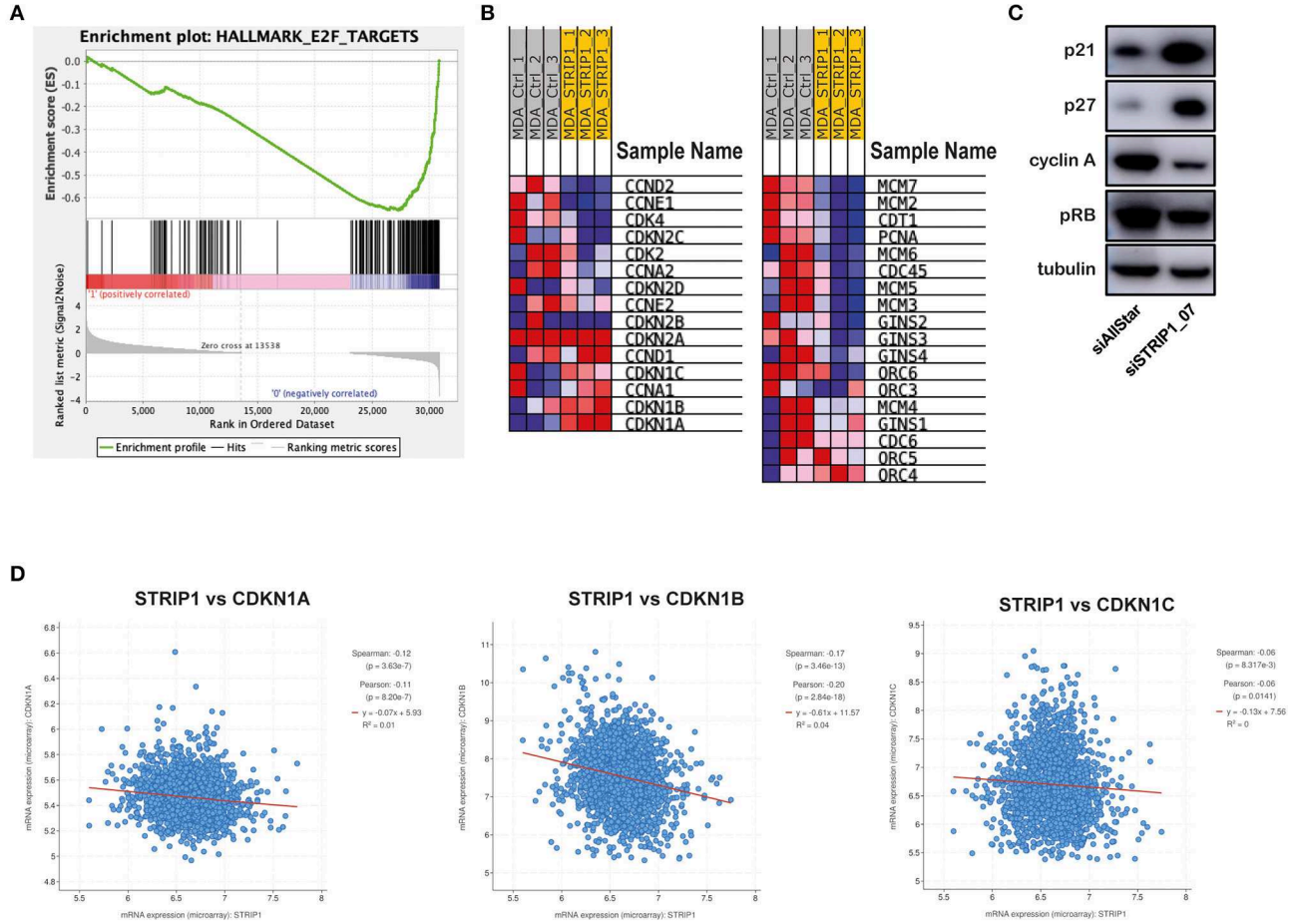

FIGURE 2 | Loss of STRIP1 induces expression of CDK inhibitors p21 and p27. **(A)** Gene Set Enrichment Analysis of RNA-sequencing data of siRNA-depleted MDA-MB-231 cells. The data shows an overall reduction in E2F targeted genes after loss of STRIP1 in MDA-MB-231 cells. **(B)** Heat map of selected genes involved in the regulation of cell cycle progression in control and STRIP1 siRNA-depleted cells. **(C)** Immunoblotting analysis of cell cycle regulating proteins p21, p27, Cyclin-A, pRB. Expression of p21 and p27 are enhanced upon STRIP1 loss while Cyclin-A and pRB are depleted upon STRIP1 loss. All immunoblotting analyses were conducted at least three independent times. **(D)** Dataset analysis showing negative correlation of STRIP1 to CDK inhibitors CDKN1A, CDKN1B, and CDKN1C.

CDKs inhibitors; CDKN2A (p16), CDKN2B (p15), CDKN2C (p18), and CDKN2D (p19) did not change after loss of STRIP1 (**Figure 2B**). The RNA sequencing analysis also demonstrated that genes encoding the functional DNA helicase machinery, responsible for unwinding the DNA template at the replication fork (Leman and Noguchi, 2013), were all downregulated after loss of STRIP1. These genes included the CDC45, the mini-chromosome maintenance (MCM2-7) proteins, and the go-ichi-ni-san (GINS) complex, as well as CDT1 and CDC6; two proteins responsible for the recruitment of the MCM complex to the replication origins (**Figure 2B**). Immunoblotting analyses confirmed that the levels of p21 and p27 were augmented upon loss of STRIP1, while the levels of cyclin A and phosphorylated RB were reduced (**Figure 2C**). These findings are in accordance with what is expected of cells arrested in the G1 phase.

To justify our cell culture experiments, we took advantage of publicly available datasets from breast cancer patients enrolled in the METABRIC [Molecular Taxonomy of Breast Cancer International Consortium] cohort (Curtis et al., 2012; Pereira et al., 2016). The clinical data supported our findings and demonstrated a significant inverse correlation between expression of STRIP1 and the three members of the CIP/Kip family of CDK inhibitors; CDKN1A (p21), CDKN1B (p27) and CDKN1C (p57) (**Figure 2D**). Interestingly, the clinical data also revealed an inverse correlation between STRIP1 and members of the INK4 family of CDK inhibitors; CDKN2A, CDKN2B, CDKN2C, and CDKN2D which the cell culture experiment could not recognize (**Supplementary Figures 1A–D**). Taken together these findings indicate that the level of STRIP1 may regulate the expression of CDK inhibitors independently of induced DNA damage and activation of p53, as MDA-MB-231 cells only express mutant p53.

p21 and p27 Induction Is Regulated by the MST3 and 4 Kinases

The molecular function of STRIP1 is to maintain close proximity between the PP2A/striatin phosphatase and its targeting substrates. These include the STRIPAK associated

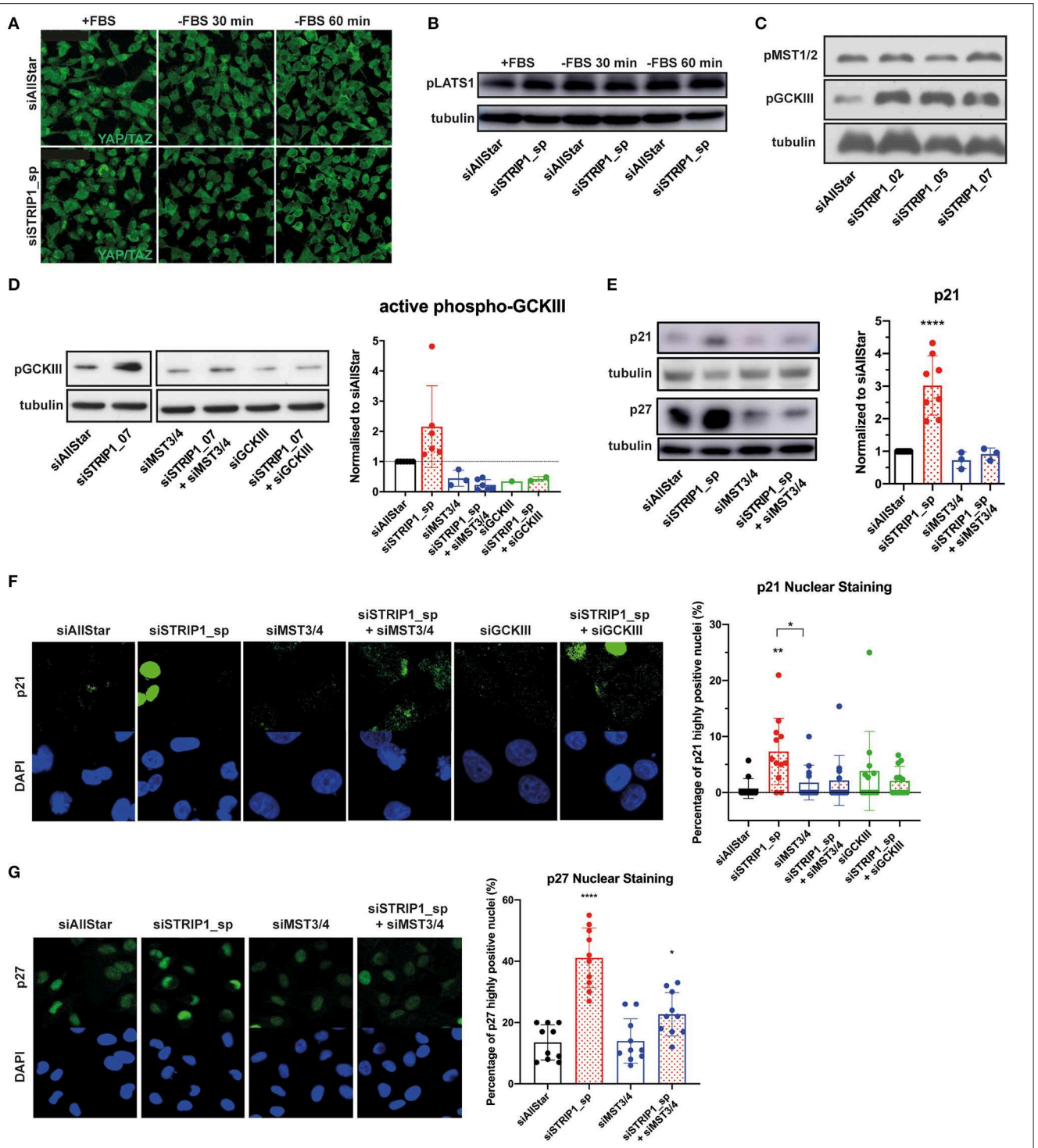

FIGURE 3 | p21 and p27 induction is regulated by activated MST3&4 kinases. **(A–C)** Depletion of STRIP1 in MDA-MB-231 cells does not affect Hippo signaling and YAP/TAZ localization. **(A)** Immunofluorescence analysis of YAP/TAZ localization after STRIP1 depletion in normal media conditions and serum starvation for 30 and 60 min. **(B)** Immunoblotting analysis of phospho-S909-LATS1 under normal media conditions and serum starvation for 30 and 60 min. **(C)** Immunoblotting analysis demonstrates that loss of STRIP1 does not induce phosphorylation of MST1/2. On the contrary, loss of STRIP1 induces strong phosphorylation of the three kinases MST3, MST4, and SOK1 forming the Germinal Center Kinase III (GCKIII) complex. **(D)** Loss of STRIP1 induces auto-phosphorylation of activation loop of the GCKIII complex, as demonstrated by rescuing the effect using kinase specific siRNAs. Included quantification of activated phospho-GCKIII in siRNA-depleted MDA-MB-231 cells. The data shows that loss of STRIP1 induces activation of GCKIII in a MST3&4 dependent manner. **(E)** Immunoblotting analysis demonstrates that siRNA

(Continued)

FIGURE 3 | depletion of MST3&4 represses p21 and p27 induction after loss of STRIP1. Included quantification of p21 levels. **(F,G)** Immunofluorescence analysis demonstrates that siRNA depletion of MST3&4 represses p21 and p27 induction after loss of STRIP1. The percentage of MDA-MB-231 cells expressing high levels of nuclear p21 and p27 was quantified. All immunoblotting and immunofluorescence analyses were conducted at least three independent times. All statistical tests were performed using one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ to siAllStar unless indicated.

kinases: MST1 and MST2 (the two hippo kinases), and MST3, MST4, and SOK1 (the three GCKIII kinases). When STRIP1 is lost, the STRIPAK complex disassembles and the kinases are no longer dephosphorylated and as a result become hyper-activated (Madsen et al., 2015; Tang et al., 2019). Depletion of STRIP1 has previously been linked to Hippo signaling and reduced cell growth. However, siRNA-depletion of STRIP1 in MDA-MB-231 cells did not alter the nuclear localization of the Hippo-controlled YAP/TAZ transcriptional regulators (**Figure 3A**). The nuclear localization of YAP/TAZ also did not vary in STRIP1-depleted cells under stress conditions of serum starvation (activating the Hippo kinases) when compared to control cells (**Figures 3A,B**). To reinforce this, we used three different siRNAs targeting STRIP1 and conclusively showed that loss of STRIP1 does not induce phosphorylation of MST1/2 but on the contrary, a strong phosphorylation of GCKIII kinases (**Figure 3C**). These findings suggest that loss of STRIP1 is not reducing cell proliferation in MDA-MB-231 cells through altered Hippo-signaling. In our previous work, we demonstrated that STRIP1 is a negative regulator of MST3 and MST4 in cancer cells (Madsen et al., 2015). Indeed, loss of STRIP1 induces auto-phosphorylation and activation of the GCKIII kinases, although the importance of SOK1 seem to be minor in MDA-MB-231 cells (**Figure 3D**). We therefore hypothesized that hyper-activated MST3&4 may be responsible for the induction of p21 and p27 seen after the loss of STRIP1. Indeed, depletion of MST3&4 completely reverted the induced p21 and p27 expression seen after loss of STRIP1 in MDA-MB-231 cells (**Figure 3E**).

AKT plays an important role in regulating cell cycle progression by phosphorylating p21, thereby reducing its interaction with CDK2/4 and PCNA thus promoting the cells to enter S-phase, and by phosphorylating glycogen synthase kinase-3 (GSK3), which hinders the degradation of β-catenin and as a result enhances cell proliferation (Rossig et al., 2001; Child and Mann, 2006; Karimian et al., 2016). Loss of STRIP1 significantly decreased phospho-AKT and phospho-GSK3β in a MST3&4 dependent manner (**Supplementary Figure 1E**), suggesting that MST3&4 kinases may contribute to the cell cycle regulation by influencing AKT-signaling.

Active MST3 and 4 Kinases Produce a Subpopulation of Cells Expressing High Levels of p21 and p27

It has been demonstrated that p21 expression can be heterogeneous in an isogenic population of cells (Overton et al., 2014). We therefore decided to examine p21 expression in single cells by immunofluorescence analysis. To our surprise, only a sub-population of MDA-MB-231 cells demonstrated strong nuclear p21 staining after loss of STRIP1 (**Figure 3F**). The quantitative analysis demonstrated that loss of STRIP1 induced high levels of p21 in around 10% of the cells, as compared to control cells having <1% of cells expressing high levels of p21 (**Figure 3F**). Importantly, co-depletion of MST3&4 almost entirely reverted the numbers of $p21^{high}$-expressing cells to levels comparable to control cells (**Figure 3F**). We then asked ourselves if MST3&4 regulated p27 in a similar way. Indeed, loss of STRIP1 induces high p27 expression in a subpopulation of the cells (**Figure 3G**). These data support a scenario where the loss of STRIP1, and the subsequent activation of MST3&4, can create a heterogeneous subpopulation of cells expressing high levels of p21 and p27 within an isogenic population of breast cancer cells.

STRIP1 Regulates DNA Damage Response

When cancer cells experience non-lethal doses of chemotherapeutics, as encountered when the drug concentration declines during treatment (Gewirtz, 1999), the cells can enter a state of therapy-induced senescence (Ewald et al., 2010). Clinically, there is evidence that therapy-induced senescence is associated with good prognosis, however there are also indications that a proliferative subpopulation can emerge with adverse effects and cancer relapse (Demaria et al., 2017). p21 is a master regulator of therapy-induced senescence, but emerging evidence also demonstrates that p21 can induce cell proliferation after chemotherapy (Abbas and Dutta, 2009; Cazzalini et al., 2010). Interestingly, the heterogeneous expression of p21 in an isogenic population of cancer cells was recently linked to cell fate decisions after non-lethal doses of chemotherapeutic treatment (Hsu et al., 2019). In that study, the authors demonstrated that the cell cycle phase and the expression level of p21 would determine if a cancer cell becomes senescent or begins to proliferate after recovering from chemotherapy. In brief, their data demonstrated that cells in G1-phase, expressing intermediate levels of p21, would become proliferative after drug recovery, but only if the cells maintained low DNA damage during treatment (Hsu et al., 2019). On the other hand, cells with too low or too high p21 levels would lead to therapy-induced senescence. More importantly, the proliferation fate of the cells also relied on the presence of intermediate p21 levels prior or during the drug treatment (Hsu et al., 2019). This "p21-goldilocks zone," as the authors called the scenario, is reminiscent to the loss of STRIP1 in MDA-MB-231 cells, where cells are arrested in G1-phase with induced levels of p21. The similarity was further emphasized by the observation that loss of STRIP1 also reduces basal levels of DNA damage response, as demonstrated by immunoblotting and immunofluorescence analyses of γH2AX (**Figures 4A,B**). Gene set enrichment analysis (GSEA) further confirmed the

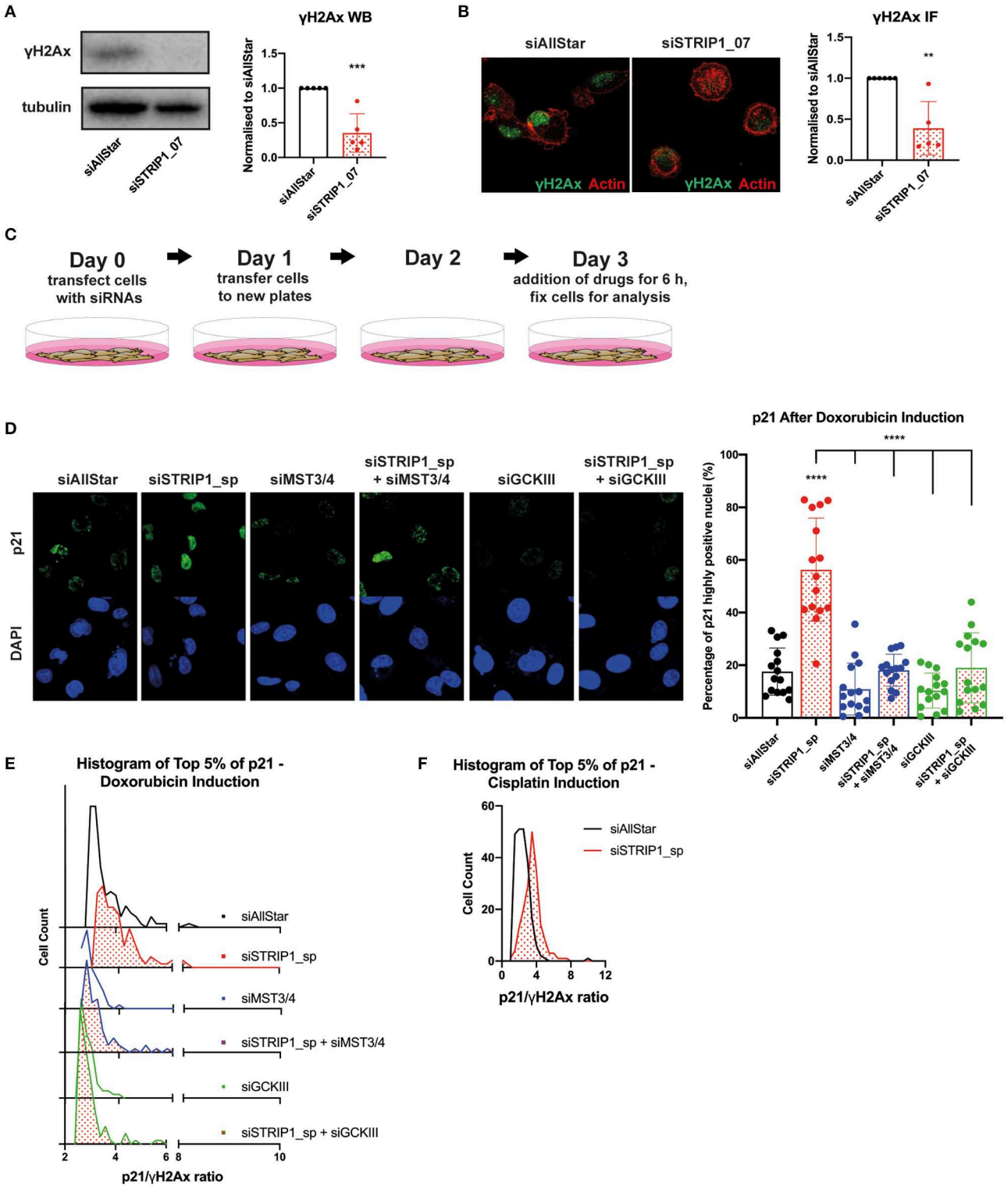

FIGURE 4 | STRIP1 regulates DNA damage response. **(A)** Loss of STRIP1 suppresses the DNA damage response as measured by γH2AX immunoblotting with quantification. **(B)** Loss of STRIP1 suppresses the DNA damage response also measured by immunofluorescence with quantification. **(C)** Experimental set-up of the drug treatment experiment. **(D–F)** siRNA-depleted MDA-MB-231 cells were treated with high dose chemotherapeutics 72 h post-transfection. **(D)** Depletion of MST3&4 represses p21 induction after loss of STRIP1 even after high dose chemotherapeutics. The percentage of siRNA-depleted MDA-MB-231 cells expressing high levels of nuclear p21 after high dose doxorubicin treatment was quantified. **(E)** Loss of STRIP1 increases the p21/γH2AX ratio in a MST3&4 dependent manner

(Continued)

FIGURE 4 | after high dose chemotherapy, indicated by the shift toward a higher ratio. Cells investigated are at the top 5% intensity of nuclear p21 using immunofluorescence analysis. **(F)** Increased p21/γH2Ax ratio is also seen in STRIP1 depleted cells after high dose treatment with another chemotherapeutic, cisplatin. Cells investigated are at the top 5% intensity of nuclear p21. All immunoblotting and immunofluorescence analyses were conducted at least three independent times. All statistical tests were performed using one-way ANOVA or parametric *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ to siAllStar unless indicated.

downregulation of genes involved in G0-G1 DNA damage checkpoint (**Supplementary Figure 1F**).

These observations prompted us to investigate if loss of STRIP1 and the subsequent activation of MST3&4 would influence cell fate decision after treatment with chemotherapy. We decided to test doxorubicin as it is one of the most commonly used chemotherapeutics in the clinic. We began by treating siRNA-depleted cells with high dose of doxorubicin (1 μM) for only 6 h (experimental set-up, **Figure 4C**). Single cell immunofluorescence analysis revealed that treatment induced high p21 expression in ∼20% of control cells, while the numbers increased to ∼60% in STRIP1-depleted cells (**Figure 4D**). Once again, the p21 levels could be reverted to that of control cells by co-depleting MST3&4 (**Figure 4D**).

The "p21-Goldilocks zone" dictates that the p21/γH2AX ratio in individual cells has to be high, if cells have to become proliferative rather than senescent (Hsu et al., 2019). We therefore quantified the p21/γH2AX ratio by immunofluorescence analysis and observed that loss of STRIP1 increased the p21/γH2AX ratio within single cells (**Figure 4E**). Importantly the amplified p21/γH2AX ratio could be reverted to control levels after depleting MST3&4 (**Figure 4E**). Consistently, loss of STRIP1 also increased the p21/γH2AX ratio in single cells treated with a second chemotherapeutic, cisplatin (**Figure 4F**). The findings indicate that hyper-activated MST3&4 can promote a "cell state", matching the "p21-Goldilocks zone," which may facilitate cell proliferation rather than senescence, if treated with sub-lethal dose of chemotherapeutics.

STRIP1 Regulates Proliferation-Senescence Cell Fate After Chemotherapy

To answer that hypothesis, we treated siRNA-depleted cells with low dose doxorubicin (50 nM) for 24 h and then allowed the cells to recover for 4 days without the presence of doxorubicin (experimental set-up, **Figure 5A**). This dosage rarely induces apoptosis but is still sufficient to be clinically relevant (Gewirtz, 1999; Hsu et al., 2019). Accordingly, the majority of cells will enter therapy-induced senescence, while a minor subpopulation may re-enter the cell cycle and start to proliferate (Hsu et al., 2019). To quantify the magnitude of cells that re-entered the cell cycle during recovery-phase, we treated the cells for 2 h with ethynyl-20-deoxyuridine (EdU). We then analyzed single cells for EdU incorporation into newly synthesized DNA, by immunofluorescence and flow cytometry. In both analyses, the loss of STRIP1 significantly increased the numbers of cells entering the cell cycle, as compared to control cells (**Figures 5B–D**). Importantly, these observations could also be reproduced using non-lethal doses of cisplatin (**Figure 5E**). As further affirmation of p21's role in a proliferative cell fate, we co-depleted STRIP1 with p21 and subjected the cells to the same low dosage chemotherapy. Indeed, p21-depletion rescued the increased proliferation of STRIP1-depleted cells (**Figure 5F**). Interestingly, we were also able to rescue the proliferative cell fate by co-depleting p27 in STRIP1-depleted cells (**Figure 5F**). Immunoblotting analysis validated that all siRNAs successfully depleted the p21 and p27 proteins (**Figure 5G**). In summary, these experiments demonstrate that STRIP1-regulation of p21 and p27 influences cell fate decisions after non-lethal doses of chemotherapy.

DISCUSSION

The STRIPAK complex has, in recent years, been linked to the progression of cancer (Wong et al., 2014; Zhang et al., 2014; Madsen et al., 2015; Huang et al., 2017). In breast cancer, the complex regulates the mode of migration of cancer cells and consequently, the ability of cells to metastasize (Madsen et al., 2015). The migration mode relies on the activation state of the two MST3&4 kinases. Hyper-activated MST3&4 couples the actomyosin network to the plasma membrane, while hypo-activated MST3&4 links actomyosin to integrins via focal adhesions. This determines whether cells move in an amoeboid or mesenchymal way, respectively (Madsen et al., 2015). In this study, we demonstrate that loss of STRIP1 in breast cancer cells also induces p21 and p27 expression in a MST3&4 dependent manner. As a consequence, the cells are arrested in the G1-phase causing a reduction in cell proliferation and tumor growth. Surprisingly, the induction of p21 was limited to a subpopulation of cells, which also exhibited low levels of DNA damage response. This phenotype of cells, arrested in G1-phase with increasing p21 and low γH2AX expression (high p21/γH2AX ratio), is reminiscent to the "Goldilocks zone" observed in lung cancer cells recovering from non-lethal dosage of chemotherapeutics (Hsu et al., 2019). These observations made us test if loss of STRIP1 would promote a "cell population" in favor of becoming proliferative rather than senescent after treatment with sub-lethal doses of chemotherapy. To our big surprise this was indeed the case, loss of STRIP1 promoted the recovery of breast cancer cells from both doxorubicin and cisplatin treatment. It is important to state that we did not examine in detail how the p21/γH2AX ratio was regulated in the heterogeneous cell population. We can conclude that the p21 expression is dependent on MST3&4, but we do not know if the levels of γH2AX also relies on these kinases.

The fact that loss of STRIP1 suppresses DNA damage response may actually have detrimental consequences, as it may result in an escaped population of cell with high levels of DNA damage and genomic instability. This has indeed

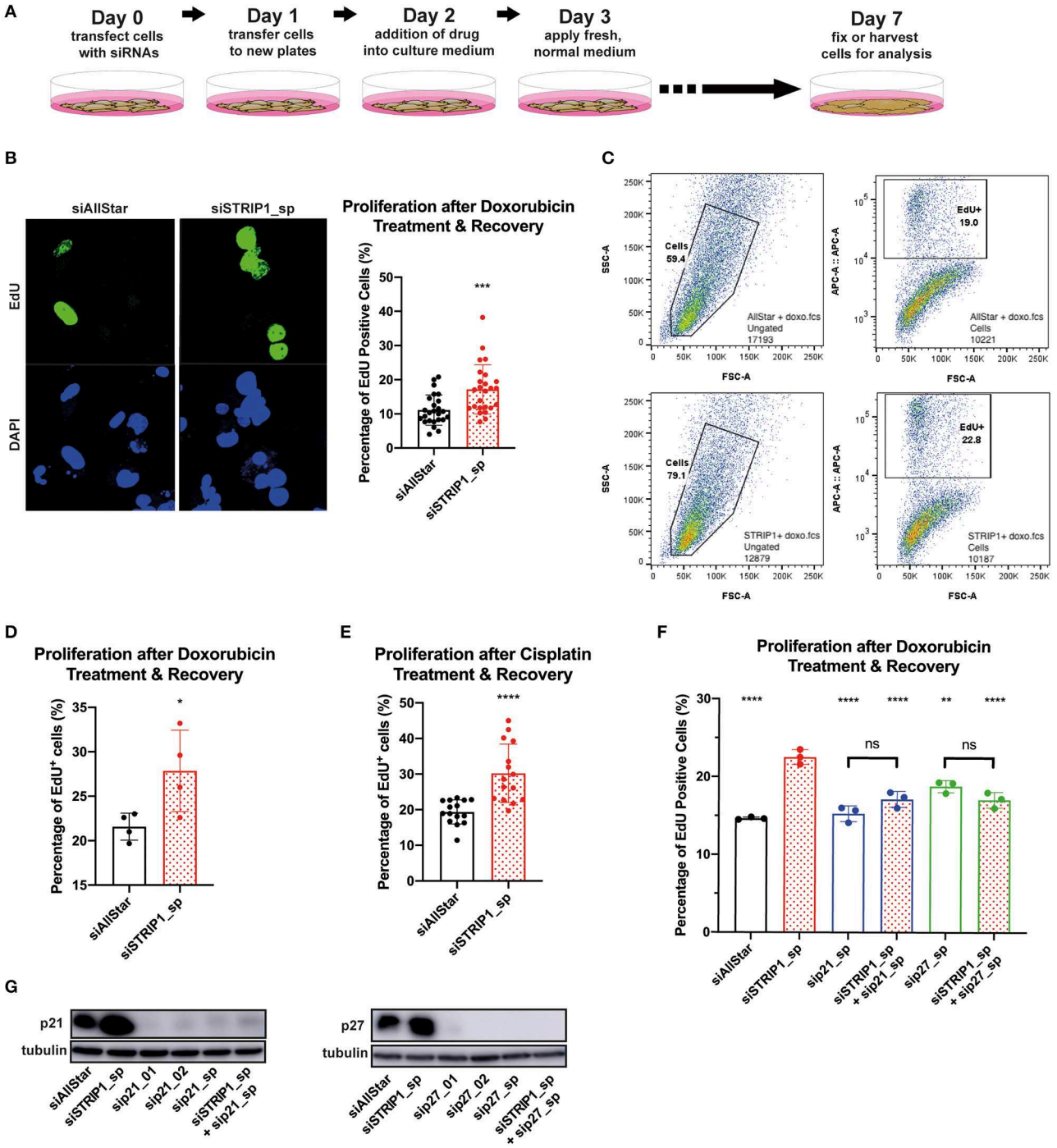

FIGURE 5 | STRIP1 regulates proliferation-senescence cell fate after chemotherapy. **(A)** Experimental set-up of the drug treatment experiment. **(B–F)** siRNA-depleted MDA-MB-231 cells were treated with low dose chemotherapy for 24 h and allowed to recover for a further 96 h. EdU was added to cells in culture 2 h prior to fixation/harvesting and analyzed for proliferation status. **(B)** Immunofluorescence quantification and representative images show increased EdU$^+$ cells after loss of STRIP1 with doxorubicin treatment. **(C)** Gating strategy for EdU proliferation flow cytometry analysis. Side scatter area and forward scatter area were used to obtain the whole cell population (Cells). The population is then analyzed according to EdU intensity (APC conjugated) against forward scatter area to identify proliferative cells (EdU+). **(D)** Flow cytometry quantification shows increased EdU$^+$ cells after loss of STRIP1 with low dose doxorubicin treatment and subsequent recovery. **(E)** Immunofluorescence quantification of EdU$^+$ cells shows increased proliferation after loss of STRIP1 with low dose cisplatin treatment and recovery. **(F)** Flow cytometry quantification of STRIP1 and p21 or p27 co-depleted cells with decreased EdU+ populations compared to STRIP1-depleted cells. Statistical analysis was compared to siSTRIP1 unless indicated. All immunofluorescence and flow cytometry analyses were conducted at least three independent times. **(G)** p21 and p27 protein knockdown efficiencies of siRNA-depleted MDA-MB-231 cells were demonstrated by immunoblotting. All statistical tests were performed using parametric *t*-test or one-way ANOVA, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ to siAllStar unless indicated.

been demonstrated in osteosarcoma cells having prolonged p53-independent expression of p21 (Galanos et al., 2016). The inverse correlation between p21 and γH2AX, with corresponding DNA instability, has also been documented in breast cancer cells (Yaglom et al., 2014).

In conclusion, our observations demonstrate a conflicting function of STRIP1 in regulating proliferation of breast cancer; low levels of STRIP1 suppress proliferation of untreated cells while inducing proliferation of cells recovering from non-lethal doses of chemotherapy. These observations can be justified by the regulation of p21 and p27, which has been shown to exhibit both tumor-suppressive and tumor-promoting functions (Abbas and Dutta, 2009).

From a speculative perspective, these observations suggest that low levels of STRIP1 may correlate with good prognosis in untreated patients, due to lower tumor growth. On the contrary, low STRIP1 levels would have a poor prognosis in patients receiving chemotherapy, due to recovery and recurrence of treated cancer cells. To investigate this idea, we took advantage of a microarray datasets of breast cancer patients using the online resource; http://kmplot.com/analysis (Gyorffy et al., 2010). First, we looked at breast cancer patients, which had never received any treatment. As the number of patients were very low, this analysis was not statistically valid, but nevertheless supported the hypothesis that low levels of STRIP1 may be beneficial (**Supplementary Figure 2A**). We then focused our attention to patients that had received systematic treatment. In stark contrast to untreated patients, low STRIP1 levels correlated with poor prognosis as hypothesized (**Supplementary Figure 2B**). We then restricted the analysis to patients that had received chemotherapy (in conjunction with endocrine therapy). The analysis also supported low levels of STRIP1 to correlate with poor prognosis (**Supplementary Figure 2C**). Although these Kaplan Meier analyses are inconclusive due to low patient numbers, they do support the notion that STRIP1 may play an important role in breast cancer. The assumption would however need further validation in animal models and clinical specimens.

From a mechanistic perspective, we demonstrate that loss of STRIP1 impedes the cell cycle progression and proliferation of breast cancer cells by inducing expression of p21 and p27, two bonafide CDK inhibitors and G1 checkpoint regulators. These observations are emphasized by the limited transcription of E2F-target genes, needed for the progression into the S-phase (Bertoli et al., 2013). The loss of STRIP1 has formerly been linked to the activation of the Hippo-kinases and the suppression of YAP/TAZ-induced proliferation (Tang et al., 2019). However, in our case we did not observe changes in Hippo-YAP/TAZ signaling, implying another mechanism of cell cycle regulation. Contrary to this, we demonstrate that the expression of p21 and p27 are strictly dependent on the stimulation of MST3&4. Although we did not look into the molecular mechanism, MST3 has been shown to regulate p21 phosphorylation and stability through the activation of NDR1/2 kinases (Cornils et al., 2011). Indeed, the phosphorylation state of p21 is an important regulator of its function, as it controls the stability and the cellular localization, as well as its direct binding to PCNA (Karimian et al., 2016). When p21 is phosphorylated, for example by AKT, the p21-PCNA bond is disrupted and the PCNA protein is now free to form a complex with the DNA polymerase δ holoenzyme to promote DNA replication (Karimian et al., 2016). Our data clearly demonstrates that loss of STRIP1 reduces AKT phosphorylation in an MST3&4 dependent manner. In yeast, the *Strip1* homolog was shown to antagonize the mTOR complex 2, thus affecting AKT activity (Pracheil et al., 2012). Thus, it seems plausible that STRIP1-MST3&4 may regulate cell proliferation through AKT regulation. An alternative explanation is that STRIP1-MST3&4 regulate p21 and p27 stability by regulating its phosphorylation state. Although we did not examine the phosphorylation of p21 and p27, we never saw any difference in their subcellular localization; the proteins were always localized to the nucleus of MDA-MB-231 cells. Hence, the link between STRIP1-MST3&4 and the phosphorylation state and stability of p21 and p27 awaits further examinations. In this regard, it is important to state that the phosphorylation of p21 has been shown to have both CDK inhibitory functions and cell proliferative promoting functions depending on the cellular context.

In conclusion, our findings suggest that STRIP1 antagonizes the two MST3&4 kinases in breast cancer cells. This may suppress tumor growth as shown in the study, but unfortunately also induce the dissemination of metastasis as previously shown (Madsen et al., 2015). On top of that, hyper-activated MST3&4 promote a subpopulation of breast cancer cells having low DNA damage response with the ability to recover from low dosage of chemotherapy.

DATA AVAILABILITY STATEMENT

The RNA-sequencing data generated in this study have been deposited in the GEO database under accession GSE145618.

ETHICS STATEMENT

The animal study was reviewed and approved by Malmø - Lund ethical experimental animal committee. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

CR-C, MD, LS, EE, PK, SW, DL, HA, and AG-M carried out the experiments. CM carried out the Kaplan–Meier and mRNA co-expression analyses and designed the project. AG-M and CM supervised the project. CR-C and CM wrote the article. All authors discussed the results and commented on the manuscript text.

FUNDING

This work was supported by the Ragnar Söderberg Foundation (N91/15, CM) BioCARE, Cancerfonden (CAN 2016/783 and CAN 2018/230, CM; 190007, SW), Åke Wiberg foundation (M16-0120 and M17-0235, CM), Swedish Research Council (2017-03389, CM), Crafoord Foundation (20160782 and 20171049, CM), Ollie and Elof Ericssons Foundation (2017, CM), Swedish Society for Medical Research (EE), Erasmus + Mobility Grants for Traineeships (2018; LS), Royal Physiographic Society of Lund (2017, AG-M).

ACKNOWLEDGMENTS

We thank The Eukaryotic Single Cell Genomics Facility, Lund University for the RNA sequencing services.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00146/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

METHODS
published: 19 March 2020
doi: 10.3389/fcell.2020.00068

Primary Mammary Organoid Model of Lactation and Involution

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Mammary gland development occurs mainly after birth and is composed of three successive stages: puberty, pregnancy and lactation, and involution. These developmental stages are associated with major tissue remodeling, including extensive changes in mammary epithelium, as well as surrounding stroma. Three-dimensional (3D) mammary organoid culture has become an important tool in mammary gland biology and enabled invaluable discoveries on pubertal mammary branching morphogenesis and breast cancer. However, a suitable 3D organoid model recapitulating key aspects of lactation and involution has been missing. Here, we describe a robust and straightforward mouse mammary organoid system modeling lactation and involution-like process, which can be applied to study mechanisms of physiological mammary gland lactation and involution as well as pregnancy-associated breast cancer.

Keywords: 3D culture, fibroblast growth factor 2, involution, lactation, mammary gland, milk production, organoid, prolactin

OPEN ACCESS

Edited by:
Jose Maria Carvajal-Gonzalez, University of Extremadura, Spain

Reviewed by:
Juan R. Viña, University of Valencia, Spain
Hidetoshi Mori, University of California, Davis, United States
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *01 November 2019*
Accepted: *24 January 2020*
Published: *19 March 2020*

Citation:
Sumbal J, Chiche A, Charifou E, Koledova Z and Li H (2020) Primary Mammary Organoid Model of Lactation and Involution. Front. Cell Dev. Biol. 8:68. doi: 10.3389/fcell.2020.00068

INTRODUCTION

Lactation, the production of milk to feed progeny, is achieved by the mammary gland. This hallmark organ of mammals mainly develops postnatally and is highly dynamic (Macias and Hinck, 2012). With each pregnancy, mammary epithelium undergoes massive proliferation, tertiary branching of the mammary ductal system, and alveoli differentiation to prepare the epithelium for proper lactation (Brisken and Rajaram, 2006; Sternlicht, 2006). After parturition, mammary epithelium fully transforms into a milk-producing factory. Alveoli expand and take up space of regressing mammary stromal adipocytes, thereby multiplying epithelial volume many times (Macias and Hinck, 2012). After weaning, when milk production is no longer required, milk-producing epithelial cells are removed, and mammary gland is remodeled into a prepregnancy state. This process is called involution, which includes programmed cell death of the epithelium, ECM remodeling, and redifferentiation of adipocytes (Hughes and Watson, 2012; Macias and Hinck, 2012; Zwick et al., 2018; Jena et al., 2019). By the end of involution, mammary gland is ready for a new cycle of pregnancy-associated growth, lactation, and subsequent involution, which can be repeated throughout the reproductive lifespan. During these changes, mammary epithelium retains its bilayered architecture with lumen-facing luminal cells and basally situated myoepithelial cells, which is essential for proper function of the organ (Adriance et al., 2005; Haaksma et al., 2011; Macias and Hinck, 2012).

Abbreviations: BOM, basal organoid medium; Csn2, Casein2–β-casein gene; ECM, extracellular matrix; EGF, epidermal growth factor; FGF2, 7, or 10, fibroblast growth factor 2, 7, or 10; LM, lactation medium; Mmp, matrix metalloproteinase; TGFα, transforming growth factor-α; TGFβ, transforming growth factor-β; Wap, whey acidic protein.

Endocrine signaling is a crucial regulator of mammary morphogenesis during pregnancy. Ovarian hormones estrogen and especially progesterone govern growth and morphogenesis of epithelium via induction of paracrine signaling between mammary stroma and epithelium, involving members of several growth factor families (Hennighausen and Robinson, 2005; Brisken and O'Malley, 2010). Pituitary hormone prolactin, on the other hand, acts directly on prolactin receptor on luminal cells and triggers alveoli maturation and lactogenic differentiation (Hennighausen and Robinson, 2005; Brisken and Rajaram, 2006). Involution is linked to cessation of hormonal stimuli and increase in inflammatory cytokines (Watson, 2006; Stein et al., 2007).

To study various aspects of mammary gland biology, three-dimensional (3D) cell culture models have been widely used for decades (Koledova, 2017a). They combine the advantages of easy manipulation of 2D cellular systems with providing complex cell–cell and cell–ECM interactions, thereby mimicking physiological conditions of *in vivo* experiments more faithfully (Shamir and Ewald, 2014; Huch and Koo, 2015; Koledova, 2017a; Artegiani and Clevers, 2018). Among the 3D culture models, primary mammary organoids have played a major role in understanding mechanisms of mammary branching morphogenesis (Ewald et al., 2008; Huebner et al., 2016; Neumann et al., 2018), including the role of ECM (Simian et al., 2001) and stromal cells (Sumbal and Koledova, 2019). Furthermore, spheroids produced from mammary cell lines were used to study tissue response to growth factors (Xian et al., 2005); organoids grown from sorted single primary mammary epithelial cells were used to study developmental potential of mammary epithelial cells (Linnemann et al., 2015; Jamieson et al., 2017), and differentiation of mammary-like organoids was achieved from induced pluripotent stem cells (Qu et al., 2017).

Despite these advances in 3D cell culture models of mammary gland, systems faithfully modeling pregnancy-associated morphogenesis and lactation have been spare. In some studies, β-casein or milk protein expression was used as a read-out of mammary epithelial functionality (Mroue et al., 2015; Jamieson et al., 2017). Several aspects of lactation and involution were captured in a coculture of mammary epithelial and preadipocyte cell lines (Campbell et al., 2014) or in hormone-treated breast cancer cell spheroids (Ackland et al., 2003; Freestone et al., 2014). However, a system modeling lactation and involution in primary mammary organoids with proper architecture of bilayered epithelium with myoepithelial cell layer has not been characterized.

Here, we report on a mammary 3D culture system for studying induction and maintenance of lactation using easily accessible and physiologically relevant murine primary mammary organoids cultured in Matrigel. Upon prolactin stimulation, the organoids produce milk for at least 14 days and maintain a histologically normal architecture with a functional contractile myoepithelial layer. Moreover, upon prolactin signal withdrawal, our system recapitulates several aspects of involution. Altogether, we describe a robust, consistent, and easy-to-do system for modeling crucial aspects of pregnancy-associated mammary gland morphogenesis and lactation.

MATERIALS AND METHODS

Isolation of Primary Mammary Epithelial Organoids

Primary mammary organoids were prepared from 7- to 10-week-old female mice (ICR or C57/BL6) as previously described (Koledova, 2017b; **Supplementary Figure 1A**). ICR strain was used for the branching morphogenesis and time-lapse imaging, cell viability and replating assays, and confocal imaging. C57/BL6 strain was used for the rest of the experiments. The animals were obtained from the Central Animal Facility of the Institut Pasteur and the Laboratory Animal Breeding and Experimental Facility of the Faculty of Medicine, Masaryk University. Experiments involving animals were approved in accordance with French legislation in compliance with European Communities Council Directives (A 75-15-01-3), the regulations of Institut Pasteur Animal Care Committees (CETEA), the Ministry of Agriculture of the Czech Republic, and the Expert Committee for Laboratory Animal Welfare at the Faculty of Medicine, Masaryk University. The study was performed by certified individuals (AC, JS, EC, and ZK) and carried out in accordance with the principles of the Basel Declaration.

Briefly, the mice were euthanized by cervical dislocation, the thoracic and inguinal mammary glands were collected, visible lymph nodes were excised, and the pooled mammary glands were finely chopped to approximately 1-mm^3 pieces and digested in a solution of collagenase and trypsin [2 mg/mL collagenase (Roche, Switzerland or Sigma, United States), 2 mg/mL trypsin (*Dutscher Dominique, France or Sigma, United States), 5 μg/mL insulin (Sigma, United States), 50 μg/mL gentamicin (Sigma, United States), 5% fetal bovine serum (Hyclone/GE Healthcare, United States) Dulbecco's in modified Eagle medium (DMEM)/F12 (Thermo Fisher Scientific, United States)] for 30 min at 37°C with shaking at 100 rpm. Next, the tissue suspension was treated with 20 U/mL DNase I (Sigma, United States) and 0.5 mg/mL dispase II (Roche, Switzerland) and exposed to five rounds of differential centrifugation at 450 × *g* for 10 s, which resulted in separation of epithelial (organoid) and stromal fractions (**Supplementary Figure 1A**). The organoids were resuspended in basal organoid medium [BOM; 1× insulin–transferrin–selenium supplement, 100 U/mL of penicillin, and 100 μg/mL of streptomycin, in DMEM/F12 (all from Thermo Fisher Scientific, United States)] and kept on ice up to 2 h before seeding for 3D culture.

3D Culture of Mammary Organoids

Freshly isolated primary mammary organoids were mixed with growth factor reduced Matrigel (Corning, United States) and plated in domes in 24-well culture plate (one dome per well, 70 μL of Matrigel per dome). 200, 400, or 1000 organoids per dome were seeded for histology, gene expression, and Western blot analysis, respectively. After setting the Matrigel for 45–60 min at 37°C, the 3D organoid cultures were overlaid with cell culture medium according to the experiment and incubated at 37°C in a humidified atmosphere with 5% CO_2 (**Supplementary Figure 1B**). The

media used were as follows: growth factor medium [BOM supplemented with different growth factors: 2.5 nM FGF2 (Peprotech, United States or Thermo Fisher Scientific, United States), 2.5 nM FGF7, 2.5 nM FGF10, 50 ng/mL EGF (all from Peprotech, United States), 5 nM TGFα (Sigma, United States), or a combination of 10 ng/mL WNT3A and 50 ng/mL R-spondin 1 (W3/R1, both from Peprotech, United States)] and lactation medium {LM; 1 μg/mL prolactin [mouse recombinant prolactin for quantitative polymerase chain reaction (qPCR), Western blot, immunohistochemistry and contraction experiments (Sigma, United States or Peprotech, United States), and sheep pituitary prolactin for confocal and time-lapse imaging, including contraction experiments (Sigma, United States)], and 1 μg/mL hydrocortisone (Sigma, United States) in BOM}. Media containing growth factors were changed every 3 days; LM was changed every 2 days. To induce contraction of lactation organoids grown with mouse recombinant prolactin, 40 μg/mL recombinant oxytocin (Sigma, United States) was used. For time-lapse imaging experiments, organoid cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C on Olympus IX81 microscope equipped with Hamamatsu camera and CellR system for time-lapse imaging. For morphological analysis of organoid development, the organoids were photographed from days 8 to 17 of culture; one image per organoid was taken every hour. The images were exported and analyzed using ImageJ (NIH, United States). For analysis of organoid contraction, the organoids were photographed from days 6 to 20 of culture. On each imaging day, the photographs were taken every second for 120 s. The images were exported to video at 10 frames per second using xCellence software (Olympus, Japan).

Replating of Organoids

To replate organoids, 3D cultures were rinsed with phosphate-buffered saline (PBS) and disintegrated by pipetting up and down in ice-cold PBS with a 1000 μL pipette. Successful disintegration of Matrigel was checked under a microscope. Organoid suspensions were centrifuged at 450 × *g* for 3 min. Organoid pellets were resuspended in fresh Matrigel and plated as described above. Organoids were maintained in BOM or in BOM supplemented with 2.5 nM FGF2; the medium was changed every 3 days. Organoid area was measured in ImageJ.

Cell Viability Assay

To asses cell viability in organoids treated with LM or LM-BOM, on the 20th day of culture, the media were changed with fresh BOM, and then resazurin (Merck, Germany) was added to the medium to the final concentration of 10 μg/mL. The plates were incubated for 6 h. Resorufin fluorescence (excitation at 560 nm, emission at 590 nm) was measured using Synergy H4 Hybrid multimode microplate reader (BioTek, United States) in technical triplicates. As a positive control of dying cells, organoids in LM-BOM conditions were treated from day 16 with 40 μM taxol (Sigma, United States) or killed on day 20 by treatment with 70% ethanol for 5 min.

Histology and Immunostaining Analysis

For histological analysis, organoids were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, United States) for 30 min and embedded in 3% low gelling temperature agarose (**Supplementary Figure 1C**). After solidification, samples were dehydrated and paraffin embedded and cut in 5-μm sections, which were dewaxed for hematoxylin and eosin staining or immunostaining. For localization of prolactin receptor expressing cells, 10-μm cryosections of mammary glands from *Prlr-IRES-Cre;ROSA26-CAGS-GFP* mice (Aoki et al., 2019) were labeled with antibodies and counterstained with 0.5 μg/mL DAPI, mounted with Vectashield (Vector Labs, United States), and images were taken on LSM800 microscope (Zeiss, Germany). The following primary antibodies were used: goat anti-GFP (Origene, United States, R1091P, 1:200), rabbit polyclonal anti-keratin 5 (BioLegend, United States, 905501, 1:200), mouse monoclonal anti-keratin 8 (BioLegend, United States, 904801, 1:200), mouse monoclonal anti-β-casein (Santa Cruz, United States, sc-166530, 1:250), and rabbit anti-mouse milk proteins (*Accurate Chemical, United States, YNRMTM, 1:500). Corresponding secondary antibodies were used: donkey anti-rabbit Dylight 488 (Immuno Reagents, United States, DkxRb-003-D594NHSX, 1:200) and donkey anti-mouse Dylight 594 (Immuno Reagents, United States, DkxMu-003-D488NHSX, 1:200), together with 1 μg/mL of Hoechst-33342 (Thermo Fisher Scientific, United States) for immunofluorescence labeling, or anti-mouse/anti-rabbit horseradish peroxidase (HRP)-associated secondary antibodies (Dako, United States).

Whole Mount Staining of Mammary Organoids

Organoids were fixed with 10% formalin for 30 min, washed with PBS and 70% ethanol, and incubated with oil red O solution [0.3% (wt/vol) oil red O (Sigma, United States) in 70% (vol/vol) ethanol (Koopman et al., 2001; Kim et al., 2015)] for 30 min in the dark. Next, organoids were washed with 70% ethanol and PBS and incubated with 0.5 μg/mL DAPI and 2 units/sample phalloidin-AlexaFluor488 (Thermo Fisher Scientific, United States) in PBS for 1 h at room temperature (RT) in the dark. Subsequently, organoids were washed and transferred to coverslip-bottom 35-mm dishes (ibidi) covered with 1% low gelling temperature agarose (Sigma, United States) and overlaid with PBS. Images were acquired using LSM800 confocal microscope (Zeiss, Germany, **Supplementary Figure 1D**) and analyzed using ZEN blue software (Zeiss, Germany).

RNA Isolation and Real-Time qPCR

Total RNA was extracted from organoid samples using RNeasy Micro Kit (Qiagen, Germany) following the manufacturer's instructions. Reverse transcription was performed using high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, United States). Quantitative real-time PCR was performed using 5 ng cDNA, 5 pmol of the forward and reverse gene-specific primers each in Light Cycler SYBR Green I Master mix (Roche, Switzerland) on LightCycler 480 II (Roche, Switzerland). All reactions were performed at least in

duplicates and in a total of at least two independent assays. Relative gene expression was calculated using the ΔΔCt method, and the values were normalized to housekeeping gene *Gapdh*. The primers of following sequences (5′–3′) were used: *Csn2*-forward (F): CCTCTGAGACTGATAGTATTT, *Csn2*-reverse (R): TGGATGCTGGAGTGAACTTTA; *Wap*-F: TT GAGGGCACAGAGTGTATC, *Wap*-R: TTTGCGGGTCCTACC ACAG; *Mmp3*-F: CCTGATGTTGGTGGCTTCA, *Mmp3*-R: TC CTGTAGGTGATGTGGGATTTC; *Mmp13*-F: ACTTCTACCCA TTTGATGGACCTT, *Mmp13*-R: AAGCTCATGGGCAGCAA CA; *Gapdh*-F: TTCACCACCATGGAGAAGGC, *Gapdh*-R: CC CTTTTGGCTCCACCCT. All primers were purchased from Sigma, United States.

Western Blot

Three-dimensional cultures were dissociated by repetitive pipetting in ice-cold PBS supplied with phosphatase inhibitor cocktail II (Merck, Germany; 2 mM imidazole, 1 mM sodium fluoride, 1.15 mM sodium molybdate, 1 mM sodium orthovanadate, 4 mM sodium tartrate dihydrate), followed by centrifugation at 450 × *g* for 3 min at 4°C. Supernatant was discarded, and pellets were lysed in ready-to-use RIPA buffer [Merck, Germany; 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0] supplied with protease inhibitor cocktail I (Merck, Germany; 500 μM AEBSF hydrochloride, 150 nM aprotinin, 1 μM protease inhibitor E-64, 0.5 mM EDTA, 1 μM leupeptin hemisulfate) and phosphatase inhibitor cocktail II. After vortexing and sonication, protein lysates were cleared by centrifugation, and protein concentration was measured using Coomassie reagent (Merck, Germany). Denatured, reduced samples were resolved on 12.5% SDS–polyacrylamide electrophoresis (Bio-Rad, United States) and blotted onto nitrocellulose membranes by Trans-blot Turbo transfer system (Bio-Rad, United States). After blotting, the membranes were blocked with 2% bovine serum albumin in PBS with 0.1% Tween-20 (Merck, Germany; blocking buffer) and incubated with primary antibodies diluted in blocking buffer overnight at 4°C. After washing in PBS with 0.05% Tween-20, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at RT. Signal was developed using an ECL substrate (Thermo Fisher Scientific, United States) and imaged with ChemiDoc MP imaging system (Bio-Rad, United States), and band density was analyzed in ImageJ. The following antibodies were used for immunoblotting: mouse monoclonal anti-β-casein (Santa Cruz, United States, sc-166530, 1:1000), mouse monoclonal anti-α-tubulin (Santa Cruz, United States, sc-5286, 1:1000), and anti-mouse secondary antibody (Merck, NA931, 1:1000).

Statistical Analysis

Statistical analysis was performed using the Prism software (GraphPad, United States); statistical test used is specified in figure legends. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. The number of independent biological replicates is indicated as *n*.

RESULTS

FGF2 Pretreatment Enhances Lactogenic Differentiation of Mammary Epithelium

During mammary gland morphogenesis, lactation is preceded by excessive branching of epithelial ducts. We hypothesized that epithelial expansion by branching morphogenesis might be required for lactogenic differentiation *in vitro*. Therefore, we first tested the impact of several growth factors on mammary epithelial morphogenesis. The primary mammary epithelial organoids were treated with FGF2, FGF7, FGF10, EGF, TGFα, or a combination of WNT3A and R-spondin 1 (W3/R1) for 7 days. Interestingly, only FGF2, a potent mammary epithelium branching-inducing factor (Ewald et al., 2008), induced extensively branched morphology (**Supplementary Figures 2A–D**).

Next, we tested if FGF2-induced epithelial expansion facilitated lactogenic differentiation. To this end, the primary mammary epithelial organoids were either treated only with LM (containing prolactin and hydrocortisone) for 4 days, or they were treated with FGF2 for 6 days and followed by 4 days of LM (**Figure 1A**). To detect lactogenic differentiation, we measured the expression of *Csn2* and *Wap* by RT-qPCR. Our results revealed that treatment of freshly isolated organoids with LM induced only expression of *Csn2* (**Figure 1B**). However, when organoids were pretreated with FGF2, the expressions of both *Csn2* and *Wap* were significantly increased (**Figure 1B**). These data suggest that mammary epithelial expansion, induced by branching morphogenesis, could enhance the lactogenic ability of mammary epithelium.

Lactation Medium Induces Production of Milk Proteins and Secretion of Lipid Droplets

Next, we compared the morphology of organoids treated with either FGF2 only or FGF2 and LM (FGF2-LM) to further characterize the phenotype of lactation organoids. On bright-field micrographs, we noticed that FGF2-LM organoids appeared to have a darker lumen, possibly due to the milk accumulation (**Figure 1C**). Interestingly, we also observed bubble-like structures at the apical site of epithelium in the same organoids, which potentially represented lipid droplets (**Figure 1C**). To further characterize these droplets, we stained the organoids for F-actin (with phalloidin), a cytoskeletal protein, or with oil red O. Confocal microscopy revealed that the droplets were negative for F-actin and strongly positive for oil red O, confirming the droplets were lipid (**Figures 1C,D**).

Next, we assessed the expression of milk proteins in the organoids. First, we detected a significant increase in *Csn2* by four orders in FGF2-LM-treated organoids compared to FGF2 alone by RT-qPCR (**Figure 1E**). Consistently, in FGF2-LM-treated organoids, we detected up-regulation of β-casein on the protein level by Western blot (**Figure 1F**) and a strong cytoplasmic signal by immunohistochemistry (**Figure 1G**), which was further confirmed by antibody against milk proteins (**Supplementary Figures 3A–C**). Taken together, these data demonstrate that

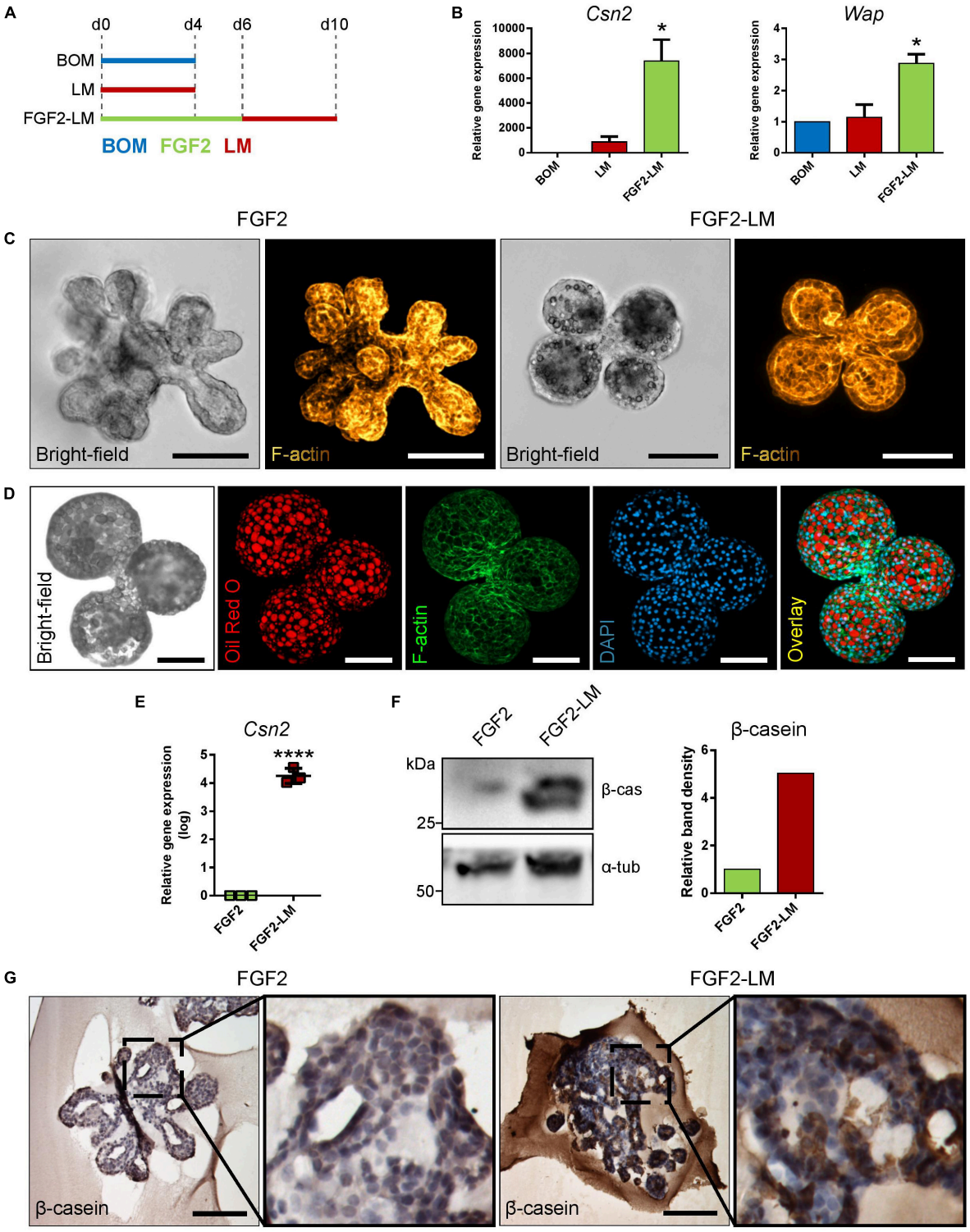

FIGURE 1 | Lactation induction in primary mammary organoids. **(A,B)** FGF2 pretreatment increases lactation capacity of primary mammary organoids. **(A)** Scheme depicting the experimental design. BOM, basal organoid medium; LM, lactation medium; FGF2, FGF2 medium. **(B)** Expression of milk genes *Csn2* and *Wap* in organoids treated with BOM, LM, or FGF2 followed by LM. The values are relative to BOM. The plot shows mean + SD; $n = 2$. One-way ANOVA, $^*p < 0.05$. **(C)** Bright-field images and maximum intensity projection images from confocal imaging of whole-mount organoids after treatment with FGF2 only or with FGF2 followed by LM. Yellow-to-brown staining shows F-actin. Scale bars represent 100 μm. **(D)** Bright-field image and maximum intensity projection images from confocal imaging of whole-mount organoid treated with FGF2 followed by LM. Red, oil red O (lipids); green, F-actin; blue, DAPI (nuclei). Scale bars represent 100 μm.

(Continued)

FIGURE 1 | Continued
(E,F) Quantification of β-casein expression in organoids treated with FGF2, or FGF2 followed by LM. **(E)** RT-qPCR analysis of β-casein gene *Csn2*. The values are relative to FGF2. The plot shows mean ± SD; $n = 3$. Unpaired Student's *t*-test, two tailed, ****$p < 0.0001$. **(F)** Western blot analysis of β-casein expression on protein level. The plot shows quantification of band density. The values are relative to FGF2. **(G)** Immunohistochemical staining of β-casein in organoids treated with FGF2 or FGF2 and then LM at days 6 and 10, respectively. Marked area is shown in higher magnification. Scale bars represent 100 μm.

mammary primary organoids are capable of milk production after prolactin treatment, which could be greatly enhanced by branching morphogenesis.

Morphology Maintenance in Long-Term Lactating Organoids

After successful induction of lactation in the primary mammary organoids with the FGF2-LM protocol, we went on to investigate the lactation-associated phenotype in long-term organoid culture. After 6 days of FGF2 treatment, the organoids were either cultured continuously with LM (FGF2-LM) or switched to BOM after 4 days of LM treatment (FGF2-LM-BOM) (**Figure 2A**). The morphogenesis of the organoids was recorded using time-lapse microscopy for 20 days. Interestingly, FGF2-LM-BOM cultured organoids regressed both in size and the complexity of the shape, whereas the organoids in FGF2-LM maintained the size and only partially lost the branched phenotype (**Figures 2B,C** and **Supplementary Figures 4A,B**). In contrast, continuous treatment with FGF2 for 20 days maintained the organoid branched morphology (**Supplementary Figures 4A,B**). In addition, unlike the organoids in FGF2-LM-BOM, the organoids in FGF2-LM retained the darker appearance, possibly due to the milk accumulation (**Figures 2B,D** and **Supplementary Figure 4A**). Morphologically, FGF2-LM-treated organoids exhibited complex architecture with multiple lumens filled with dense eosinophilic material, which was maintained throughout the experiment (**Figure 2E**, upper panel). However, upon LM withdrawal, the complex architecture was lost rapidly, and organoids involuted into small spheroids with much simpler structures (**Figure 2E**, lower panel).

Milk Production in Long-Term Lactating Organoids

Of note, we detected strong β-casein signal in the intraluminal of long-term lactating organoids by immunohistochemistry. Closer observation revealed that cytoplasmic β-casein signal was sustained in long-term LM culture (**Figure 3A**, upper panel), but lost after LM withdrawal (**Figure 3A**, lower panel). In addition, RT-qPCR revealed that FGF2-LM-treated organoids maintained a high level of *Csn2* expression, which was dramatically reduced by four to five orders of magnitude in FGF2-LM-BOM-treated organoids (**Figure 3B**). The same change was confirmed in the protein level by Western blot (**Figure 3C**). Therefore, the production of β-casein depended on the prolactin signaling.

Altogether, these data suggest that these organoids have a proper epithelial architecture and the capacity to maintain milk production over prolonged culture time in response to the prolactin signaling.

Lactating Organoids Retain Functional Myoepithelial Layer With Contractility

Next, we co-stained the lactating organoids for keratin 5 and keratin 8, markers of myoepithelial and luminal cells, respectively, to confirm that the organoids contain proper bilayer epithelial architecture. We found that FGF2-LM-treated organoids contained a continuous layer of myoepithelial cells, similar to FGF2-treated organoids (**Figure 4A**). Moreover, the myoepithelial cell layer was retained during the long-term culture in LM treatment, as well as after LM withdrawal (**Figure 4B**), suggesting the luminal–myoepithelial cell homeostasis was stable during long-term culture.

Importantly, FGF2 treatment induced stratification of the luminal layer, which is in agreement with published work (**Figure 4A**; Ewald et al., 2008). Upon LM treatment, the organoids showed resolution of the stratified epithelium to a predominantly bilayer structure, with luminal cells (keratin 8 positive) lining the luminal space (**Figures 4A,B**), which is important for producing milk. Remarkably, we observed the LM-treated organoids could contract periodically (**Supplementary Movie 2**). In comparison, organoids never treated with LM showed relatively static structures (**Supplementary Movie 1**). Of note, the contracting phenotype maintained during the long-term LM treatment and quickly ceased after LM withdrawal (**Figure 4C**). This result is somewhat puzzling because prolactin receptor is present only in the luminal cells (**Supplementary Figure 5A**). Of note, the prolactin used in our contraction experiments was isolated from sheep pituitary, which contains oxytocin (Vorherr et al., 1978). To test whether the contraction of myoepithelial cells is a direct effect of prolactin signaling, we compared contraction induction upon LM containing either sheep pituitary prolactin or mouse recombinant prolactin. Interestingly, only sheep pituitary prolactin induced organoid contraction; mouse recombinant prolactin did not induce contraction (**Supplementary Figure 5B** and **Supplementary Movie 3**). However, when the organoids cultured with mouse recombinant prolactin were treated with recombinant oxytocin, they did contract (**Supplementary Movie 4**), demonstrating that oxytocin is required for myoepithelial cell contraction. Taken together, these results demonstrate that myoepithelial layer is present in the lactating organoids. And more importantly, these myoepithelial cells can contract in response to LM treatment, suggesting they are functionally similar to the *in vivo* counterpart.

LM Withdrawal Triggers Involution-Like Phenotype in Lactating Organoids

Involution is characterized by the regression of the lactating epithelium through programmed cell death and remodeling of the mammary gland, which is induced upon weaning of the

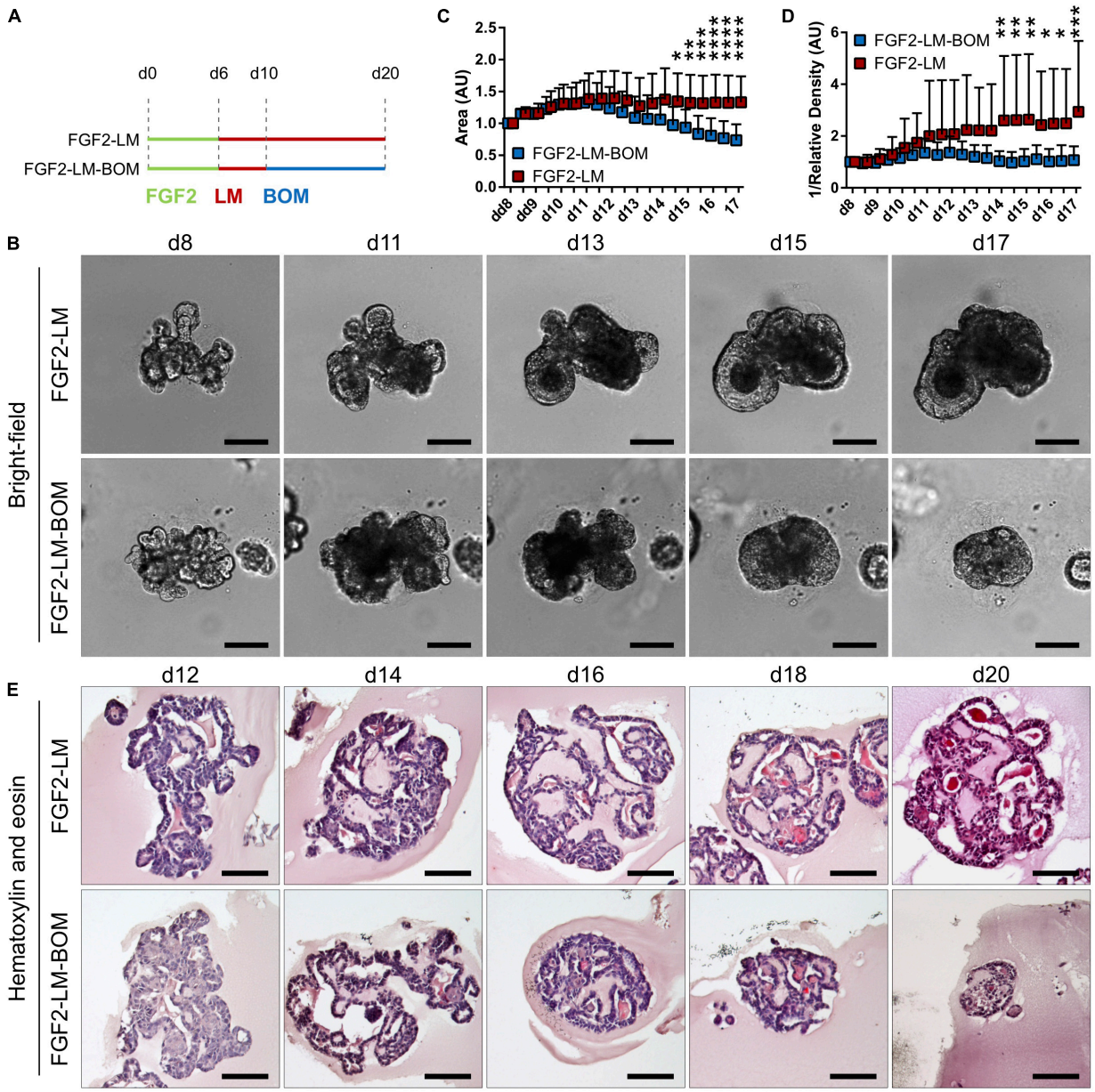

FIGURE 2 | Morphology of organoids undergoing long-term lactation. **(A)** Scheme depicting experimental design. FGF2, FGF2 medium; LM, lactation medium; BOM, basal organoid medium. **(B)** Bright-field images from time-lapse imaging of organoid morphogenesis under continuous LM treatment (FGF2-LM) or under LM withdrawal and replacement with BOM (FGF2-LM-BOM). Scale bars represent 100 μm. **(C,D)** Morphometric analysis of organoid area **(C)** and density **(D)** from the time-lapse experiment. The plots show mean + SD; $n = 2$, $N = 20$ organoids per condition. Two-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. **(E)** Hematoxylin and eosin staining of organoids at different time points of long-term lactation. Scale bars represent 100 μm.

pups (Jena et al., 2019). Interestingly, withdrawal of LM from lactating organoids also induced a size regression and loss of the branched morphology with luminal architecture (**Figures 2B–E**). Using cell viability assay that is based on conversion of non-fluorescent resazurin to fluorescent resorufin by viable cells, we found that lactating organoids upon LM withdrawal (FGF2-LM-BOM) showed reduced viability in comparison to lactating organoids in LM (FGF2-LM) (**Figure 5A**), most likely due to increased cell death in response to LM withdrawal, which is a characteristic of involution. Yet the viability of organoids upon LM withdrawal was higher than that of organoids undergoing taxol- or ethanol-induced cell death (**Figure 5A**). Furthermore, replating of the involution-like organoids (FGF2-LM-BOM) to fresh Matrigel and FGF2 treatment reversed the size regression (**Figure 5B**) and, more importantly, induced branching morphogenesis (**Figures 5C,D**). This demonstrates that involuting organoids are viable and that the morphological changes induced upon LM withdrawal are reversible.

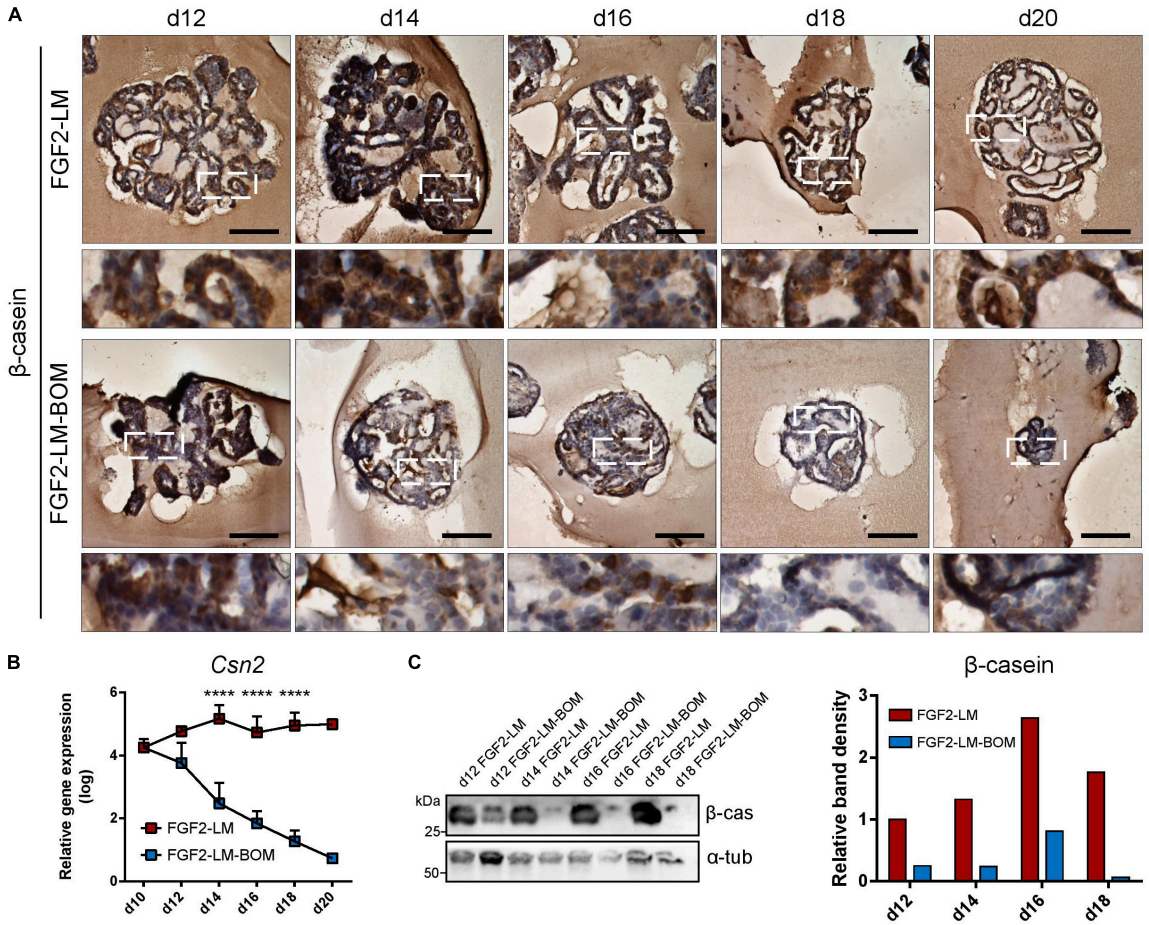

FIGURE 3 | Milk production during long-term lactation. **(A)** Immunohistochemical staining of β-casein in organoids during long-term LM treatment or after LM withdrawal (LM-BOM), according to experimental scheme in **Figure 2A**. Marked area is shown in higher magnification. Scale bars represent 100 μm. **(B)** *Csn2* expression during long-term lactation with continuous lactation medium (FGF2-LM) or with hormonal withdrawal (FGF2-LM-BOM). The plot shows mean + SD; $n = 3$ for d12 to d18, $n = 1$ for d20. Two-way ANOVA, $^{****}p < 0.0001$. **(C)** Western blot analysis and band density quantification of β-casein expression in organoids during long-term lactation.

Furthermore, cessation of milk production and ECM remodeling are two hallmarks of involution. Consistently, we detected a reduced β-casein signal (**Figures 3A,C**) and *Csn2* expression (**Figure 3B**) in the organoids upon LM withdrawal. Interestingly, we also found that the expression of *Mmp2* and *Mmp13*, two important Mmps for the ECM remodeling process during involution, was up-regulated in organoids after LM withdrawal (**Figures 5E,F**). Together, these results demonstrate that upon withdrawal of hormonal stimulation lactating organoids stop milk production and enter an involution-like process, thereby mimicking the *in vivo* situation upon weaning.

DISCUSSION

In this work, we described the use of primary mammary epithelial organoids to model pregnancy-associated morphogenesis and lactation. In our 3D culture system, primary mammary organoids exposed to LM with prolactin recapitulated several aspects of lactation process. Upon LM withdrawal, organoids regressed in a manner similar to the involution process *in vivo*.

Our data showed that FGF2 primes mammary epithelium for lactation. This is consistent with *in vivo* studies that noted morphological abnormalities in pregnancy-associated tertiary branching of mammary epithelium with attenuated FGF receptor signaling (Lu et al., 2008; Parsa et al., 2008). However, it remains to be elucidated what of the FGF2-mediated processes, including epithelial expansion, branching, and maturation, are essential contributors to milk production efficiency.

While several previous studies reported lactation induction in mammary epithelial organoids in response to prolactin *in vitro*, they did so only at a single time point (Mroue et al., 2015; Jamieson et al., 2017). Long-term lactation in organoid cultures has not been reported before. In this study, we documented milk production maintenance and stable morphology of lactating organoids over 14 days' culture period. Physiological lactation in mouse lasts for circa 3 weeks (König and Markl, 1987),

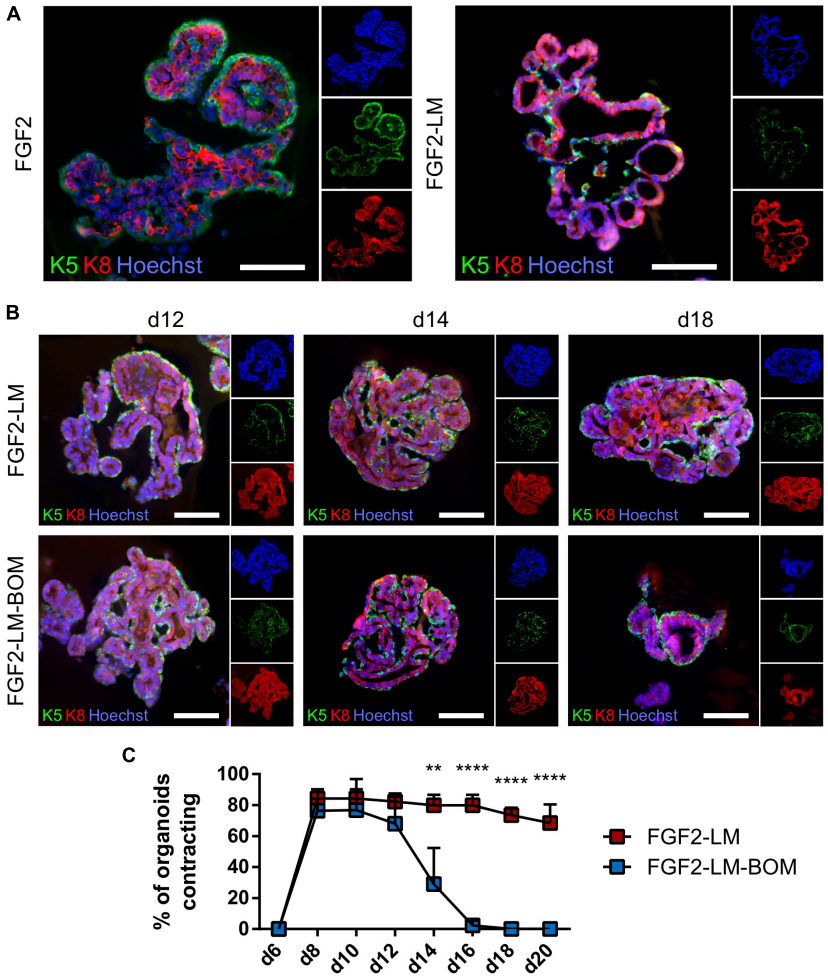

FIGURE 4 | Lactating organoids retain functional myoepithelial layer. **(A)** Immunofluorescent staining shows distribution of myoepithelial (keratin 5 positive, green) and luminal cells (keratin 8 positive, red) in organoids treated with FGF2 or FGF2 followed by LM. Hoechst, blue (nuclei). Scale bars represent 100 μm. **(B)** Immunofluorescent staining shows distribution of myoepithelial (keratin 5 positive, green) and luminal cells (keratin 8 positive, red) in organoids during long-term lactation. Hoechst, blue (nuclei). Scale bars represent 100 μm. **(C)** Quantification of contracting organoids from movies recorded at indicated time-points. The plot shows mean + SD; $n = 2$, $N = 50$ organoids per experiment. Two-way ANOVA, $^{**}p < 0.01$, $^{****}p < 0.0001$.

and milk composition and production rate vary during the lactation period to accommodate the needs of the offspring (Knight et al., 1986). We propose that our model would be suitable to study factors that influence dynamic changes in milk composition and quantity in the long term. Among others, insulin is used in our model to support cell survival and growth and has been implicated in milk production (Nommsen-Rivers, 2016) both in rodent and human. Our model could help to further elucidate how insulin signaling impacts on milk production. Moreover, while previous studies used sample-destructive methods to detect lactation, such as organoid fixation and immunodetection of milk proteins (Mroue et al., 2015; Jamieson et al., 2017), we propose approaches for observing changes in milk production in the same organoid over time. They include morphological changes accompanying lactation in organoids, namely, appearance of lipid droplets in luminal space, increase in organoid darkness (integrated density), and the intriguing contraction of myoepithelial cells, which are easily observable by light microscopy and traceable by time-lapse imaging.

Myoepithelial cells form a layer of mammary epithelium that is situated basally to the luminal cells (Macias and Hinck, 2012). Besides the recently elucidated role in keeping epithelial homeostasis and integrity (Adriance et al., 2005; Goodwin and Nelson, 2018; Sirka et al., 2018), the key function of myoepithelial cells is to enable milk ejection by contraction when pups are suckling (Haaksma et al., 2011). In response to tactile stimuli, oxytocin is released from pituitary, and it binds to oxytocin receptor on myoepithelial cell to induce contraction (Nishimori et al., 1996; Froemke and Carcea, 2017). Therefore, oxytocin was used to induce myoepithelial contraction in single cells (Raymond et al., 2011), as well as in an organoid system (Mroue et al., 2015). However, organoid contraction was shown only as a decrease in organoid area

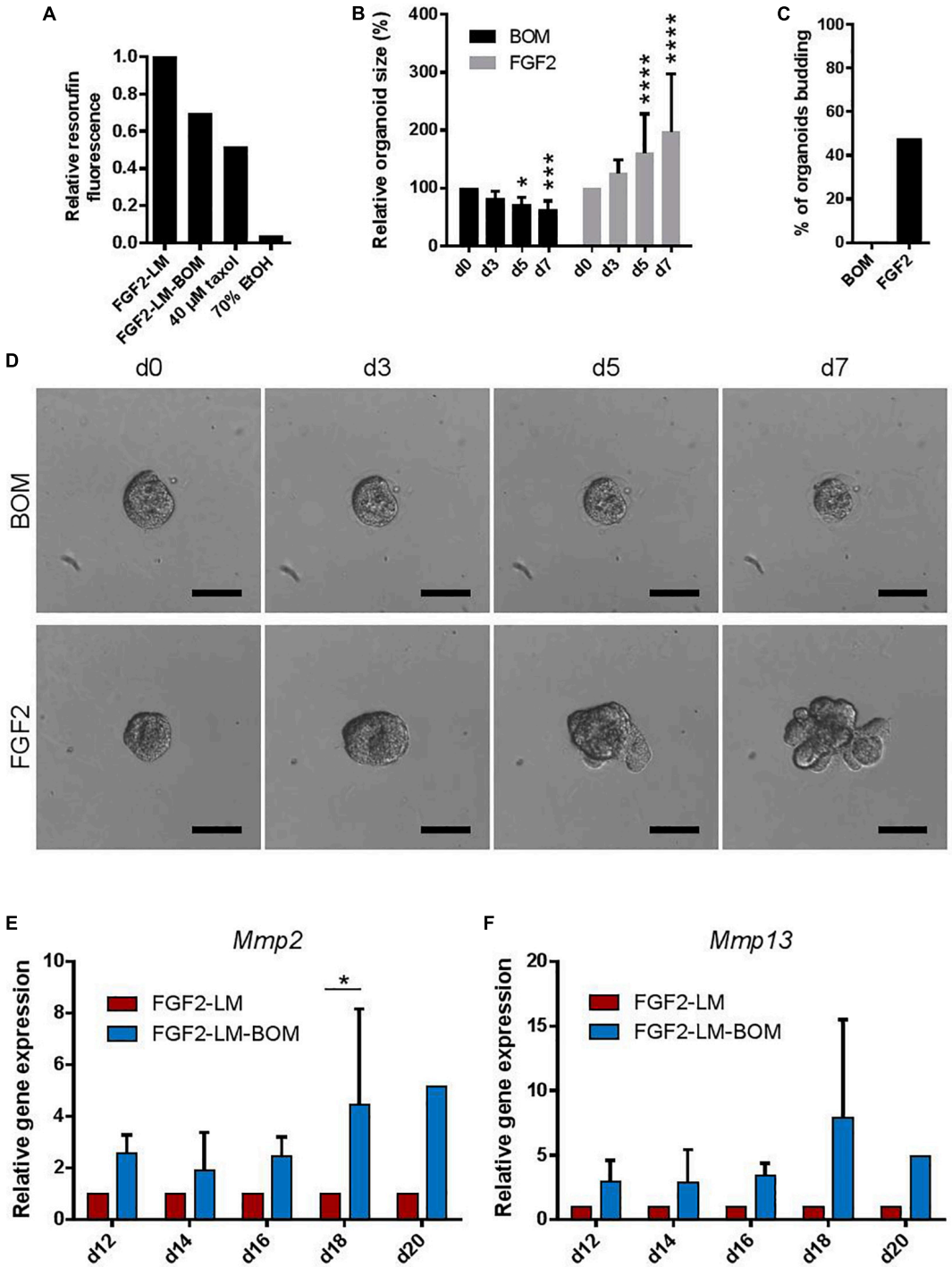

FIGURE 5 | Withdrawing hormones induces an involution-like phenotype in lactating organoids. **(A)** The viability of the lactating and involuting organoids using resazurin assay. The plot shows relative resorufin fluorescence of organoids with continuous LM treatment (FGF2-LM), LM withdrawal and replacement with BOM (FGF2-LM-BOM), and FGF2-LM-BOM organoids treated with 40 μM taxol for 4 days (40 μM taxol) or 70% ethanol for 5 min (70% EtOH) to induce cell death. Values are relative to FGF2-LM. **(B–D)** Analysis of FGF2-LM-BOM organoids after replating to BOM or FGF2 medium. **(B)** Quantification of the size of the FGF2-LM-BOM organoids that were replated and cultured with BOM or FGF2 for the number of days as indicated. The plot shows mean + SD; $n = 1$, $N = 25$ organoids per condition. Two-way ANOVA, asterisks indicate change in comparison to d0; $^{*}p < 0.5$, $^{***}p < 0.001$, $^{****}p < 0.0001$. **(C)** Quantification of the number of budding FGF2-LM-BOM organoids after replating and culture with BOM or FGF2 for 7 days. **(D)** Bright-field images showing morphogenesis of FGF2-LM-BOM organoids after replating and culture with BOM or FGF2 for 7 days. Scale bars represent 100 μm. **(E,F)** RT-qPCR analysis of *Mmp2* and *Mmp13* expression in organoids during long-term lactation with continuous lactation medium (LM) treatment or with hormonal/LM withdrawal (LM-BOM). The values are relative to FGF2-LM at each time point. The plots show mean + SD; $n = 3$ for d12–d18, $n = 1$ for d20. Two-way ANOVA, $^{*}p < 0.05$.

over 20 min (Mroue et al., 2015). In contrary, we observed that contraction of a lactating organoid is a very fast process, and the dynamic changes in organoid shape and size are visible to human eye. From videos of contracting organoids, recorded at the rate of one frame per second, we calculated that the frequency is about one contraction per 10 s, which is very similar to the recently reported alveoli warping frequency of lactating mammary tissue upon oxytocin stimulation (Stewart et al., 2019). Therefore, our model provides a suitable *in vitro* system for studying the regulation of the contractile function of myoepithelial cells.

Upon LM withdrawal, lactating organoids underwent involution-like process: regression in size and complexity, which is reversible by FGF2 treatment upon reseeding; and up-regulation of the expression of MMPs, the proteases typically found in mammary gland during involution (Lund et al., 1996; Green and Lund, 2005). Involution-like morphological changes upon prolactin withdrawal were documented also in the 3D coculture model of lactation using mammary epithelial and preadipocyte cell lines. However, epithelial cells cultured without preadipocytes were not reported (Campbell et al., 2014). Thus, for the first time in organoid culture, we show that involution-like regression of epithelium occurs, at least in part, in an epithelium-intrinsic manner. Our observations do not contradict the crucial role of paracrine signaling required for proper involution, including the leukemia inhibitory factor and TGFβ signaling that activate STAT3-mediated regression of epithelium (Nguyen and Pollard, 2000; Kritikou et al., 2003; Hughes and Watson, 2012). Our results point to the existence of epithelial-intrinsic mechanisms of involution, for study of which our epithelial-only organoid model could be advantageous. Certainly, more work is required to establish this model as a valid system for studying physiological involution. In this study, we did not evaluate the onset of programmed cell death and its regulation. In addition, optimization of the culture conditions with cytokine cocktail would be required to further mimic physiological involution.

Several human diseases, developmental defects, or insufficiencies in mammary epithelial tissue are linked to lactation and involution period. Among others, inadequate milk production affects many women after giving birth, especially after premature deliveries and with obese mothers (Olsen and Gordon, 1990; Kent et al., 2012; Nommsen-Rivers, 2016). We propose that human breast tissue, gained from reduction mammoplasties, could be utilized to isolate primary human breast organoids for an analogous lactation assay. Furthermore, findings from murine organoids could be translated into human organoids to identify physiological barriers for lactation, which will provide valuable information for developing novel interventions to support lactation success and provide health benefit across two generations. Moreover, our organoid model could be used to investigate mechanisms of pregnancy-associated breast cancer, an aggressive form of breast cancer with peak of incidence within 5 years after delivery (Schedin, 2006). Mammary organoids isolated from genetic mouse models, such as animals carrying mutations in oncogenes or tumor suppressors, or organoids exposed to carcinogens could be used in our lactation model to unveil mechanisms and signaling pathways leading to epithelial cell carcinogenesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the French legislation in compliance with European Communities Council Directives (A 75-15-01-3), the regulations of Institut Pasteur Animal Care Committees (CETEA),the Ministry of Agriculture of the Czech Republic, and the Expert Committee for Laboratory Animal Welfare at the Faculty of Medicine, Masaryk University.

AUTHOR CONTRIBUTIONS

JS, AC, EC, and ZK performed the experimental work. AC, JS, ZK, and HL contributed to the experimental design and data analysis. AC, ZK, and HL supervised the study. All the authors interpreted the data. ZK and HL acquired funding for the study. AC, JS, and ZK wrote the manuscript. All authors discussed the results and approved the final version of the manuscript.

FUNDING

Work in the laboratory of HL is funded by the Pasteur, Centre National pour la Recherche the Agence Nationale de la Recherche (ANR-10-LABX-73 and ANR-16-CE13-0017-01), Fondation ARC (PJA 20161205028 and 20181208231), and AFM-Telethon Foundation. Work in the laboratory of ZK was funded by the Grant Agency of Masaryk University (grant no. MUNI/G/1446/2018). AC was funded by the postdoctoral fellowships from the Revive Consortium. JS was funded by the P-Pool (Masaryk University, Faculty of Medicine), Amgen Scholars Europe and Erasmus+ programs and by the Grant Agency of Masaryk University (grant no. MUNI/A/1565/2018). EC was funded by the Ph.D. fellowship from Sorbonne Université.

ACKNOWLEDGMENTS

We are particularly grateful to Katarina Mareckova and Anas Rabata for their excellent technical support, and to Dr. Mari Aoki and Dr. Ulrich Boehm for providing cryosections of *Prlr-IRES-Cre;ROSA26-CAGS-GFP* mammary glands. We thank the Central Animal Facility of the Institut Pasteur and the Laboratory Animal Breeding and Experimental Facility of the Faculty of Medicine,

Masaryk University. We also thank the Revive Consortium for funding the exchange program. We acknowledge the core facility CELLIM of CEITEC supported by the Czech-BioImaging large RI project (LM2015062 funded by MEYS CR) for their support with obtaining scientific data presented in this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00068/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 31 March 2020
doi: 10.3389/fcell.2020.00203

Multidimensional Imaging of Mammary Gland Development: A Window Into Breast Form and Function

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An in-depth appreciation of organ form and function relies on the ability to image intact tissues across multiple scales. Difficulties associated with imaging deep within organs, however, can preclude high-resolution multidimensional imaging of live and fixed tissues. This is particularly challenging in the mammary gland, where the epithelium lies deeply encased within a stromal matrix. Recent advances in deep-tissue and live imaging methodologies are increasingly facilitating the visualization of complex cellular structures within their native environment. Alongside, refinements in optical tissue clearing and immunostaining methods are enabling 3D fluorescence imaging of whole organs at unprecedented resolutions. Collectively, these methods are illuminating the dynamic biological processes underlying tissue morphogenesis, homeostasis, and disease. This review provides a snapshot of the current and state-of-the-art multidimensional imaging techniques applied to the postnatal mammary gland, illustrating how these approaches have revealed important new insights into mammary gland ductal development and lactation. Continual evolution of multidimensional image acquisition and analysis methods will undoubtedly offer further insights into mammary gland biology that promises to shed new light on the perturbations leading to breast cancer.

Keywords: mammary gland development, breast cancer, 3D imaging, 4D imaging, intravital microscopy, mammary stem cells, lactation

OPEN ACCESS

Edited by:
Zuzana Koledova,
Masaryk University, Czechia

Reviewed by:
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Specialty section:
This article was submitted to Stem Cell Research, a section of the journal Frontiers in Cell and Developmental Biology

Received: *18 December 2019*
Accepted: *09 March 2020*
Published: *31 March 2020*

Citation:
Lloyd-Lewis B (2020) Multidimensional Imaging of Mammary Gland Development: A Window Into Breast Form and Function. Front. Cell Dev. Biol. 8:203. doi: 10.3389/fcell.2020.00203

INTRODUCTION

Life is underpinned by a series of dynamic biological events tightly coordinated in space and time. Consequently, real-time visualization of cellular processes unfolding in their most relevant contexts is paramount for an in-depth understanding of tissue development and disease (Follain et al., 2017). Recent advances in rapid, high-resolution imaging methodologies, genetically-encoded fluorophores and *in vivo* models are enabling this endeavor, illuminating the dynamic cellular and subcellular events that underpin life (Follain et al., 2017). This mini-review focuses on the application of multidimensional imaging methods to the mammary gland, a secretory organ essential for mammalian offspring survival.

The adult mammary gland comprises of a branched ductal epithelium sheathed by an adipocyte-rich stroma. Two principle cell lineages form the mammary epithelial bi-layer; an inner layer of luminal cells enveloped by a layer of myoepithelial (basal) cells. Mammary gland development

is a multi-stage process, occurring during embryogenesis, puberty and repeated pregnancy cycles (Watson and Khaled, 2008; Macias and Hinck, 2012). This dynamicity was first depicted in 1933 via a sequence of camera lucida drawings of murine mammary gland morphology at different stages of development (Cole, 1933). Subsequent advances in light and electron microscopy rapidly revealed the intricate architecture of the mammary epithelium, laying the groundwork for future studies into the molecular mechanisms that underlie mammary gland form and function (reviewed in Neville, 2009). The mouse is an excellent model for investigating processes regulating human mammary gland biology, providing relevant insights into the perturbations that give rise to breast cancer (Sreekumar et al., 2015).

Historically, detailed microscopic analyses of mammary gland tissues have been restricted to thin, two-dimensional (2D) sections. While informative, with enduring relevance, tissue sections lack architectural context and are hampered by assumptions regarding the uniformity of a particular 2D plane (Sale and Pavelic, 2015; Lloyd-Lewis et al., 2016). Moreover, biological entities are intrinsically three-dimensional (3D), and their true nature cannot be ascertained by a thin section (Richardson and Lichtman, 2015). Volumetric 3D imaging, therefore, is necessary to reveal the spatially complex topology of the branched mammary epithelium. In addition, as fixed tissue analyses are limited to snapshots in time, four-dimensional (4D, *x*-, *y*-, *z*-, *t*-) live cell imaging is required to interrogate the inherently dynamic processes underpinning the development and function of this complex tissue.

Herein, this mini-review provides an overview of the available strategies for high-resolution multidimensional fluorescence imaging of mammary gland tissues at the microscopic scale. Due to space constraints, technologies for imaging at the nano-, meso-, and macro-scale will not be discussed here. Subsequently, this article will briefly highlight recent 3D and 4D imaging studies that have provided important insights into mammary gland ductal development and lactation, which could not have been resolved using conventional histological techniques.

FLUORESCENCE LIGHT MICROSCOPY PLATFORMS FOR HIGH-RESOLUTION MULTIDIMENSIONAL IMAGING

High-resolution fluorescence 3D and 4D microscopic imaging can be performed using optical sectioning techniques such as confocal (Conchello and Lichtman, 2005), multiphoton (Helmchen and Denk, 2005; Dunn and Young, 2006) and light sheet microscopy (LSFM) (Huisken et al., 2004; Keller et al., 2008). Broadly, optical sectioning acquires images of thin focal planes within thick specimens by eliminating the contribution of out-of-focus light and scatter in each image plane. This provides greater contrast, allowing stacks of images captured at serial focal planes to be computationally combined for 3D reconstruction (Conchello and Lichtman, 2005). The universal utility of these imaging approaches for multidimensional microscopy, particularly for *in vivo* cell biology, are discussed in detail elsewhere (Timpson et al., 2011; Follain et al., 2017).

In general, confocal microscopy is the most commonly used optical sectioning technique for fluorescence 3D imaging. However, confocal modalities rely on excitation wavelengths in the visible range that suffer from tissue light absorption and scattering, limiting imaging depths to superficial regions ($\sim$100 μm) in most specimens (Conchello and Lichtman, 2005; Follain et al., 2017). Nevertheless, when important biological information can be garnered from near-surface tissue areas, confocal microscopy is associated with a number of advantages, including widespread accessibility, relatively fast acquisition speeds and flexible multicolor acquisition capabilities (Egeblad et al., 2008; Ebrahim and Weigert, 2019).

For deep tissue fluorescence imaging, multiphoton microscopes equipped with pulsed infrared lasers are frequently used. This approach relies on the simultaneous absorption of two or more low-energy infrared photons for fluorophore excitation. In turn, this confines two-photon excitation to a limited focal volume, enabling optical sectioning alongside reduced phototoxicity and bleaching (Helmchen and Denk, 2005; Dunn and Young, 2006). Moreover, long-wavelength excitation by infrared lasers are associated with decreased tissue scattering and light absorption, facilitating deeper light penetration and imaging depths of up to 1 mm in many tissues. In addition, by exploiting the physical and auto-fluorescent properties of endogenous molecules, nonlinear multiphoton-excitation facilitate second (SHG) (Campagnola et al., 2002) or third (THG) harmonic generation imaging of non-labeled cellular components, such as collagen and lipids (Friedl et al., 2007; Weigelin et al., 2016).

Light sheet fluorescence microscopy (LSFM) is a powerful method that performs optical sectioning using a thin plane of light, allowing focal planes to be captured in a single exposure (Huisken et al., 2004; Keller et al., 2008). This facilitates rapid and long-term 3D imaging of specimens, including live mouse embryos, at high spatiotemporal resolution with minimal photodamage (Power and Huisken, 2017; Katie McDole et al., 2018; Wan et al., 2019). Similarly to confocal microscopy, however, LSFM is constrained by tissue light scattering, limiting its application to relatively transparent or thin samples (Wan et al., 2019). In addition, the unique optical geometry inherent to most current configurations pose significant barriers for sample maintenance during acquisition (Benninger and Piston, 2013), precluding *in vivo* imaging of adult mice by LSFM. Nevertheless, when combined with optical tissue clearing (discussed below), LSFM facilities rapid whole-organ 3D imaging of fixed specimens (Keller and Ahrens, 2015; Susaki and Ueda, 2016), including the mammary gland (Lloyd-Lewis et al., 2016).

3D IMAGING STRATEGIES FOR FIXED TISSUES

All light microscopy methods are hampered by tissue light scattering and absorption, which ultimately defines the limit of depth penetration (Wan et al., 2019). The mammary gland is a case in point, as the adipocyte-rich stroma poses significant barriers for high-resolution, deep tissue 3D imaging. Consequently, a number of strategies are used to improve

mammary gland wholemount immunostaining and depth of imaging in fixed tissues, including microdissection (Rios et al., 2014, 2016b), enzymatic digestion (Wuidart et al., 2016, 2018; Scheele et al., 2017; Lilja et al., 2018), and optical tissue clearing (Davis et al., 2016; Lloyd-Lewis et al., 2016, 2018; Elias et al., 2017; Seong et al., 2018; Chen et al., 2019; Hitchcock et al., 2019; Rios et al., 2019; Stewart et al., 2019). Tissue microdissection facilitates high-resolution 3D imaging of large areas of the ductal epithelium within stroma-divested mammary glands (Rios et al., 2014). Conversely, proteolytic digestion of mammary tissues prior to immunostaining results in improved antibody penetrations, enabling whole-gland 3D imaging of slide-mounted tissues (Wuidart et al., 2016, 2018; Scheele et al., 2017; Lilja et al., 2018). This approach, however, risks damaging or depleting epithelial and stromal cell populations within the mammary fat pad (Rios et al., 2016a), prohibiting its widespread utility. Alternatively, tissue clearing techniques can be harnessed to improve optical access and depth of imaging in intact mammary gland tissues (Richardson and Lichtman, 2015; Lloyd-Lewis et al., 2016).

Recent innovations in optical sectioning microscopy, particularly LSFM, have precipitated the development of numerous optical tissue clearing techniques aimed at rendering biological specimens transparent (Richardson and Lichtman, 2015; Tainaka et al., 2016). These methods seek to increase tissue imaging depths by minimizing light scattering caused by mismatches in refractive indices (RIs) between heterogeneous cellular components. Broadly, optical clearing methods rely on organic solvent-based (e.g., 3DISCO; Erturk et al., 2012) or aqueous reagent-based clearing agents (e.g., Scale, Hama et al., 2015; SeeDB, Ke et al., 2013; CUBIC, Susaki et al., 2014; FRUIT, Hou et al., 2015; C_e3D, Li et al., 2017; UbasM, Chen et al., 2017) to equilibrate RIs within a tissue (**Table 1** and recently reviewed in Matryba et al., 2019). Samples may also be hydrogel-embedded prior to clearing to preserve cellular structures (e.g., "active" and "passive" CLARITY methods; Chung and Deisseroth, 2013; Yang et al., 2014).

By testing a number of these techniques in the mammary gland, a recent study demonstrated that SeeDB (Ke et al., 2013) and CUBIC (Susaki et al., 2014) protocols enable high-resolution 3D imaging of expansive regions of the mammary epithelium within its native stroma (**Figure 1A** and **Table 1**; Lloyd-Lewis et al., 2016). These protocols have subsequently been further developed (Ke et al., 2016; Tainaka et al., 2018), although they remain to be tested in mammary tissues. A recent study also determined the compatibility of CLARITY tissue clearing with 3D imaging of human breast tumor biopsies and archived paraffin embedded samples, highlighting the utility of this approach for enhanced visualization of intra-tumoral heterogeneity in breast cancers (Chen et al., 2019). Thus, optical tissue clearing and 3D imaging of surgically-resected breast tumors holds great potential for improved tumor classification, and thereby treatment strategies, in breast cancer patients. Nonetheless, several tissue clearing methods are disadvantaged by long incubation times, particularly when combined with immunostaining protocols (Richardson and Lichtman, 2015). Difficulties associated with sample mounting, in addition to antibody penetration and performance, also pose challenges for comprehensive deep tissue 3D imaging of mammary gland wholemounts and tumors (Lloyd-Lewis et al., 2016). To address these constraints, a recent study developed a new aqueous-reagent-based tissue clearing reagent (FUnGI) that renders human and murine mammary tissues transparent in 2 h (Rios et al., 2019). When combined with immunolabeling, this protocol spans 3 days, achieving uniform antibody staining that enables large-scale 3D imaging of the mammary epithelium and tumors at single-cell resolution (Rios et al., 2019). The continual development of tissue clearing reagents and 3D image analysis pipelines adapted for human organs (Zhao et al., 2020) will undoubtedly help facilitate the transfer of high-resolution 3D imaging to clinical practice.

Thus far, high-resolution deep tissue and/or whole-gland 3D imaging has mostly been harnessed in genetic fate-mapping studies in the mammary gland (Rios et al., 2014; Davis et al., 2016; Wuidart et al., 2016, 2018; Elias et al., 2017; Lilja et al., 2018; Lloyd-Lewis et al., 2018; Seong et al., 2018) and tumors (Van Keymeulen et al., 2015; Rios et al., 2019), where the ability to visualize expansive regions of the mammary epithelium is paramount for accurate and quantitative clonal analysis. Notably, in contrast to enzymatic digestion or mechanical dissection, most optical tissue clearing protocols preserve tissue and matrix architecture (Lloyd-Lewis et al., 2016). This provides opportunities, therefore, to explore interactions between mammary epithelial cells and their surrounding cellular and non-cellular [e.g., extracellular matrix (ECM)] niche by deep tissue 3D imaging (Inman et al., 2015). In this vein, two recent studies used optical tissue clearing and deep tissue 3D imaging to characterize mammary resident CD45+ leucocyte (Hitchcock et al., 2019), and more specifically macrophage (Stewart et al., 2019), populations at different stages of mammary gland development. Whilst CD45+ cells/macrophages were observed at all developmental stages, their prevalence, morphology, localization and interactions with the mammary epithelial bilayer exhibited stage-specific differences (Hitchcock et al., 2019; Stewart et al., 2019). These interesting findings suggest a surprisingly dynamic interplay between immune cells and the mammary epithelium, which could not have been revealed using conventional histological techniques.

4D INTRAVITAL IMAGING IN THE MAMMARY GLAND: TECHNICAL CONSIDERATIONS

In vivo imaging is an indispensable tool in basic, pre-clinical and clinical research, and is routinely used in medical practice (Condeelis and Weissleder, 2010). While low-resolution imaging approaches (including computed tomography, magnetic resonance imaging, and positron emission tomography) provide valuable anatomic and physiological information into biological tissues and tumors, these imaging modalities lack the resolution to visualize individual cells *in vivo*. By contrast, high-resolution intravital microscopy (IVM) facilitates real-time microscopic imaging of individual cells within intact tissues in live

TABLE 1 | An overview of the tissue clearing methods applied to mammary gland tissues and/or tumors.

Method	Method overview	Key components	RI	Time to clear[b]	Clearing capability	IHC	Preservation		Mammary gland references	Original references
							Structure	FP		
3DISCO	Organic solvent based	Dichloromethane/ dibenzyl ether	1.56	2 days	Strong	Difficult (iDisco)[c]	Compromised – shrinkage	Rapid loss	Erturk et al., 2012; Lloyd-Lewis et al., 2016	Erturk et al., 2012
CLARITY	Aqueous solution based – hydrogel embedding	SDS/acrylamide/Rapiclear/ 80% glycerol	1.52	10 days	Strong	Compatible	Preserved	Preserved	Chen et al., 2019	Chung and Deisseroth, 2013
PACT[a]	Aqueous solution based – hydrogel embedding	SDS/acrylamide/ sRIMS/Rapiclear	1.45–1.46	10–14 days	Weak	Compatible	Preserved – mild expansion	Preserved	Lloyd-Lewis et al., 2016	Yang et al., 2014
Ce3D	Aqueous solution based – simple immersion	*N*-methylacetamide/ Histodenz	1.49–1.5	2 h	Strong	Not tested	Not analyzed	Not tested	Rios et al., 2019	Li et al., 2017
SeeDB	Aqueous solution based – simple immersion	Fructose/thioglycerol	1.49	5 days	Moderate	Compatible	Preserved – mild shrinkage	Preserved	Davis et al., 2016; Lloyd-Lewis et al., 2016, 2018; Elias et al., 2017	Ke et al., 2013
FRUIT	Aqueous solution based – simple immersion	Fructose/Urea	1.49–1.5	3 days	Poor	Not tested	Not analyzed	Not tested	Rios et al., 2019	Hou et al., 2015
ScaleS	Aqueous solution based – simple immersion	Urea/Sorbitol	1.38	3 days	Strong	Not tested	Not analyzed	Not tested	Rios et al., 2019	Hama et al., 2015
FUnGI	Aqueous solution based – simple immersion	Urea/fructose/ glycerol	1.46	2 h	Strong	Compatible	Preserved	Preserved	Rios et al., 2019	Rios et al., 2019
UbasM	Aqueous solution based – simple immersion	Urea/Amino-sugars	Not provided	7–12 days	Not shown	Not tested	Not analyzed	Preserved	Chen et al., 2017	Chen et al., 2017
CUBIC	Aqueous solution based – simple immersion	Urea/sucrose	1.48–1.49	5 days	Strong	Semi-compatible[d]	Preserved – mild expansion	Some loss[d]	Davis et al., 2016; Lloyd-Lewis et al., 2016, 2018; Seong et al., 2018; Hitchcock et al., 2019; Stewart et al., 2019	Susaki et al., 2014

[a]*PACT (passive clarity technique) performed using either Rapiclear or sRIMS (sorbitol RI-matching solution) for imaging in Lloyd-Lewis et al. (2016).* [b]*Including fixation time.* [c]*3DISCO protocol combined with optimized whole-mount immunolabeling procedures (iDISCO). Fluorescence signal is rapidly quenched using benzyl alcohol benzyl benzoate (BABB) and specialized imaging chambers are required for imaging in dibenzyl ether.* [d]*May be improved using second generation CUBIC protocol (R1A, unpublished, protocol available at http://cubic.riken.jp/) and newer derivatives (Tainaka et al., 2018). RI, refractive index; FP, fluorescent protein; IHC, immunohistochemical analysis. Not analyzed/tested means not assessed in mammary gland tissues.*

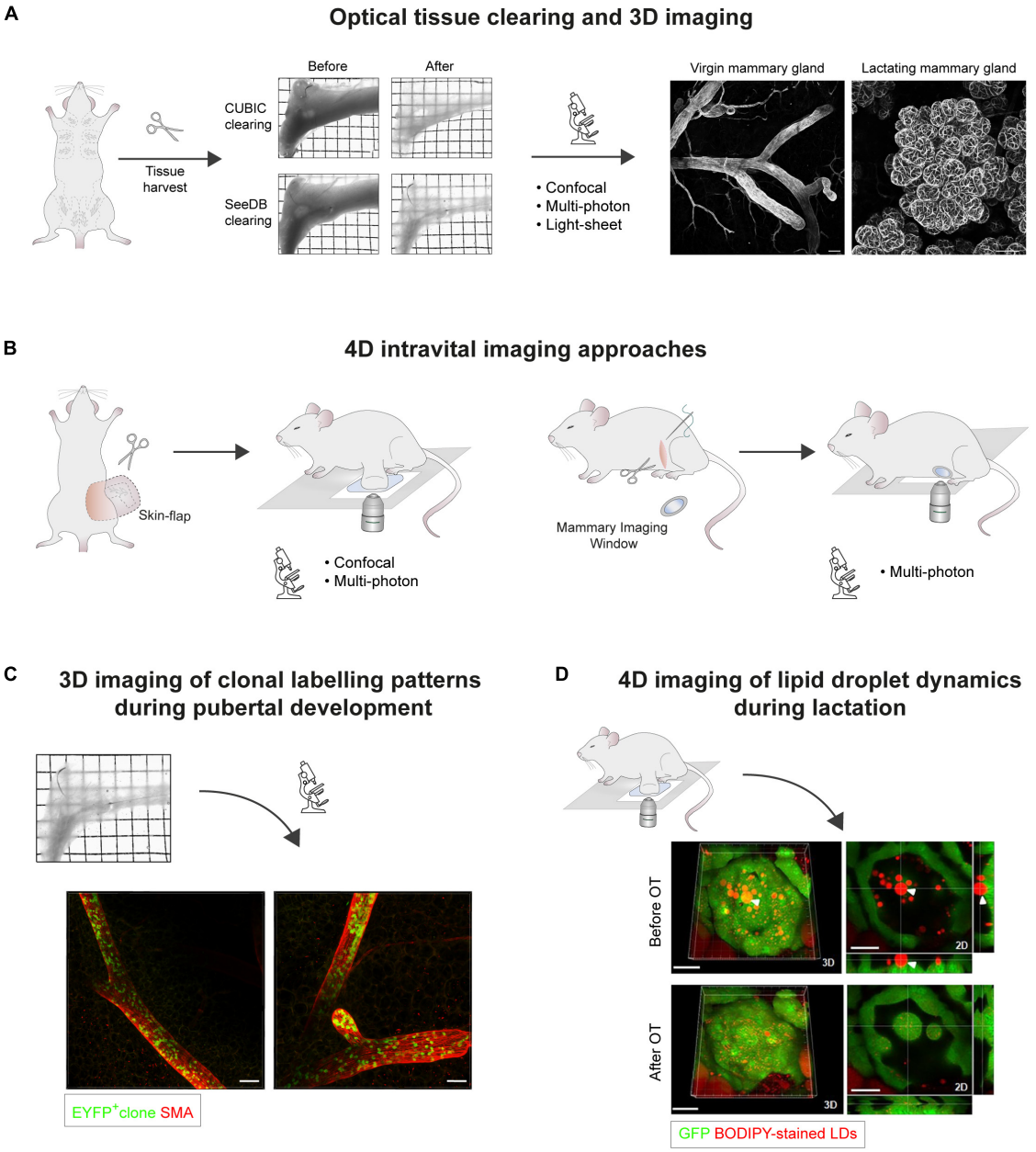

FIGURE 1 | Microscopic 3D and 4D imaging of mammary gland ductal development and lactation. **(A)** Optical tissue clearing and 3D imaging of fixed mammary tissues. Transmission images of harvested abdominal mammary glands before and after tissue clearing using CUBIC or SeeDB protocols. Grid width: 2 mm. 3D confocal imaging of mammary epithelial structures immunostained for Smooth Muscle Actin (SMA) in cleared virgin and lactating mammary tissues. Scale bars, 100 μm. **(B)** 4D intravital imaging approaches. Intravital microscopy can be performed either by surgically exposing the tissue via a skin-flap incision for multiple hours (non-recovery imaging, <40 h), or by implanting optical imaging windows for longitudinal imaging spanning multiple days to weeks. While confocal microscopy is suitable for imaging superficial tissue regions, multiphoton excitation is required for deep-tissue imaging, particularly through mammary imaging windows. **(C)** Clonal patterns arising from the genetic labeling of a single EYFP+ epithelial cell in the mammary gland of a ∼7 week old *R26*$^{[CA]30EYFP}$ mouse. SeeDB tissue clearing and immunostaining were performed prior to 3D imaging by confocal microscopy. Labeled progeny span multiple ducts and branches, and exhibit a sporadic, interspersed labeling pattern, emphasizing the importance of performing whole-gland and/or deep tissue 3D imaging for accurate clonal analysis. These patterns likely arise from the proliferation and intermixing of both labeled and unlabeled terminal end bud (TEB)-resident precursors, which have equipotent potential to contribute to ductal elongation. Scale bars, 100 μm. **(D)** Confocal intravital imaging of fluorescent BODIPY-stained lipid droplets (LDs) in surgically-exposed lactating mammary glands. Release of LDs from the apical surface is mediated by oxytocin (OT)-induced myoepithelial cell contractions. 3D images and 2D sections of the same alveolus before and after OT exposure are shown. White arrowhead points to an LD that was embedded in the cytoplasm prior to alveolus contraction. Scale bars, 30 μm. Images in **(A,C)** adapted from Davis et al. (2016) Nature Communications, under https://creativecommons.org/licenses/by/4.0/. Images in **(D)** adapted from Masedunskas et al. (2017) Mol Biol Cell, under https://creativecommons.org/licenses/by-nc-sa/3.0/.

animals (Pittet and Weissleder, 2011). This powerful approach is increasingly harnessed in experimental and pre-clinical studies in fields spanning developmental biology, immunology, neuroscience, and cancer research (Condeelis and Weissleder, 2010; Nobis et al., 2018). Although currently limited, the utility of high-resolution IVM for clinical use (e.g., in dermatology, laser endomicroscopy) is an active area of research (Coste et al., 2019).

To undertake high-resolution IVM of internal organs, they must be made available to the microscope's objective. The superficial location of the mammary gland makes it amenable to IVM via a "skin-flap" incision, which exposes the tissue for imaging while maintaining its structure and perfusion in the anesthetized mouse (**Figure 1B**; Ewald et al., 2011a,c). This strategy is appropriate for short-to-medium-term IVM of mammary glands for up to 40 h under non-recovery anesthesia (Egeblad et al., 2008; Ewald et al., 2011b,c). For consecutive IVM in longitudinal studies, however, surgical implantation of an optical mammary imaging window is required (**Figure 1B**; Kedrin et al., 2008; Gligorijevic et al., 2009; Ritsma et al., 2012; Zomer et al., 2013). This facilitates tracking of individual cells in live tissues over extended periods of time in near physiological conditions (Alieva et al., 2014). Cell type-specific fluorescent reporters, optogenetic tools and dyes can be combined for simultaneous imaging by multi-color IVM, allowing dynamic interactions between different mammary cell types and cellular structures to be visualized *in situ* (Ellenbroek and Van Rheenen, 2014; Nobis et al., 2018; Perrin et al., 2019). The majority of IVM studies rely on multiphoton modalities for deep tissue imaging (Pittet and Weissleder, 2011; Ellenbroek and Van Rheenen, 2014; Perrin et al., 2019). Nevertheless, the increased surface epithelial mass and lower adipocyte content of lactating mammary tissues and tumors, for example, make these contexts more acquiescent to confocal IVM (Ebrahim and Weigert, 2019).

For visualizing biological phenomenon that remain beyond the capabilities of current IVM tools, alternative *ex vivo* approaches may be used. For example, limited 4D imaging can be performed on excised mammary gland tissue pieces (Davis et al., 2015). Inadequate diffusion of extracellular molecules into thick adult tissues, however, results in artifacts such as tissue hypoxia, restricting this approach to short-term imaging (Shamir and Ewald, 2014; Davis et al., 2015; Lloyd-Lewis et al., 2017). Conversely, many fetal tissues, including the embryonic mammary gland, are able to be maintained *ex vivo* in explant cultures for extended periods (Kratochwil, 1969; Hens et al., 2007; Voutilainen et al., 2012, 2013). Embryonic mammary buds and their surrounding mesenchyme can be established in culture from embryonic day E11.5, allowing real-time *ex vivo* visualization of mammary embryonic branching morphogenesis (Voutilainen et al., 2012, 2013). Mammary embryonic explant cultures, therefore, represent a powerful and accessible tool for dissecting the cellular mechanisms underlying embryonic mammogenesis, an often overlooked phase in mammary gland development. Alternatively, 3D *in vitro* mammary cell culture systems – including mammary organoids that recapitulate the organization and epithelial hierarchy observed *in vivo* – can be used for real-time imaging of mammary epithelial cell behaviors in an experimentally tractable setting (Simian et al., 2001; Debnath et al., 2003; Fata et al., 2007; Ewald et al., 2008; Pasic et al., 2011; Jardé et al., 2016). As this mini-review is focused on imaging mammary gland tissues, these systems will not be discussed further here (for further details see; Shamir and Ewald, 2014; Rios and Clevers, 2018).

MULTIDIMENSIONAL INSIGHTS INTO MAMMARY GLAND DEVELOPMENT

3D and 4D imaging of the mammary gland is increasingly used to address fundamental questions relating to breast biology and cancer. The *in vivo* accessibility of this tissue makes it a particularly excellent model system for high-resolution intravital imaging of tumorigenic processes. The application of IVM to study tumorigenesis, including mammary, has been extensively reviewed elsewhere (Condeelis and Weissleder, 2010; Ellenbroek and Van Rheenen, 2014; Suijkerbuijk and van Rheenen, 2017; Nobis et al., 2018). The following, instead, highlights recent IVM and 3D imaging studies focused on physiological mammary gland development and function, and the insights revealed using these approaches.

Multidimensional Imaging of Mammary Ductal Morphogenesis

While the mammary epithelium begins its morphogenetic journey in the embryo, the majority of its development occurs postnatally. Hormonal stimulation during puberty promotes the elongation and branching of a rudimentary ductal tree, fueled by the proliferative activity of adult mammary stem/progenitor cells housed in terminal end bud (TEB) structures (Watson and Khaled, 2008; Macias and Hinck, 2012). The differentiation potential of these cells – i.e., their ability to generate one or both of the mammary epithelial cell lineages – is an area of intense interest. Early population-based genetic fate-mapping studies in the postnatal mammary gland generated conflicting results, providing evidence in support of both unipotent and bi/multipotent capacities of adult stem/progenitor cells under physiological conditions (for a detailed overview see Lloyd-Lewis et al., 2017; Seldin et al., 2017; Rodilla and Fre, 2018). Discrepancies between these studies may be, in part, attributable to the temporal and promiscuous labeling of cells by selected pathway-specific or lineage-specific promoters. Misleading results may also have arisen due to the limited power of population-based lineage tracing to accurately detect single clones using 2D mammary tissue sections, particularly when labeling is performed above clonal density (Lloyd-Lewis et al., 2017).

To resolve these inconsistencies, more recent genetic fate-mapping studies in the mammary gland – encompassing single cell, neutral, or saturation lineage tracing techniques – have relied on deep tissue and/or whole-gland 3D imaging for quantitative clonal analyses. By combining fate-mapping techniques with the 3D imaging strategies described above, it was established that unipotent luminal and basal progenitors maintain the mammary epithelial lineages during postnatal mammary gland development (Davis et al., 2016; Wuidart et al., 2016; Scheele et al., 2017;

Lloyd-Lewis et al., 2018). Moreover, 3D imaging revealed that the progeny of a single labeled cell can be distributed in a stochastic, interspersed pattern throughout the length of the branching epithelium (**Figure 1C**). These studies indicate that, despite displaying heterogeneity in gene expression at the single cell level (Scheele et al., 2017), proliferative, unipotent TEB-resident cells actively and stochastically contribute to mammary ductal development (Davis et al., 2016; Lloyd-Lewis et al., 2017, 2018; Scheele et al., 2017). Static lineage tracing methods, however, are limited in their ability to reveal the dynamics of individual clone behaviors, necessitating the use of IVM in this context (Scheele et al., 2017; Fumagalli et al., 2019). Interestingly, time-lapse IVM of mammary gland ductal development revealed that TEB-resident mammary epithelial cells continually divide and intermix, with each lineage-restricted cell type maintaining equipotent potential to contribute to ductal elongation (Scheele et al., 2017; Fumagalli et al., 2019). Notably, these quantitative 3D and 4D imaging methods provide avenues for biostatistical modeling of mammary stem/progenitor cell fate, and how this translates into organ structure (Paine et al., 2016; Wuidart et al., 2016; Scheele et al., 2017; Lilja et al., 2018). Thus, when combined with genetic lineage-tracing, the ability to image the mammary epithelium in multiple dimensions (Davis et al., 2016; Wuidart et al., 2016, 2018; Scheele et al., 2017; Lilja et al., 2018; Lloyd-Lewis et al., 2018) has provided important insights into clonal dynamics and cell behaviors during mammary gland development that could not have been attained by examining thin tissue sections (Sale and Pavelic, 2015; Lloyd-Lewis et al., 2017).

While recent genetic fate-mapping studies have demonstrated the unipotency of postnatal mammary lineage precursors in physiological conditions, the durable plasticity of these cells is becoming increasingly apparent (Seldin et al., 2017; Wahl and Spike, 2017; Rodilla and Fre, 2018). Unipotent precursors have been shown to reacquire multi-lineage differentiation capacity in transplantation assays (Stingl et al., 2006; Van Keymeulen et al., 2011; van Amerongen et al., 2012), in response to oncogenic induction (Liu et al., 2007; Koren et al., 2015; Van Keymeulen et al., 2015; Tao et al., 2017) and upon ectopic expression of critical fate determinants of the opposing lineage (Lilja et al., 2018; Wuidart et al., 2018). A recent study also demonstrated that genotoxic exposure results in mammary epithelial cell hyperplasia and lineage infidelity, possibly mediated by signals from the tissue microenvironment (Seldin and Macara, 2019). The future application of IVM in this context is fundamental for revealing the dynamic cellular processes and behaviors underlying mammary epithelial cell plasticity (Fumagalli et al., 2019). Moreover, as this plasticity is likely exploited during mammary tumorigenesis (Liu et al., 2007; Koren et al., 2015; Van Keymeulen et al., 2015; Hein et al., 2016; Tao et al., 2017) – possibly via reactivation of embryonic developmental programs in adult breast tissues (Spike et al., 2012; Zvelebil et al., 2013; Rodilla and Fre, 2018) – an improved understanding will provide important insights into the critical steps leading to breast cancer initiation.

Mammary ductal morphogenesis is heavily dependent on reciprocal interactions between epithelial cells and the microenvironment (Inman et al., 2015; Lloyd-Lewis et al., 2019). Mammary tissue resident macrophages, for example, are recruited to TEB structures during puberty, and have been shown to be essential for normal ductal development (Gouon-Evans et al., 2000, 2002). Preliminary IVM studies in pubertal *Csf1r-EGFP* macrophage reporter mice (Sasmono et al., 2003) revealed that macrophages adjacent to putative TEB structures move rapidly along collagen fibrils, where they promote collagen fibrillogenesis to steer TEB invasion through the mammary fat pad (Ingman et al., 2006). Interestingly, recent 3D deep tissue imaging in optically-cleared mammary tissues revealed that macrophages envelop and infiltrate TEB structures (Stewart et al., 2019), and can intercalate between the epithelial bilayer within ductal regions (Hitchcock et al., 2019; Stewart et al., 2019). Collectively, these 3D and 4D imaging studies suggest a close functional relationship between macrophages and the mammary epithelium, supporting recent findings that established macrophages as important components of the mammary basal stem/progenitor cell niche (Chakrabarti et al., 2018). Detailed insights into these intriguing results awaits further IVM studies of mammary ductal development in *Csf1r-EGFP* mice (Stewart et al., 2019).

Multidimensional Imaging of the Lactating Mammary Gland

Pregnancy is marked by a distinct phase of mammary epithelial growth, branching, and differentiation, resulting in the formation of abundant secretory (milk-producing) lobuloalveolar structures (Watson and Khaled, 2008). Milk secreted into the alveolar lumen is expelled for the suckling neonate by the contraction of alveolar basal cells in response to maternally-produced oxytocin, a process dependent on calcium ions (Gimpl and Fahrenholz, 2001; Haaksma et al., 2011; Davis et al., 2015). Lipids, particularly triacylglycerols, are major milk constituents (Oftedal, 1984) that are packaged and secreted in the form of membrane-coated lipid droplets (LDs) during lactation (Walther et al., 2017). While classical biochemical and morphological analyses have revealed valuable insights into LD assembly, fusion and secretion, the kinetics underlying this dynamic process remained unclear (Mather and Keenan, 1998; McManaman, 2012).

To address this, a recent study performed time-lapse IVM of fluorescent BODIPY-stained LDs in lactating mammary glands to measure their dynamics at peak lactation (Masedunskas et al., 2017). This approach showed that LDs transit to the cell apex by relatively slow and intermitted rates of directed motion (∼0–2 μm/min) and that, regardless of size, fusion of pre-existing LD underlined their growth. Notably, it was observed that oxytocin-induced myoepithelial cell contraction is required to release mature LDs from secretory cells into luminal spaces (**Figure 1D**; Masedunskas et al., 2017). This suggests that LD droplet secretion is intermittently stimulated by milk let-down (Masedunskas et al., 2017), and is not a continuous process as previously assumed from static observations (Mather and Keenan, 1998; Neville, 2009; McManaman, 2012). Intriguingly, alveolar cells switch their cellular function from LD secretion to uptake during mammary gland involution, triggering a complex program of cell death that returns the mammary gland to a near pre-pregnant state

(Kreuzaler et al., 2011; Sargeant et al., 2014). Although fraught with difficulties, IVM studies investigating the mechanisms and dynamics of LD uptake during involution is an aim for the future.

Seeking to assess the spatiotemporal dynamics of oxytocin-induced alveolar contractions, a recent study performed 4D *ex vivo* imaging of mammary tissue pieces from lactating mice engineered to express a Ca^{2+} fluorescent indicator in myoepithelial cells (Stevenson et al., 2019). This approach revealed that Ca^{2+} oscillations couple to myoepithelial cell contractions, which physically deform the inner luminal cell layer for milk ejection (Stevenson et al., 2019). Interestingly, 4D *ex vivo* imaging showed that Ca^{2+}-contraction coupling similarly occurs in ductal myoepithelial cells, indicating that they actively participate in milk ejection during lactation (Stevenson et al., 2019). Together, these recent 4D *in vivo* and *ex vivo* imaging studies (Masedunskas et al., 2017; Stevenson et al., 2019) have provided valuable insights into the dynamic mechanisms underlying milk lipid production, secretion, and expulsion during lactation, building on findings obtained using static measures (Mather and Keenan, 1998; Gimpl and Fahrenholz, 2001; Neville, 2009).

The benefits of 3D imaging over conventional 2D histological techniques is particularly evident when imaging densely packed tissues such as the lactating mammary gland (Rios et al., 2016b). For example, while binucleated secretory luminal cells are readily discernible by 3D imaging (Rios et al., 2016b; Hitchcock et al., 2019) their prevalence is likely underestimated when analyzing mammary tissue sections (Oliver et al., 2012; Hughes and Watson, 2018). The impact of polyploidy – a consequence of the requirement for DNA synthesis for lactation (Banerjee et al., 1971; Banerjee and Wagner, 1972; Smith, 2016) – on LD frequency and dynamics, however, remains unclear. Moreover, recent 3D imaging of optically-cleared lactating tissues revealed that macrophages closely mirror the stellate morphology of adjacent and contacting alveolar myoepithelial cells, a phenotype that is indistinguishable in thin tissue sections (Hitchcock et al., 2019; Stewart et al., 2019). The functional significance of this behavior, however, remains to be elucidated (Hitchcock et al., 2019; Stewart et al., 2019).

CONCLUDING REMARKS

Tissue development and function depend on highly co-ordinated programs of cell proliferation, differentiation, migration, communication, and death. Static 2D measurements alone are insufficient to unravel this complexity. Deep tissue 3D imaging approaches are providing avenues to obtain detailed, spatially integrated insights into the inner workings of the mammary gland, and possess great potential for improving breast tumor classification and characterization in future clinical practice. In addition, the advent of high resolution IVM is transforming the ability to explore the dynamic cellular behaviors governing tissue physiology and dysfunction in near native contexts. High-resolution IVM is increasingly harnessed in experimental and translational breast cancer research, providing valuable dynamic information into mammary tumor growth, progression, metastasis and therapeutic response that ultimately may impact patient care (Condeelis and Weissleder, 2010; Ellenbroek and Van Rheenen, 2014; Suijkerbuijk and van Rheenen, 2017). In contrast, the application of IVM to study mammary gland postnatal development is lagging. Indeed, the light-scattering adipose stroma that shrouds the mammary epithelial tree poses significant challenges for high-resolution *in vivo* imaging of normal and pre-neoplastic ductal structures. Nevertheless, continual improvements in imaging tools, including multiphoton lasers (Andresen et al., 2009), adaptive optics (Rueckel et al., 2006), sensitive detectors and image processing methods (Gligorijevic et al., 2014; Weigert et al., 2018; Perrin et al., 2019) hold great promise for future IVM studies into mammary gland development. The burgeoning application of the multidimensional imaging approaches described herein to the mammary gland will undoubtedly herald a new era in our investigation and understanding of breast biology.

AUTHOR CONTRIBUTIONS

BL-L conceived and wrote the entire manuscript.

FUNDING

This work was supported by the University of Bristol Vice-Chancellor's Fellowship scheme.

ACKNOWLEDGMENTS

Thanks to Prof. Ann Williams, Dr. Mark Jepson and James Handy for helpful discussions and valuable feedback, and apologies to all investigators whose work could unfortunately not be cited owing to space limitations.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 02 April 2020
doi: 10.3389/fcell.2020.00160

Fanconi Anemia Pathway: Mechanisms of Breast Cancer Predisposition Development and Potential Therapeutic Targets

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OPEN ACCESS

Edited by:
Alexandra Van Keymeulen, Université libre de Bruxelles, Belgium

Reviewed by:
James Korkola, Oregon Health and Science University, United States
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Specialty section:
This article was submitted to Molecular Medicine, a section of the journal Frontiers in Cell and Developmental Biology

Received: 11 October 2019
Accepted: 28 February 2020
Published: 02 April 2020

Citation:
Fang C-B, Wu H-T, Zhang M-L, Liu J and Zhang G-J (2020) Fanconi Anemia Pathway: Mechanisms of Breast Cancer Predisposition Development and Potential Therapeutic Targets. Front. Cell Dev. Biol. 8:160. doi: 10.3389/fcell.2020.00160

The maintenance of genomic stability is crucial for species survival, and its failure is closely associated with tumorigenesis. The Fanconi anemia (FA) pathway, involving 22 identified genes, plays a central role in repairing DNA interstrand cross-links. Importantly, a germline defect in any of these genes can cause Fanconi's anemia, a heterogeneous genetic disorder, characterized by congenital growth abnormalities, bone marrow failure, and predisposition to cancer. On the other hand, the breast cancer susceptibility genes, *BRCA1* and *BRCA2*, also known as *FANCS* and *FANCD1*, respectively, are involved in the FA pathway; hence, researchers have studied the association between the FA pathway and cancer predisposition. Here, we mainly focused on and systematically reviewed the clinical and mechanistic implications of the predisposition of individuals with abnormalities in the FA pathway to cancer, especially breast cancer.

Keywords: breast cancer, Fanconi anemia, susceptibility, SNP, predisposition

INTRODUCTION

Fanconi Anemia (FA), a rare autosomal or x-chromosomal recessive human genetic disease, was first described by Guido Fanconi in 1927 (Nalepa and Clapp, 2018), and is characterized by congenital growth abnormalities, bone marrow failure, and predisposition to cancer. During the last 2–3 decades, we have gained remarkable insight into the clinically and biologically complex cancer predisposition syndrome. Although FA occurs rarely (1–5 per million), the heterozygous carriers are present at a much higher frequency (1/300) (D'Andrea, 2010). Biallelic mutations in the genes of the FA pathway reportedly cause FA.

The FA pathway, also called the FA-BRCA pathway, is a fundamental DNA repair pathway that recognizes DNA damage and orchestrates DNA damage responses, especially for DNA interstrand crosslink (ICL) repair (Su and Huang, 2011). Owing to the functional complementation of ICL sensitive cells, 22 FA or FA-like genes have been identified (**Box 1**; Knies et al., 2017; Nalepa and Clapp, 2018). Among these, 8 genes (*FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL*, and *FANCM*) were reported to assemble into a nuclear E3 ubiquitin ligase complex, named the FA core complex, which can monoubiquitinate the FANCD2/FANCI heterodimer (I-D heterodimer). The monoubiquitinated I-D heterodimer localizes to the damaged chromatin,

BOX 1 | List of genes in the FA pathway.
The FA pathway, also called the FA-BRCA pathway, is a fundamental DNA repair pathway, with 22 genes, i.e., *FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN, FANCO, FANCP, FANCQ, FANCR, FANCS, FANCT, FANCU, FANCV,* and *FANCW.*

and interacts with DNA-repair proteins and other downstream FA proteins (FANCD1, FANCDN, FANCJ, and FANCS), to perform repair via homologous recombination (HR) (Kim and D'Andrea, 2012). After the repair process is completed, the de-ubiquitylation enzyme, Ubiquitin Specific Peptidase 1 (USP1), removes the monoubiquitin from the I-D complex, to turn off the network, for recycling to be performed (Kim and Kim, 2016; **Figure 1**).

A typical cellular feature of cells derived from FA patients is that they are specifically hypersensitive to ICL anti-tumor agents, such as mitomycin C, cisplatin, anddiepoxybutane (D'Andrea and Grompe, 2003), which subsequently increase chromosomal breakage, arrest numerous cells at the G2/M phase, and induce apoptosis (Bhattacharjee and Nandi, 2017). Clinically, even without classical physical findings, the high accumulation of chromosomal breakage products, which occurs during the diepoxybutane chromosome fragility assay, could enable the diagnosis of FA (Auerbach, 2009). More importantly, increased chromosomal breakage predisposes FA patients to cancer. Malignancies develop in about 20% of FA patients with homozygous mutations, such as acute myelogenous leukemia, squamous-cell carcinomas of the head and neck, gynecologic squamous-cell carcinoma, and esophageal carcinoma (Dluhy et al., 2005). Interestingly, heterozygous mutations in FA genes, i.e., *BRCA1/FANCS* and *BRCA2/FANCD1*, confer an increased risk of cancer occurrence, especially breast cancer (Petrucelli et al., 2010). Hence, this article mainly focuses on and systematically reviews the clinical and mechanistic implications of the predisposition of individuals with abnormalities in the FA pathway to cancer, especially breast cancers.

FA PATHWAY AND BREAST CANCER PREDISPOSITION

It is accepted that mutations in the FA pathway are strongly associated with a predisposition to breast cancer (Chen et al., 2014). Representatively, homozygous mutations in *BRCA2* cause a severe form of FA disease (Svojgr et al., 2016). King et al. (2003) found that carriers with inherited heterozygous mutations in *BRCA2* have a high risk for developing breast and ovarian cancer. Similarly, heterozygous *BRCA1* mutations can also cause hereditary breast and ovarian cancer syndromes and the biallelic loss of *BRCA1* genes would cause FA development. Other FA genes, such as *BRIP1/FANCJ* and *PALB2/FANCN*, were also identified as breast cancer susceptibility genes (Seal et al., 2006; Rahman et al., 2007).

Breast cancer is the most common cancer affecting women, and has become the leading cause of cancer-related deaths in females worldwide (Siegel et al., 2018). The incidence of breast cancer are various in different ethnicities, but genetic factors caused by family history influence the occurrence of breast cancer (Brewer et al., 2017). Nevertheless, pathogenic mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2* only account for 25–40% of familial breast cancers (FBCs) cases (Mahdavi et al., 2019). Another 5–10% FBC cases are attributed to mutations in other rare susceptibility genes, such as *TP53, ATM, PALB2, BRIP1,* and *CHEK2* (Chen and Parmigiani, 2007).

Unsurprisingly, women with inherited pathogenic mutations in *BRCA1* or *BRCA2* have up to an 85% risk of breast cancer development; hence, risk reduction measures, such as intensive radiological screening, prophylactic surgery, or chemoprevention were suggested for these candidates (Thompson and Dixon, 1992). However, the genetic pathogenesis of the major FBC cases remains unknown. Besides *BRCA1* and *BRCA2*, it is extremely important to identify new breast cancer susceptibility genes, for the prevention and treatment of FBCs.

THE MECHANISMS OF THE FA PATHWAY ARE ASSOCIATED WITH THE OCCURRENCE OF CANCERS

DNA repair, an active cellular process that responds to constant DNA damage, is essential for maintaining genomic integrity. Inherited mutations in DNA repair genes were identified to predispose carriers exhibiting genomic instability to cancer. For example, ATM serine/threonine kinase is recruited and activated by DNA double-strand breaks, leading to cell cycle arrest. And the mutations in *ATM* are responsible for the disorder Ataxia telangiectasia (Rotman and Shiloh, 1998). Bloom syndrome protein exhibits both DNA-stimulated ATPase and ATP-dependent DNA helicase activities, and mutations in *BLM* cause Bloom syndrome (Kaneko and Kondo, 2004).

The following section will describe the mechanisms of the FA pathway involved in the repair of the ICL damage, and the corresponding mutations that cause a genomic integrity deficit and promote tumorigenesis (Joenje and Patel, 2001; **Figure 2**).

Impaired Interphase DNA Damage Response (DDR)

FA proteins are involved in DDR at multiple levels. First, the DNA damage sensor, ataxia-telangiectasia, and RAD3-related (ATR) kinases, together with its downstream kinase checkpoint kinase 1 (CHK1), detect DNA lesions (mainly stalled replication forks in ICLs), and initiate a response from the FA pathway, by phosphorylating the FA core complex and I-D heterodimer (Ishiai et al., 2017). Subsequently, the activated DDR-stabilized TP53 protein boosts the transcription of cyclin-dependent kinase inhibitor 1A (CDKN1A), to inhibit proliferation and facilitate repair progression (Warfel and El-Deiry, 2013). Meanwhile, the FA core complex monoubiquitinates the I-D heterodimer and promotes ICL repair by causing nucleases, such as FANCP (SLX4), Fanconi-associated nuclease 1 (FAN1), and XPF-ERCC1 to cleave injured DNA strands (Yamamoto et al., 2011; Pizzolato

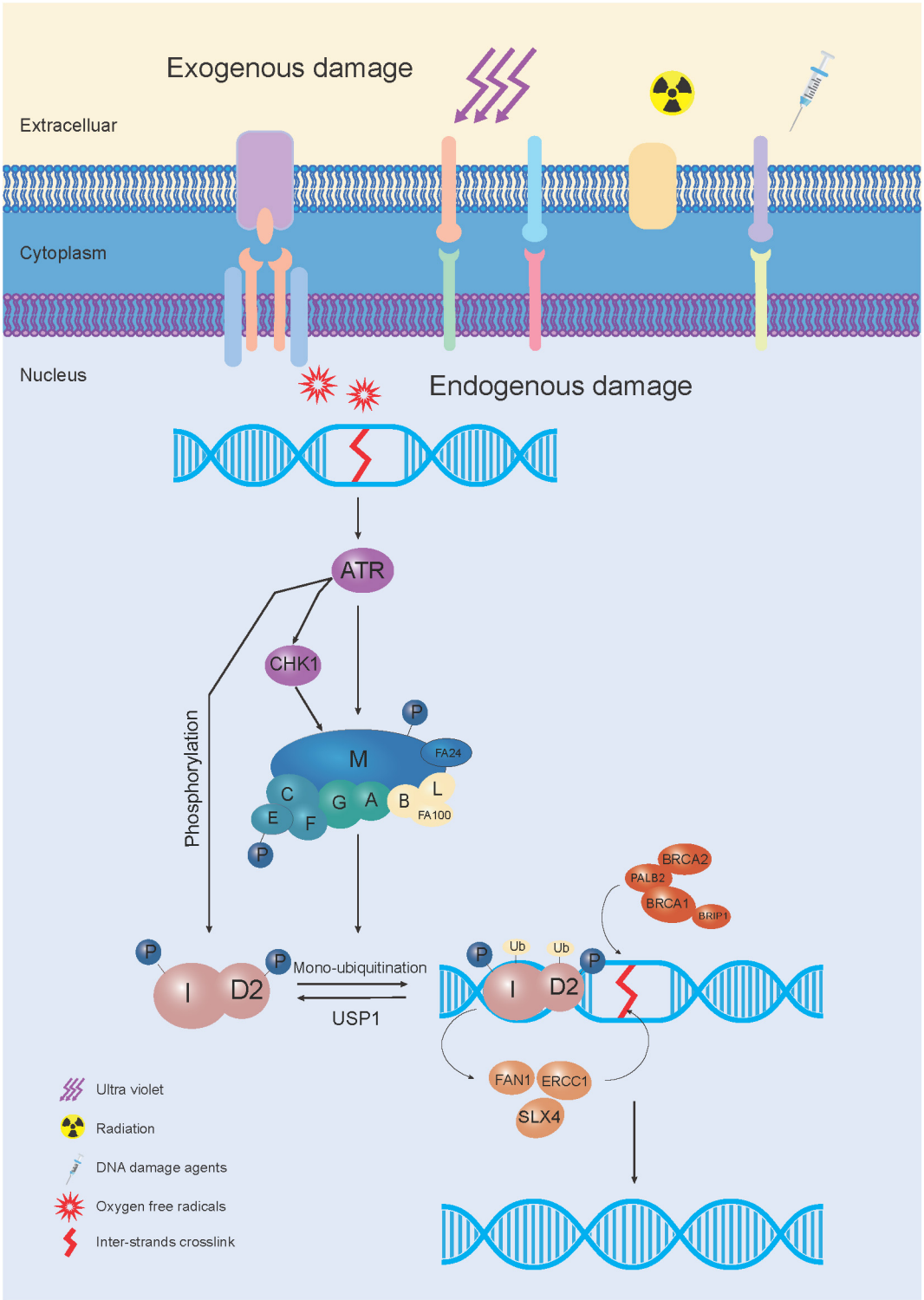

FIGURE 1 | The schematic elucidation of the FA pathway mechanism used during DNA repair. In response to exogenous and/or endogenous damage, 8 FA genes (*FANCA*, *FANCB*, *FANCC*, *FANCE*, *FANCF*, *FANCG*, *FANCL*, and *FANCM*) were assembled into the FA core complex, which functioned as a nuclear E3 ubiquitin ligase complex, to monoubiquitinate the I-D heterodimer. The monoubiquitinated I-D heterodimer was localized to the damaged chromatin, and interacted with DNA-repair proteins and other FA proteins (FANCD1, FANCDN, FANCJ, and FANCS) in the FA pathway, to conduct the repair process through homologous recombination (HR). After the damage was repaired, monoubiquitin was removed from the I-D complex by a de-ubiquitylation enzyme, Ubiquitin Specific Peptidase 1 (USP1), to "turn off" the network.

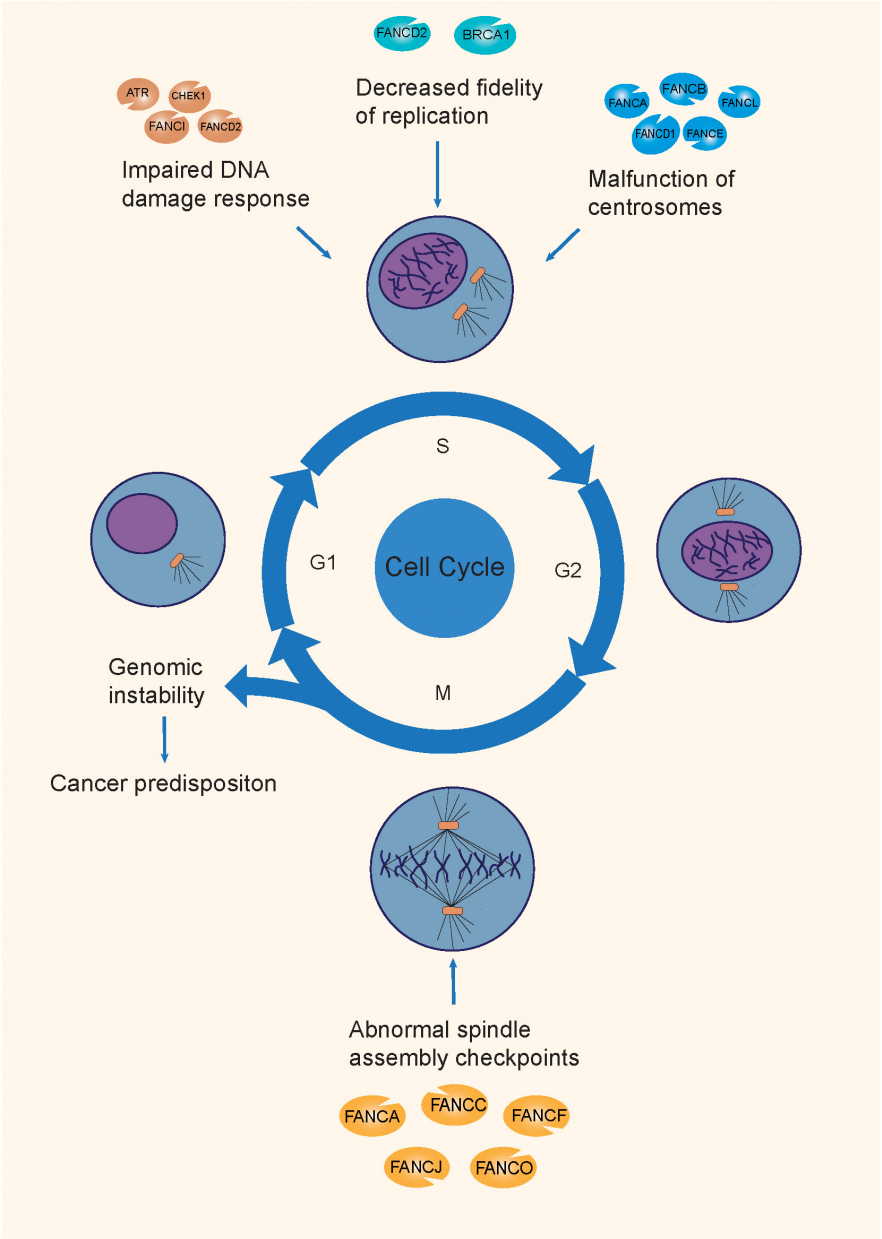

FIGURE 2 | The mechanisms of tumorigenesis attributable to FA mutations. FA genes maintain genomic integrity through the different phases of the cell cycle, by participating in the DDR process, replication fork protection, normal centrosome function, and spindle assembly checkpoints. Mutations on different FA genes are involved in different mechanisms during the cell cycle, causing genomic instability, and causing a predisposition to cancer.

et al., 2015). Finally, the repair process is completed through HR, mainly by the FA downstream genes *FANCS*, *FANCD1*, and *FANCO* (Kim and D'Andrea, 2012). Mutations in such FA genes would impair the DDR process, leading to genomic instability.

Decreased Replication Fork Protection and Fidelity

Besides the DDR process, Schlacher et al. (2012) reported a novel repair-independent mechanism, that is *FANCD2*- or *BRCA1*-mediated stalling of replication forks, in order to promote the degradation of replication forks and increase replication fidelity, thereby maintaining genomic stability during DNA replication. Normally, BRCA1 interacts with RAD51 during the process of repair of DNA double-strand breaks (Boulton, 2006). The regular level of FANCD2 and FANCM prevented replication fork damage caused by poor coordination between DNA replication and transcription. Surprisingly, in *FANCD2*-deficient cells, increased RAD51 expression levels enhanced the protection of replication forks. Moreover, *FANCD2*-mediated fork protection showed epistatic effects with *RAD51*, which

were indicative of an unanticipated fork protection process, and a repair-independent pathway of FA proteins that prevented genomic instability (Schlacher et al., 2012).

Supernumerary or Over-Replication of Centrosomes

The centrosome serves as the main microtubule-organizing center and regulator of cell cycle progression in animal cells. During mitosis, the nucleated microtubule of the centrosome promotes mitotic spindle assembly, via chromosomal interactions (Nalepa et al., 2013). The presence of extra centrosomes was linked to chromosomal instability, and caused aneuploidy and cancer, by promoting merotelic kinetochore-spindle association (Ganem et al., 2009).

Nalepa et al. (2013) performed RNAi screening and the results showed that 8 FA proteins (FANCA, FANCB, FANCD1, FANCD2, FANCE, FANCG, FANCL, and FANCN) distinctively localized to centrosomes during mitosis, while FANCC and FANCA localized to the mitotic spindle in a cell-cycle dependent manner. Interestingly, an abnormally high number of centrosomes was observed in the primary fibroblasts of FA patients, as compared to that in the controls. Moreover, the accumulation supernumerary centrosomes were observed in artificial FA-deficient cells (Nalepa et al., 2013), suggesting that besides FANCD1, other FA proteins are also essential for maintaining normal centrosome numbers during mitosis. Zou et al. (2014) discovered that in non-stressed Hs587T cells, deficiency of BRCA1 induces centrosome amplication and aneuploidy. However, in hydroxyurea and mitomycin C-treated Hs587T cells experiencing prolonged genotoxic stress, they found that BRCA1 and FANCJ cooperatively promotes DNA damage-induced centrosome amplification (DDICA), by activating polo-like kinase (Zou et al., 2014). On the other hand, in non-invasive breast cancer cell line MCF-7, BRCA1 nuclear export stimulates its regulation of centrosome duplication, which is mediated by the major nuclear export receptor, CRM1 (chromosome region maintenance protein 1) under irradiation treatment (Brodie and Henderson, 2012), predicting the diverse mechanism of BRCA1 function in regulating centrosome amplication in different types of breast cancer cells. Additionally, it was found that cells with deficiencies or dysfunctions of in FA genes promoted error-prone mitosis, along with chromosome missegregation and interphase DNA damage (Abdul-Sater et al., 2015), which contributed to genomic instability, and subsequently, to tumorigenesis.

Abnormal Spindle Assembly Checkpoint (SAC)

It is known that the capture of the kinetochore by the spindle is a critical step for correct segregation during mitosis, and SAC prevents the separation of duplicated chromosomes until their proper attachment to the spindle apparatus. The SAC can monitor the interactions between kinetochores and spindle microtubules, and be activated by diverse kinds of defects, such as spindle depolymerization (Li and Murray, 1991), dicentric chromosomes (Neff and Burke, 1992), aberrant segregation of centromeres (Wells and Murray, 1996), dysfunctions of kinetochores (Wang and Burke, 1995), or mutations in centromeric DNA (Wang and Burke, 1995), resulting in anaphase arrest, via the inhibition of the anaphase-promoting complex. Hence, the misfunctioning of the SAC can lead to chromosome missegregation, aneuploidy, and even tumorigenesis (Musacchio and Salmon, 2007).

The localization of FA proteins to the mitotic spindle in a cell cycle-dependent manner reveals that FA signaling is essential for the SAC during cell division (Nalepa et al., 2013). It was reported that multiple FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCD2, FANCI, FANCL, FANCJ, FANCO, and FANCP) are essential for the normal functioning of the SAC (London and Biggins, 2014). Deficiencies in such FA proteins may weaken the functions of SAC, subsequently resulting in genomic instability.

Using all the above mechanisms, the mutations in FA genes finally resulted in genomic instability and subsequent tumorigenesis, however, it is still unclear why germline mutations in certain FA genes lead to tissue-specific tumors. Despite its critical role in ICL repair, the loss of the *BRCA1* function affected specific tissues in the breast and ovaries (Rebbeck et al., 2015). The BRCA1 suppressor hypothesis was put forward, stating that these particular tissues had unique genetic factors or special physiological environments that enhanced cell survival in the absence of *BRCA1*, such as those resulting from the expression of estrogen or other hormones targeting the breast and ovaries. Upon exhibiting additional survival-promoting genetic changes, the cells would be transformed into a malignant tumor (Elledge and Amon, 2002).

FA GENES AND THEIR ASSOCIATION WITH BREAST CANCER PREDISPOSITION

Based on the mentioned mechanisms, certain FA genes have been identified as breast cancer susceptibility genes, while further evidence is needed to identify others such potential genes (**Table 1**).

Identified Breast Cancer Susceptibility Genes in the FA Pathway

BRCA1/FANCS and BRCA2/FANCD1

The human breast cancer type1 susceptibility protein (*BRCA1*) (FA alias *FANCS*) and breast cancer type 2 susceptibility protein *(BRCA2)* (FA alias *FANCD1*) are the most important hereditary breast cancer genes, as identified by linkage studies in 1994 and 1995, respectively (Hall et al., 1990; Miki et al., 1994; Wooster et al., 1994, 1995). *BRCA1* and *BRCA2* are essentially tumor suppressor genes, which mainly help to repair damaged DNA or destroy cells if DNA cannot be repaired, thereby ensuring genomic stability (Gudmundsdottir and Ashworth, 2006). Taken together, mutations in *BRCA1/2* account for 25–40% of FBCs (Antoniou et al., 2001), and up to 10% of all breast cancers (Pfeffer et al., 2017) (**Figure 3**). Deleterious variants in *BRCA1/2* confer a strong predisposition

TABLE 1 | Classification of FA genes that confer to breast cancer susceptibility.

FA gene	Alias	Estimated frequency in FA	Chromosomal location	Molecular function	Breast cancer susceptibility	References
FANCS	*BRCA1*	Rare	17q21.31	DNA repair via homologous recombination	Identified	Hall et al., 1990; Miki et al., 1994; Wooster et al., 1994, 1995
FANCD1	*BRCA2*	Rare	13q12–13	• DNA repair control and effector recruitment; • Regulates RAD51	Identified	Hall et al., 1990; Miki et al., 1994; Wooster et al., 1994, 1995
FANCJ	*BRIP1*	<2%	17q22–24	• 5′-to-3′ DNA helicase; • Binds BRCA1; • Phosphorylated following DNA damage	Identified	Guenard et al., 2008; Ouhtit et al., 2016
FANCN	*PALB2*	About 2%	16p12.1	Partner for BRCA2 stability and nuclear localization	Identified	Southey et al., 2010; Blanco et al., 2013; Foo et al., 2017
FANCO	*RAD51C*	Rare	17q22	DNA repair via homologous recombination	Identified	Meindl et al., 2010
FANCM		<0.2%	14q21.3	• FA core complex assembly • DNA helicase involved in repair of Holliday junctions and replication forks • Recruits the BLM helicase during the DDR	Potential	Kiiski et al., 2014; Peterlongo et al., 2015; Neidhardt et al., 2017
FANCC		10%	9q22.3	FA core complex assembly	Potential	Thompson et al., 2012
FANCD2		About 2%	3q25.3	• FA I-D complex assembly • Monoubiquitylate and phosphorylate following DNA damage	Potential	Barroso et al., 2006; van der Groep et al., 2008; Mantere et al., 2017
FANCP	*SLX4*	Rare	16p13.3	• Resolution of Holliday junctions • Interacts with several nucleases, including FANCQ	Potential	Landwehr et al., 2011; Surowy et al., 2018

to breast cancer, and increase the relative risk to carriers by about 10- to 20-fold, as compared to that for the general population (Stratton and Rahman, 2008). During their lifetime, breast cancer carriers have a breast cancer developmental risk of up to 50 and 80% at 70 and 90 years (Chen and Parmigiani, 2007). Besides breast cancer, a dysfunction in *BRCA1/2* is also proven to be associated with an elevated risk of occurrence of other cancers, such as ovarian, pancreatic, prostate, and stomach cancers (Roy et al., 2011). Although the frequencies of *BRCA1/2* mutations vary significantly in different populations, based on geographic regions and ethnicities (Fackenthal and Olopade, 2007), they tend to occur infrequently in most populations; hence, *BRCA1/2* genes are classified as rare high-penetrance breast cancer susceptibility genes (Stratton and Rahman, 2008).

BRCA1, encoded by the *BRCA1* gene on 17q21, contains four major domains, i.e., a zinc ring finger (RING) domain, BRCA1 serine cluster domain (SCD), and two BRCA1 C Terminus (BRCT) domains (Rosen et al., 2003). *BRCA1* is mainly involved in repairing double-stranded breaks in DNA and cell cycle checkpoint activation (Caestecker and Van de Walle, 2013), along with transcriptional regulation and chromatin modification (Venkitaraman, 2002; Yoshida and Miki, 2004). Hundreds of mutations have been identified in *BRCA1*, and most of the disease-causing variants of *BRCA1* are present in the BRCT and RING domains, which are essential for the repair function (Nelson and Holt, 2010).

BRCA2 protein, encoded by the *BRCA2* gene on 13q12.3, is responsible for repairing DNA via the specific regulation of the HR pathway, and has a significantly different structure, as compared to that of *BRCA1* (Orelli and Bishop, 2001). It mainly contains eight BRC repeats and the BRCA2 DNA-binding domain, which includes a helical domain (H), three oligonucleotide binding (OB) folds and a tower domain (T) (Roy et al., 2011). Not surprisingly, different mutations in *BRCA1/2* cause variant subtypes of breast cancers. It was reported that pathogenic mutations in *BRCA1* normally result in triple-negative breast cancers (TNBC) (Lee, 2008), while *BRCA2* mutations typically cause the development of ER + luminal subtypes exhibiting a slow proliferation and low level of aggression (Talens et al., 2017).

However, the tumor suppressor mechanism of *BRCA1* and *BRCA2* was thought to have no association with the FA pathway, until Howlett et al. (2002) identified the *FANCD1* gene as *BRCA2* in 2002. The study was based on the fact that cell lines homozygous for *BRCA1/2* mutations are hypersensitive to mitomycin-C (Moynahan et al., 2001) and that homozygous *BRCA2* mutant mice have phenotypic features similar to those observed in the mice with FA (Connor et al., 1997). These findings urged Howlett et al. (2002) to screen mutations within *BRCA1/2* in FA patients without mutations in known FA genes. They found that they were heterozygous for truncating *BRCA2* mutations in one FA-B and two unassigned FA cases. Moreover, the reference FA-D1

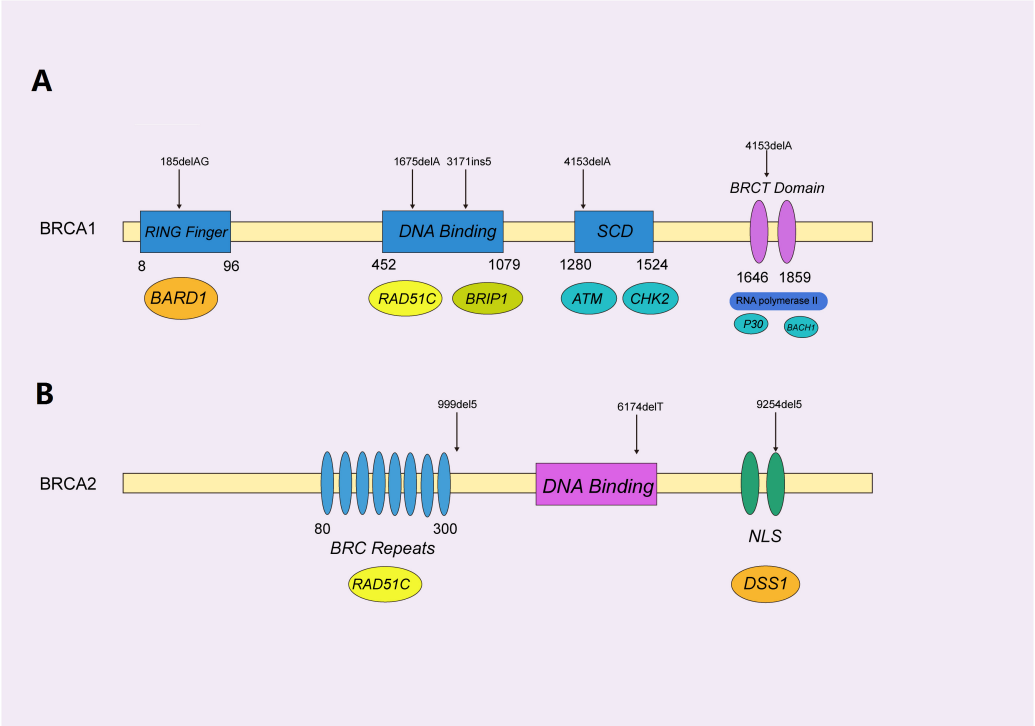

FIGURE 3 | Functional domains of BRCA1/2 protein with pathogenic mutations. **(A)** The functional domains of the BRCA1 protein, mainly containing the RING-finger, SQ-cluster, and BRCT (middle) domains, functionally interacted with BARD1, RAD51C, and ATM (beneath), to orchestrate homologous recombination. The selected reported pathogenic mutations are indicated with black arrows, as shown. **(B)** BRCA2 was represented by a similar schematic figure with different functional domains (middle) and binding partners (beneath); confirmed pathogenic mutations are also shown above.

cell line was homozygous for a *BRCA2* splicing mutation that resulted in an in-frame deletion of four amino acids, and an additional FA-D1 patient carried two truncating *BRCA2* mutations (Howlett et al., 2002). All these findings strongly suggested that *BRCA2* caused FA-D1. This surprising but significant discovery not only enabled us to determine an important connection between the FA genes and breast cancer, but also provided an attractive model for identifying more breast cancer susceptibility genes and exploring their underlying mechanism. Thus, a new role of the FA pathway in breast cancer predisposition was discovered.

Similarly, *BRCA1* was identified as FANCS by Sawyer et al. (2015), with a missense mutation in the C terminal, and a frameshift mutation in exon 11 in a 23-year-old female with breast cancer exhibiting multiple congenital abnormalities and an FA-like presentation. Together with a preceding report describing the biallelic loss of *BRCA1* in a young ovarian cancer patient with multiple congenital abnormalities (Domchek et al., 2013), this proof contributed to the identification of *BRCA1* as *FANCS*.

BRIP1/FANCJ

BRIP1 is a member of the RecQ DEAH helicase family, and is encoded by *BRIP1*, a tumor suppressor gene involved in the DNA repair pathway, via its interaction with *BRCA1* (Ouhtit et al., 2016). In Levitus et al. (2004) reported 2 new genetic subtypes excluded from 9 known subtypes (A, B, C, D1, D2, E, F, G, and L), including FA-J, based on 8 unrelated FA patients, and defined FA-J cell line with mono-ubiquitinated FANCD2, which complemented group FA-I but did not complement each other, indicating a downstream defect in FA-J cells (EUFA1289 cells). However, they did not identified the genes defective in complementation groups FA-I nor FA-J. Levitus et al. (2005) successfully identified *BRIP1* as an FA gene in a sub-group of mutated FA-J patients, named *FANCJ* A recurrent nonsense mutation 2392C→T/R798X was identified in 4 individuals with different ethnic backgrounds, as well as three splice mutations in the intervening sequence (IVS), i.e., IVS3 + 5G→T, IVS17 + 2insT, and IVS11-498A→T, which demonstrated the relationship between *BRIP1* and the onset and development of FA-J.

Soon, Seal et al. (2006) identified *BRIP1* as a breast cancer susceptibility gene by detecting several truncating mutations in *BRIP1* that were associated with the onset of breast cancer in high-risk families without mutations in *BRCA1/2*. Further studies illustrated that *BRIP1* was a low/moderate-penetrance breast cancer susceptibility gene (Guenard et al., 2008). Several other mutations, such as C47G/rs4988351, 2971C > G/Q944E/, rs7213430, and rs4986764 (49-51) were reported to cause the elevated risk of breast cancer in different populations, supported the role of *BRIP1* in breast cancer development.

PALB2/FANCN

PALB2 was first identified as an interactor of BRCA2 in the DDR process (Xia et al., 2006). PALB2, which is physically bound to BRCA1/2, forms a BRCA complex and maintains genomic integrity via the FA and HR pathways (Sy et al., 2009; Zhang et al., 2009a,b). Unsurprisingly, it was found that mutations in *PALB2* could cause the appearance of FA subtype N, i.e., *FANCN*, presented with skin, thumb, heart and kidney abnormalities and growth retardation like other FA subtype, however, the presention of FANCN patients is similar to the phenotype of biallelic BRCA2 mutations and differs from other FA subtypes, most notably with respect to the high risks of childhood solid tumors, particularly Wilms tumor and medulloblastoma (Reid et al., 2007; Xia et al., 2007). So, the mutations in *PALB2* normally not only resulted in typical FA phenotypes, but also increased the occurrence of pediatric malignancies, Wilm's tumors, and medulloblastomas (Reid et al., 2007). Importantly, the cancer spectrum caused by mutations in *PALB2* is quite similar to that induced by mutations in *BRCA2*, thereby validating the direct interaction between *PALB2* and *BRCA2* (Nepomuceno et al., 2017).

Almost simultaneously, Rahman et al. (2007) first reported that *PALB2* is a breast cancer susceptibility gene, thereby establishing the fact that mutations in *PALB2* cause a predisposition to breast cancer. Monoallelic truncating *PALB2* mutations were identified in 10/923 individuals with FBCs, conferring a 2.3-fold higher risk for breast cancer, as compared to 0/1,084 controls (Rahman et al., 2007). However, the penetrance of mutations in *PALB2* varied significantly in different populations, ranging in a 2–30-fold higher risk, as compared to that in non-carriers (Southey et al., 2010; Antoniou et al., 2014; Slavin et al., 2017). Several missense variants with an unknown significance have also reportedly been associated with breast cancer (Blanco et al., 2013; Damiola et al., 2015; Li et al., 2015; Nakagomi et al., 2016), among which L35P was identified as the pathogenic variant in a family with a strong history of breast cancer (Foo et al., 2017).

RAD51C/FANCO

RAD51C, which belongs to the *RAD51* family, is crucial for maintaining genome stability in the HR pathway by binding to single-stranded DNA and unwinding duplex DNA, and forming helical nucleoprotein filaments at the DNA breakage site (Suwaki et al., 2011). As biallelic germline mutations in *RAD51C* were associated with an FA-like syndrome, in 2010, *RAD51C* was demonstrated to be the same as *FANCO* in the FA pathway (Vaz et al., 2010). Meindl et al. (2010) discovered *RAD51C* to be a cancer susceptibility gene, and discovered 6 pathogenic mutations in 1,100 families with breast/ovarian cancer, and not discovering it either in 620 pedigrees with breast cancer alone, or in 2,912 healthy controls. Interestingly, the penetrance level of *RAD51C* is similar to that in BRCA1/2, indicating the important cellular function of *RAD51C* as a tumor suppressor gene in the DNA repair process (Meindl et al., 2010). Osorio et al. (2012) screened the mutations in the *RAD51C* gene in a large series of 785 Spanish families with breast and/or ovarian cancer, and identified that 1.3% exhibited mutations, thus supporting the fact that *RAD51C* played a role as a susceptibility gene.

Potential Breast Cancer Susceptibility Genes in the FA Pathway

FANCM

FANCM is the most conserved protein in the FA pathway, and plays an important role in promoting branch migration in Holliday junctions and DNA repair structures at replication forks (Blackford et al., 2012). With its translocase and endonuclease activities, *FANCM* functions as a tumor suppressor gene, by suppressing spontaneous sister chromatid exchanges and maintaining chromosomal stability (Gari et al., 2008). Kiiski et al. (2014) first reported a nonsense mutation in *FANCM*, c.5101C > T (p.Q1701X); it was associated with the breast cancer risk in the Finnish population, and was significantly more frequent among breast cancer patients than in controls, with a particular enrichment observed in TNBC patients. The second variant associated with breast cancer risk, c.5791C > T, was discovered (Peterlongo et al., 2015), followed by several heterozygous loss of function (LoF) mutations in *FANCM* (Neidhardt et al., 2017). All these observations provided evidence that *FANCM* is a candidate breast cancer susceptibility gene.

FANCC

The FANCC protein, which is present in the Fanconi anemia complementation group, is involved not only in DNA repair and genome integrity maintenance (Kitao et al., 2006), but also in metabolic disorders (Nepal et al., 2018) and provision of protection against oxidative stress-induced apoptosis (Kulanuwat et al., 2018). In Berwick et al. (2007) found that 6 out of 33 carriers with *FANCC* mutations developed breast cancer, and a 2.4-fold increase in standardized incidence ratios (SIR) was noted among carrier grandmothers. Another three truncating mutations in *FANCC* were observed in 438 breast cancer families, while 1 pathogenic mutation was identified in an additional 957 breast cancer families; no deleterious mutation was reported in 464 healthy controls nor in 1,000 genomic data (Thompson et al., 2012). However, the role of mutations reportedly occurring during breast carcinogenesis remains unclear. Further research is needed, to confirm the possible susceptibility alleles of *FANCC* mutations.

FANCD2

As mentioned above, FANCD2 can combine with FANCI to form the I-D heterodimer, which would be monoubiquitinated by E3 ubiquitin ligase; this is regarded as the central step in the activation of the FA pathway (Ishiai et al., 2017). More importantly, it was found that FANCD2 co-localized with BRCA1/2 in DNA damage-inducible foci (Wang et al., 2004; Montes de Oca et al., 2005), suggesting a strong potential association between FANCD2 and FBC. Further studies provided more evidence that established their association. Although Lewis et al. (2005) first reported that there was no evidence regarding the fact that highly penetrant exonic or splice site mutations in

FANCD2 contributed to FBCs, an article published during the next year predicted that mutations in *FANCD2* were associated with an increased risk of sporadic breast cancer (Barroso et al., 2006). Then, van der Groep et al. (2008) discovered that the somatic inactivation of (epi)genetic events in *FANCD2* might be important in both sporadic and hereditary breast carcinogenesis. Rudland et al. (2010) further illustrated that the cytoplasmic loss of *FANCD2* in primary breast carcinomas might allow the selection of cells overexpressing proteins that could induce metastases before surgery. In 2017, truncating mutations in *FANCD2* were discovered, which connected this FA gene with hereditary breast cancer susceptibility during case-control analysis (Mantere et al., 2017), indicating that *FANCD2* is a potential breast cancer susceptibility gene.

SLX4/FANCP

SLX4, a DNA repair protein, encoded by the *SLX4* gene, regulates three structure-specific endonucleases (SLX1, XPF-ERCC1, and MUS81-EME1), and is necessary for providing resistance to DNA crosslinking agents, topoisomerase I(TOPI), and poly (ADP-ribose) polymerase (PARP) inhibitors (Shah et al., 2013). Biallelic mutations of *SLX4* (also known as *FANCP*) have been identified in patients with a new subtype of FA, termed as FA-P (Kim et al., 2011; Stoepker et al., 2011). Ponce et al. (2012) detected an *SLX4* missense change, i.e., c.1114C > T (p.Arg372Trp), segregated along with breast cancer genes within the family, which supported the potential role of *SLX4* in conferring breast cancer susceptibility. Even though several studies failed to verify the role of *SLX4* mutations in breast cancer (Landwehr et al., 2011; Bakker et al., 2013; Shah et al., 2013), a potential link between SLX4 and breast cancer predisposition was strongly recommended by Surowy et al. (2018), through the successful identification of a variant rs3810813 in the *SLX4/FANCP* gene, which was significantly associated with both breast cancer and decreased DNA repair capacity.

IMPLICATIONS FOR BREAST CANCER THERAPY

The disruption of the FA pathway results in defective DNA repair, genomic instability, and tumorigenesis, and provides promising targets for breast cancer therapy, by inducing completely different biological characteristics in tumor cells. Strategies for targeting these deficiencies are summarized in the following section.

Synthetic Lethality and Parp Inhibitors

DNA damage in the human genome mainly involves single-strand breaks (SSBs), double-strand breaks (DSBs), and inter-strand crosslinks. DSBs are restored via double-strand break repair (DSBR), which involves HR and non-homologous end joining (NHEJ). SSBs are restored by single-strand break repair (SSBR), which involves base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). BER plays the most important role in SSBR, by recruiting other DNA repair players to the site of DNA damage, through the activity of poly (ADP-ribose) polymerases (PARPs) (Caldecott, 2019).

PARPs are involved in various cellular processes, such as DNA repair, DNA replication, recombination, and chromatin remodeling. Among the 17 types of PARPs, PARP-1 plays the most important role during DNA damage, mainly in SSBR (Keung et al., 2019). During SSBR, PARP1 detects the damaged site and binds damaged DNA through its N-terminal zinc finger motifs; then, the catalytic C-terminal domain is activated, to hydrolyze NAD + and produce linear and branched PAR chains, which can extend over hundreds of ADP-ribose molecules (Langelier et al., 2012). Subsequently, several DNA repair proteins, including topoisomerases (TOP), DNA ligase III, DNA polymerase β, and scaffolding proteins, such as X-ray cross-complementing protein 1 (XRCC1) are recruited, to finish the repair process (Rouleau et al., 2010; Palazzo and Ahel, 2018). When PARP-1 is defective or inhibited, SSB cannot be repaired, which results in stalled replication forks and DSBs (O'Neil et al., 2017). In cells exhibiting normal HR, these DSBs can be repaired, to compensate for the loss of PARP1 function. However, in cells exhibiting defective HR, such as breast cancer cells with pathogenic BRCA1/2 mutations, defects cannot be repaired, leading to tumor-specific cell death (Lord and Ashworth, 2017). This is explained by synthetic lethality, originally referring to a lethal phenotype that results from the simultaneous disruption of two genes, while the disruption of either gene alone causes the cell to remain viable (Ashworth and Lord, 2018). Specifically, the simultaneous loss of PARP-1 induced SSBR and BRCA1/2 induced HR would result in cell death, while cells exhibiting a disruption in either of these could survive.

Besides inhibiting PARP catalytic activities, PARP trapping on DNA, a formation of non-covalent protein-DNA adducts was illustrated in the molecular mechanism of the cytotoxicity of PARP inhibitors, considering single-agent activities (Murai et al., 2012). During the repair process, PARP inhibitors effectively induce PARP1 and PARP2 trapping onto DNA and forbid the utilization of NAD + and auto-PARylation, associated with catalytic inhibition of PARylation (Murai et al., 2014). A novel implementation of the proximity ligation assay developed by Hopkins et al. (2019), showed high sensitivity and throughput at single-cell resolution to detect trapped PARP-DNA complexes. Importantly, the toxicity of trapped PARP complexes is not restricted to cancer cells with HR deficiency, but also drive single-agent cytotoxicity in healthy human bone marrow, suggesting the inverse relationship between trapping potency and tolerability (Hopkins et al., 2019). Based on CRISPR screening, a high-confidence set of 73 genes was confirmed to increase PARP inhibitor sensitivity when mutated (Zimmermann et al., 2018). Pommier et al. (2016) systematically reviewed the mechanism of PARP trapping and its relationship with chemoresistance in clinical, provided the implication of PARP trapping for chemotherapy combination. To better understand the two pathways in mediating the cytotoxicity of PARP inhibitors, Wang et al. (2019) designed and constructed a series of small molecule PARP degraders to mimic PARP1 genetic depletion and decouple PARP1

catalytic inhibition from PARP1 trapping, showing promising approaches to suppress PARP1 hyperactivation in various pathological conditions.

Based on the mechanisms for inhibition of PARP catalytic activities and PARP trapping, several PARP inhibitors (PARPi), such as Olaparib (KuDOS/AstraZeneca) (Mateo et al., 2015), Veliparib (Abbvie) (Kummar et al., 2012), Rucaparib (Pfizer/Clovis) (Swisher et al., 2017), and Niraparib (Merck/Tesaro) (Scott, 2017) have been developed and applied in clinical studies. PARPi were particularly effective in the treatment of patients with breast, ovarian, or other cancers, who were *BRCA1* and/or *BRCA2* deficient. For example, Olaparibis the first PARPi approved by the FDA for the treatment of breast cancer patients carrying *BRCA* germline mutations (Tutt et al., 2010). Compared with standard therapy, olaparib monotherapy provided a significant benefit for metastatic breast cancer patients with a germline *BRCA* mutation, with 2.8 months prolonged median progression-free survival (PFS) and 42% reduced risk of disease progression or death (Robson et al., 2017). Recently, a randomized, open-label, phase 3 trial was conducted in advanced breast cancer and a germline *BRCA* mutation to evaluate therapeutic effect of talazoparib, another PARPi, showing the significant benefit of single-agent talazoparib over standard chemotherapy, with respect to 3 months prolonged PFS and 35.4% increased objective response rate (Litton et al., 2018). Apart from *BRCA1/2* mutations, individuals with deficiencies in other FA genes and tumor suppressor genes involved in HR could benefit from the potential therapeutic capacities of PARPi; as the subsequent effects were unclear, they are being investigated (Lord and Ashworth, 2016).

Hypersensitivity to ICL Agents

Besides synthetic lethality, cells defective in several FA pathway genes, especially those involved in HR, were found to be hypersensitive to certain chemotherapeutic reagents, particularly ICL agents (Van Der Heijden et al., 2003; Chirnomas et al., 2006). Representatively, triple-negative breast cancer patients with germline or somatic pathogenic BRCA1/2 mutations are sensitive to cisplatin or carboplatin, which are recommended as the preferred regiments for HER2-negative breast cancer patients, as per the NCCN Guideline Version 1.2019. It provides an alternative to neoadjuvant chemotherapy or adjuvant chemotherapy treatment in patients with late advanced triple-negative breast cancer. Therefore, researchers have hypothesized that the inactivation of the FA pathway could act as a predictive biomarker of the chemotherapeutic response. Easy and reproducible methods that could be widely adopted for understanding the viability of the pathway need to be developed. Mukhopadhyay et al. (2010) successfully developed a method to determine the HR status by studying RAD51 focus formation in primary cell cultures. The identification of novel agents to which FA pathway-deficient cells are hypersensitive could provide additional therapeutic targets.

In terms of the above two aspects, the FA pathway shows promising clinical implications in cancer therapy. The biochemical mechanisms of the FA pathway need to be studied further, to identify novel biomarkers and develop effective therapeutic targets.

DISCUSSION

The identified breast cancer susceptibility genes in the FA pathway, including *BRCA1, BRCA2, BRIP1, PALB2,* and *RAD51C*, are essential genes involved in HR, the error-free pathway for DSB repair during physiological cell cycle progression, which repairs replication-associated DNA damage (Michl et al., 2016; Wright et al., 2018). HR is also involved in the final steps of ICL repair, primarily in the S and G2 phase, when a sister chromatid is available as the repair template and provides a high fidelity and error-free solution for repair. Additionally, it is illustrated that deficiencies in the common genes in the FA and HR pathway result in unrepaired DNA damage and sequential genomic instability, and eventually increase the risk of breast cancer and predisposition to certain kinds of cancer (**Box 2**).

In summary, the identified susceptibility gene *BRCA2* is required for the loading of *RAD51* onto ssDNA during the repair process (Davies et al., 2001). During HR, *PALB2* (*FANCN*) and *BRIP1* (*FANCJ/BACH1*) functions as the binding partner and regulator for *BRCA1* and *BRCA2*, respectively (Hiom, 2010; Park et al., 2014). On the other hand, the potential breast cancer susceptibility gene in the FA pathway, *FANCM*, is also needed for recruiting CtIP (C-terminal binding protein interacting protein) and MRN (MRE11-RAD50-NBS1) at the site of ICL, during the HR process (Daley et al., 2013). These findings have not only

BOX 2 | Facts.

- The germline mutations in the Fanconi anemia pathway partially elucidate the functional basis of genomic instability, predisposition to cancer, and tumorigenesis in diverse human cancers, especially breast cancer.
- The potential underlying mechanisms of the FA pathway involved in tumorigenesis included the impaired interphase DNA damage response, decreased replication fork protection and fidelity, supernumerary or over-replication of centrosomes, and abnormal spindle assembly checkpoints.
- Several FA genes, such as *BRCA1/FANCS*, *BRCA2/FANCD1*, *PALB2/FANCN,* and *RAD51C/FANCO* have been confirmed to be breast carcinoma susceptibility genes at present.

BOX 3 | Open questions.

- Why did heterozygous germline mutations in certain FA genes predisposed carriers to tissue-specific cancers, such as breast cancer?
- Besides the reported susceptibility and potential breast cancer susceptibility genes, are mutations in other FA genes associated with breast cancer, or other types of cancers?
- Besides DNA damage repair, were any other underlying mechanisms involved in the association between FA pathway and breast cancer?
- The search for potential cancer therapy targets and treatment strategies associated with the FA pathway are important research hotspots and have implications in clinical practice.

elucidated the crosstalk between the FA and HR pathways, but also provided an insight into the possible mechanism by which mutations in the FA pathway cause a predisposition to breast cancer.

Moreover, other known breast cancer susceptibility genes are either associated with the FA pathway or involved in DNA repair. For example, *ATM*, a rare moderate-penetrance breast cancer susceptibility gene, is responsible for phosphorylation and chromatin recruitment in *FANCM* (Sobeck et al., 2009). *CHEK2*, a serine/threonine kinase, is activated upon DNA damage and implicated in pathways governing DNA repair, cell cycle arrest or apoptosis in response to the initial damage (Apostolou and Papasotiriou, 2017). *TP53* is the most frequent mutational target in human cancers, and mutations in *TP53* are associated with different types of malignancies and adverse prognoses, including during breast cancer (Bellazzo et al., 2018). In conclusion, among all the DNA repair pathways, the FA pathway has the strongest association with increased risk of developing breast cancer. Hence, the FA pathway is also termed as the FA/BRCA pathway.

However, the underlying mechanism remained unclear (**Box 3**). Is it possible for other FA genes to predispose some specific ethnic group cancer? Why are the roles of *FANCD2* and *FANCI* in cancer predisposition not identified, though they are central participants in the FA pathway? Is it possible for the FA pathway and HR process to be the same, as more and more genes of each are identified to be identical? All these issues still need to be addressed by researchers.

During the past two decades, we have witnessed great advancements in the study of FA, with the identification of more and more FA genes and the biological mechanism of FA was elucidated. It was believed that more and more genes will be identified as FA genes, especially for those involved in HR. This would enable us to gain greater insight into breast cancer susceptibility and the FA pathway, which would provide clinical benefits to patients with FA and breast cancer.

AUTHOR CONTRIBUTIONS

JL and G-JZ contributed conception and design of the study. C-BF, H-TW, and JL organized the database, searched the literature, structured, and drafted the manuscript, figures, and table carefully. M-LZ organized the database and drafted the manuscript carefully. JL and G-JZ revised the original manuscript critically. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (Nos. 81501539 and 81320108015), the Natural Science Foundation of Guangdong Province (No. 2016A030312008), and Li Ka Shing Foundation Grant for Joint Research Program between Shantou University and Technion-Israel Institute of Technology (No. 43209501). G-JZ was a recipient of the Chang Jiang Scholar's award granted by the Ministry of Education of China.

ACKNOWLEDGMENTS

We are thankful to Luke Shen for his valuable advice and English editing.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 16 June 2020
doi: 10.3389/fcell.2020.00461

Expression of ncRNAs on the DLK1-DIO3 Locus Is Associated With Basal and Mesenchymal Phenotype in Breast Epithelial Progenitor Cells

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Stem Cell Research, a section of the journal Frontiers in Cell and Developmental Biology

Received: *12 December 2019*
Accepted: *18 May 2020*
Published: *16 June 2020*

Citation:
Budkova Z, Sigurdardottir AK, Briem E, Bergthorsson JT, Sigurdsson S, Magnusson MK, Traustadottir GA, Gudjonsson T and Hilmarsdottir B (2020) Expression of ncRNAs on the DLK1-DIO3 Locus Is Associated With Basal and Mesenchymal Phenotype in Breast Epithelial Progenitor Cells. Front. Cell Dev. Biol. 8:461. doi: 10.3389/fcell.2020.00461

Epithelial-to-mesenchymal transition (EMT) and its reversed process mesenchymal-to-epithelial transition (MET) play a critical role in epithelial plasticity during development and cancer progression. Among important regulators of these cellular processes are non-coding RNAs (ncRNAs). The imprinted DLK1-DIO3 locus, containing numerous maternally expressed ncRNAs including the lncRNA maternally expressed gene 3 (*MEG3*) and a cluster of over 50 miRNAs, has been shown to be a modulator of stemness in embryonic stem cells and in cancer progression, potentially through the tumor suppressor role of *MEG3*. In this study we analyzed the expression pattern and functional role of ncRNAs from the DLK1-DIO3 locus in epithelial plasticity of the breast. We studied their expression in various cell types of breast tissue and revisit the role of the locus in EMT/MET using a breast epithelial progenitor cell line (D492) and its isogenic mesenchymal derivative (D492M). Marked upregulation of ncRNAs from the DLK1-DIO3 locus was seen after EMT induction in two cell line models of EMT. In addition, the expression of *MEG3* and the maternally expressed ncRNAs was higher in stromal cells compared to epithelial cell types in primary breast tissue. We also show that expression of *MEG3* is concomitant with the expression of the ncRNAs from the DLK1-DIO3 locus and its expression is therefore likely indicative of activation of all ncRNAs at the locus. *MEG3* expression is correlated with stromal markers in normal tissue and breast cancer tissue and negatively correlated with the survival of breast cancer patients in two different cohorts. Overexpression of *MEG3* using CRISPR activation in a breast epithelial cell line induced partial EMT and enriched for a basal-like phenotype. Conversely, knock down of *MEG3* using CRISPR inhibition in a mesenchymal cell line reduced the mesenchymal and basal-like phenotype of the cell line. In summary our study shows that maternally expressed ncRNAs are markers of EMT and suggests that *MEG3* is a novel regulator of EMT/MET in breast tissue. Nevertheless, further studies are needed to fully dissect the molecular pathways influenced by non-coding RNAs at the DLK1-DIO3 locus in breast tissue.

Keywords: DLK1-DIO3 locus, *MEG3*, ncRNAs, epithelial plasticity, breast progenitor cells

INTRODUCTION

Breast cancer is the most common cancer in women and the second most common cancer overall (Ghoncheh et al., 2016). Despite major advances in diagnosis and treatment of cancer in recent years, metastasis and development of resistance to cancer therapies continues to be a challenge, causing over 90% of all cancer-related deaths (Ben-Jacob et al., 2012). A major contributing factor to metastasis and drug resistance is the heterogeneity and plasticity of the cells within tumors (Dagogo-Jack and Shaw, 2018). Epithelial-to-mesenchymal transition (EMT), is a developmental process that can be hijacked by cancer cells (Zeisberg and Kalluri, 2004; Moustakas and Heldin, 2007; Radisky et al., 2007). Generally, cells undergoing EMT, acquire increased migration and invasive properties and show increased resistance to apoptosis (Robson et al., 2006; Cao et al., 2016). Through these processes, EMT is considered a major mediator of phenotypic plasticity in cancer cells, metastatic formation and drug resistance (Mani et al., 2008; Scheel and Weinberg, 2012; Ansieau, 2013; Nieto et al., 2016; Lu and Kang, 2019). Recently, hybrid E/M (or partial EMT) cells have been shown to have even more metastatic and stem cell potential compared to the full epithelial or mesenchymal phenotype (Pastushenko et al., 2018). A reversed program, mesenchymal-to–epithelial transition (MET) is considered to facilitate colonization in secondary sites and reverse the plastic mesenchymal phenotype back to an epithelial state (Lu and Kang, 2019). This, however, is debated and further studies will increase our knowledge of the role of EMT/MET in cancer progression and metastasis.

EMT can be initiated through intrinsic factors such as expression of EMT related transcription factors (SNAI1, SNAI2, TWIST1, ZEB1, or ZEB2), cadherin switch from E-cadherin (CDH1) to N-cadherin (CDH2) or through epigenetic mechanisms. It can also be brought on by extrinsic factors derived from the microenvironment, such as secreted soluble factors: transforming growth factor-β (TGF-β), epidermal growth factor (EGF), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF) or Wnt signaling factors (Moustakas and Heldin, 2007; Peinado et al., 2007; De Craene and Berx, 2013; Wang and Zhou, 2013; Williams et al., 2019).

Non-coding RNAs (ncRNAs) are among intrinsic regulators of EMT (Zaravinos, 2015). It is increasingly apparent that the ncRNAs are crucial in normal development and disease, but its mechanistic mode of action is largely unknown (Liz and Esteller, 2016). The two major classes of non-coding RNAs are long non-coding RNA (lncRNAs) and microRNA (miRNAs). Accumulating evidence suggests that lncRNAs function in a broad range of cellular processes such as cell growth, survival, migration, invasion and differentiation (Mercer et al., 2009; Sun et al., 2013; Di Gesualdo et al., 2014; Fatica and Bozzoni, 2014). LncRNAs are defined by the size of their transcripts and are longer than 200 nucleotides (nt), with no protein-coding function (Eades et al., 2014). Unlike microRNAs, lncRNAs are poorly conserved, but function in a regulatory network at the transcriptional, post-transcriptional, and translational level. miRNAs are 22 nt long RNA molecules that regulate expression post-transcriptionally primarily by binding to three prime untranslated region (3′UTR) of target genes (Bartel, 2009).

The imprinted DLK1-DIO3 locus located on chromosome 14 contains three paternally expressed protein-coding genes (*DLK1*, *RTL1*, *DIO3*) and numerous maternally expressed non-coding genes, including the lncRNA maternally expressed gene 3 (*MEG3*), and a cluster of over 50 miRNAs (Zhang et al., 2010; Dill and Naya, 2018; Baulina et al., 2019; Li et al., 2019).

The DLK1-DIO3 locus has been described as an important contributor to pluripotency and stemness in embryonic stem cells (ESCs) (Kaneko et al., 2014). It discriminates between mouse induced pluripotent stem cells (iPCS) and mouse ESCs, where genes from the locus were strongly repressed in iPSC clones compared to ES clones (Liu et al., 2010; Stadtfeld et al., 2010a). Furthermore, activation of maternally expressed genes from the locus is a strong indicator of the developmental potential of iPSC (Kang et al., 2009). miRNAs from the DLK1-DIO3 locus have been shown to promote pluripotency by inhibition of differentiation and stimulation of self-renewal in mouse ES cells (Moradi et al., 2017) and were found to be increased in tumor-originating cancer cells from lung adenocarcinoma (Valdmanis et al., 2015).

MEG3 is a potential tumor suppressor gene in several cancer types, mainly through the observation that *MEG3* expression is lower in various tumor tissues compared with non-tumor tissues of the same origin (Sheng et al., 2014; Sun et al., 2014, 2016; Yin et al., 2015; Chak et al., 2017; Molina-Pinelo et al., 2018). The tumor suppressor role of *MEG3* is ascribed to stabilization of p53 with inhibition of proliferation and promotion of apoptosis (Zhang et al., 2003, 2010; Zhou et al., 2007; Wang et al., 2012; Sun et al., 2016).

MEG3 was reported to positively regulate EMT in lung (Terashima et al., 2017) and ovarian (Mitra et al., 2017) cancer. Furthermore, *MEG3* has been shown to contribute to the development of osteosarcoma through increased migration, invasion and decreased apoptosis (Wang and Kong, 2018). Higher levels of *MEG3* were detected in plasma from colorectal cancer patients compared with non-cancerous controls (Liu et al., 2019).

D492 is a primary breast epithelial cell line, immortalized with the E6 and E7 oncogenes from the human papilloma virus 16 (Gudjonsson et al., 2002). Therefore, the p53 protein, which mediates the previously described tumor suppressor role of *MEG3*, is repressed in this cell line. D492 can generate both luminal and basal/myoepithelial cells in monolayer and 3D culture, expressing luminal or myoepithelial keratins such as keratin 19 and keratin 14, respectively. Furthermore, when D492 cells are co-cultured with endothelial cells, they, can generate spindle-shaped colonies with EMT phenotype. D492M (mesenchymal) was established from one such spindle-shaped colony (Sigurdsson et al., 2011). D492M is a phenotypically stable EMT cell line. It has lost epithelial markers such as keratins, E-cadherin and TP63, and gained expression of mesenchymal markers such as N-cadherin (Sigurdsson et al., 2011; Hilmarsdottir et al., 2015). D492M has acquired classical properties of cancer stem cells, such as increased CD44/CD24 ratio, anchorage independent growth, resistance to apoptosis

and increased migration/invasion (Sigurdsson et al., 2011). D492 serves as a model for branching morphogenesis and together D492 and D492M represent a unique EMT model of isogenic cell lines with an epithelial and mesenchymal phenotype, respectively (Briem et al., 2019b). The ability of D492 to undergo mesenchymal transition upon endothelial stimulation makes it a valuable cell model to study EMT induced by extrinsic factors, although it is important to note that neither D492 nor D492M are tumorigenic in mice.

In this study, we describe a new role for the DLK1-DIO3 locus in EMT and phenotypic plasticity of breast cells. Following EMT in breast epithelial cell lines, expression of the ncRNAs at the DLK1-DIO3 locus was increased. In addition, *MEG3* was highly expressed in stromal cells in breast tissue and its expression correlated with decreased survival in breast cancer. Moreover, increased expression of the ncRNAs at the DLK1-DIO3 locus in a breast epithelial progenitor cell line promoted cellular plasticity and induced partial EMT. Collectively, our study provides a further understanding of the role of the DLK1-DIO3 locus in cellular phenotype of breast cells and might provide important insight into novel therapeutic targets aimed at overcoming heterogeneity and therapy resistance in breast cancer.

MATERIALS AND METHODS

Cell Lines

Both D492 and D492M were cultured in H14 medium, as described previously (Gudjonsson et al., 2002; Sigurdsson et al., 2011) in flasks coated with collagen I (Advanced BioMFatrix, 5005-B). HEK-293T cell were cultured in Dulbecco's Modified Eagle Medium (DMEM), high glucose, GlutaMAX (TM), pyruvate (Gibco, 31966), supplemented with 10% Fetal bovine serum (FBS), penicillin and streptomycin (Gibco, 15140-122). Primary Human umbilical vein endothelial cells (HUVECs) were obtained from Landspitali, University Hospital in Reykjavik, Iceland, (with informed consent, approved by Landspitali Ethical Committee No. 35/2013), cultured in Endothelial Growth Medium 2 (EGM2) media (Lonza, CC-3162) supplemented with growth factors and 5% FBS, further referred to as EGM5 medium as previously described (Sigurdsson et al., 2011). HMLE (Elenbaas et al., 2001) is epithelial progenitor cell line, from which was derived mesenchymal cell line HMLEmes after stable induction of EMT-TF (Mani et al., 2008). HMLE and HMLEmes were cultured in chemically defined HMLE media, containing DMEM/F12 with penicillin and streptomycin and growth factors Insulin (Sigma, I1882) 10 μg/ml, EGF (Peprotech, AF-100-15) 10 ng/ml, Hydrocortisone (Sigma, H0888) 500 ng/ml.

Primary human luminal-epithelial cells (LEP), myoepithelial cells (MEP), breast endothelial cells (BRENCs) and fibroblast were isolated from breast reduction mammoplasties (with informed consent, approved by the Icelandic National Bioethics Committee VSN-13-057) as previously described (Sigurdsson et al., 2011) and maintained in chemically defined medium 3 (CDM3) and chemically defined medium 4 (CDM4) as previously described (Pechoux et al., 1999; Ingthorsson et al., 2010). All cells were maintained in an incubator with 5% CO_2 at 37°C.

3D Cultures/Mammosphere Assays

3D cultures were carried out in a 48-well plate format (Corning, 353078) in growth factor reduced reconstituted basement membrane rBM (further referred to as Matrigel, Corning, 354230). 5–10 × 10^3 cells were seeded in 150 μl of Matrigel per well. Plate was incubated in 5% CO_2 at 37°C for 15 min to solidify the Matrigel and then 300 μl of H14 media was added on top. The cells were grown for 3 weeks and pictures were taken on day 1, 7, 14, and 21. Cell culture media was changed three times per week. The colonies were quantified at day 14. The total number of cells was converted into percentage.

For co-culture experiments, 0.5 × 10^3 of the epithelial cells were co-cultured with 1 × 10^5 of endothelial cells (HUVECs) and were resuspended in 150 μl of Matrigel. Plate was incubated in 5% CO_2 at 37°C for 15 min to solidify the Matrigel and then 300 μl EGM5 media was added on top. HUVECs cultured in Matrigel are viable, however, quiescent, having supporting role in the epithelial cells' proliferation. The effect of *MEG3* was quantified by counting all colonies bigger than 100 μm.

Total RNAseq and Analysis of the Data

The gene microarray expression analysis from D492 and D492M was published previously from our group by Sigurdsson and colleagues (Sigurdsson et al., 2011) and the total RNA-sequencing comparing D492 and D492M was published by Halldorsson and colleagues (Halldorsson et al., 2017).

The RNA was extracted using Tri-Reagent (Thermo Fisher Scientific, AM9738) from 5 replicates for each cell line. Whole Transcriptome Sequencing of $D492M^{KD-CTRL}$ and $D492M^{KD-MEG3}$ was performed in deCODE genetics (Reykjavik, Iceland). RNA sequencing reads were mapped to the reference genome (Ensembl primary assembly, version GRCh38) using STAR version 2.6.1 (Dobin et al., 2013). The program htseq-count (Anders et al., 2015) was used to quantify how many reads match each gene in an annotation file (Ensemble version GRCh38.96). The data from htseq-count was imported into R (R Development Core Team, 2015) and differential expression (DE) analysis on $D492M^{KD-CTRL}$ vs $D492^{KD-MEG3}$ was performed using DESeq2 (Love et al., 2014). Prior to DE analysis, genes with expression less than two reads were discarded. *P*-values were corrected for multiple testing using the false discovery rate (FDR) method. To compare gene expression from $D492M^{KD-CTRL}$ vs $D492M^{KD-MEG3}$ a volcano plot was generated. *P* value cut-off of 0.05 was applied. Volcano plot over all data ($p < 0.05$) was made in R using the EnhancedVolcano package from BioConductor. The top ten most upregulated and downregulated genes according log2 fold change were labeled. Gene Set Enrichment Analysis (GSEA) was applied to identify enrichment of gene signatures. Comparative analysis was investigated using the "Hallmark" database. The list of significantly expressed pathways is presented as a bar plot.

Quantitative RT-PCR Analysis

Total RNA was extracted with Tri-Reagent (Thermo Fisher Scientific, AM9738). 1 μg of RNA of each sample was reverse transcribed into complementary DNA (cDNA), using Random

Hexamers (Thermo Fisher Scientific, N8080127) and SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, 18090-200) kit and subjected to quantitative real time PCR (qRT-PCR) using Sybr Green dye Luna® Universal qPCR Master Mix (NEB, M3003L) or TaqMan probes Luna® Universal Probe qPCR Master Mix (NEB, M3004L) according to manufacturer's protocol. *GAPDH* was used as control for gene expression. For assaying the relative expression of each gene, the $2^{-\Delta\Delta Ct}$ was determined using an ABI 7500 instrument (Applied Biosystems).

List of Primers

TaqMAN: *ZEB1* (Thermo Fisher Scientific, Hs00232783_m1), *ZEB2* (Thermo Fisher Scientific, Hs00207691_m1), *SNAI1* (Thermo Fisher Scientific, Hs00195591_m1), *SNAI2* (Thermo Fisher Scientific, Hs00950344_m1), *TWIST1* (Thermo Fisher Scientific, Hs01675818_s1), *GAPDH* (Thermo Fisher Scientific, 4326317E).

SYBR Green: *KRT14* (IDT, Hs.PT.58.4592110), *KRT19* (IDT,Hs.PT.58.4188708), *MEG3* ex 10-11 (IDT, Hs.PT.58.25190740), *GAPDH* (IDT, Hs.PT.39a.22214836), *KRT5* (IDT, Hs.PT.58.14446018), *TP63* (IDT, Hs.PT.58.2966111), *CDH3* (IDT, Hs.PT.58.39234242).

Small RNAseq

The Microarray of small RNA data was published previously by our group by Hilmarsdottir and colleagues (Hilmarsdottir et al., 2015) and the small RNAseq data was published previously by Briem and colleagues (Briem et al., 2019a).

miRNA qRT PCR

Total RNA was extracted with Tri-Reagent (Thermo Fisher Scientific, AM9738). The RNA was reverse transcribed using miRCURY LNA RT Kit (Qiagen, 339340) for cDNA synthesis reactions, according to manufacturer's protocol. Quantitative RT-PCR analysis of miRNAs was performed using miRCURY LNA SYBR Green PCR Kit (Qiagen, 339346), according to manufacturer's protocol. Gene expression levels were quantified using primers for: hsa-miR-127-3p (Qiagen, YP00204048), hsa-miR-409-3p (Qiagen, YP00204358), hsa-miR-411-5p (Qiagen, YP00204531), hsa-miR-493-3p (Qiagen, YP00204557). Normalization was done with U6 snRNA (Qiagen, YP00203907). The $2^{-\Delta\Delta Ct}$ was used determined using ABI 7500 instrument (Applied Biosystems) to calculate the relative expression of each gene.

Allele Specific Expression Analysis

Total RNA was extracted with Tri-Reagent (Thermo Fisher Scientific, AM9738) and reverse transcription done using 1 μg of DNase I-treated total RNA using random hexamers (Thermo Fisher Scientific, N8080127) and SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, 18064022) according to the manufacturer's instructions. PCR primers were designed using Primer3 and Pyrosequencing primers were designed using PyroMark Assay Design 2.0 (Qiagen). The reverse PCR primer had a 5′-biotin modification and was HPLC-purified. Primers were synthesized by IDT 5′-TGGCCTTTTCTTCTCCTGAA, 5′-/5Biosg/TGACACATGGAAAGCACCAT and sequencing primer 5′-TCCGGGGTTACTGCCCT-3′. Polymerase chain reactions were performed in 50 μl using 10 ng of diluted cDNA or 10 ng of DNA, 1 U DreamTaq DNA polymerase (Fermentas, EP0701), 1X PCR buffer, 200 μM of dNTPs and 0,5 μM of each PCR primer. The following PCR protocol was used: 94°C for 2 min, followed by 50 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and 72°C for 9 min. To check the quality of the amplification, PCR products were analyzed by gel electrophoresis. Pyrosequencing were sequenced using the PyroMark Q24 system (Qiagen), following the manufacturer's instructions. For the ASE SNP, DNA and RNA (cDNA) were pyrosequenced simultaneously. The proportions of individual alleles for the SNP were obtained using the PyroMark Q24 software version 1.0.10 (Qiagen). Genomic DNA from D492M was examined to confirm the heterozygosity.

Clinical Cohort

RNA from breast cancer patients (diagnosed in the years 1987–2003) and relevant patient data was obtained from the Department of Pathology Landspitali – The National University Hospital of Iceland. Informed consent was obtained from patients involved in this study according to the national guidelines. The study was approved by The Icelandic Data Protection Commission (2001/523 and 2002/463) as well as the National Bioethics Committee of Iceland (VSN-11-105-V2). 119 samples were used in the study assigned to the following subgroup: 33 luminal A, 24 luminal B, 22 Basal, 12 ErbB2, 10 Normal and 18 not classified. cDNA was synthesized from 2 μg of total RNA using Random Hexamers primers (Thermo Fisher Scientific, N8080127) and RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). *MEG3* mRNA expression level was measured with the previously described qRT-PCR primers and TBP (Applied Biosystems, 4326322E) was used as a reference gene.

Western Blot Assay

Cells were washed with cold Phosphate Buffered Saline (PBS) and lysed in radio immunoprecipitation assay (RIPA) buffer with phosphatase and protease inhibitors (Halt Protease Inhibitor Cocktail, Thermo Fisher Scientific, 78430) for 10 min on ice and scraped with cell scraper. Protein concentration was measured using Bradford reagent (BioRad, 5000002). Equal amounts of protein (5–15 μg) were separated on 10% NuPage Bis-Tris gels (Invitrogen, NP0301PK2) with NuPage MES running buffer (Thermo Fisher Scientific, NP0002) and transferred with NuPage Transfer buffer (Thermo Fisher Scientific, NP0006-1) to polyvinylidene fluoride (PVDF) membranes Millipore Imobilion-FL transfer membrane, pore size 0,45 μM (Millipore, IPFL00010). The membranes were blocked with Odyssey Blocking buffer (TBS) (LiCor, 927-500) and incubated with primary antibodies overnight at 4°C. List of antibodies: keratin 14 (KRT14; Abcam, Ab15461), keratin 19 (KRT19; Abcam, Ab7754), P-cadherin (CDH3; Cell signaling, CS2130), tumor protein p63 (TP63, Abcam, Ab124762), keratin 5/6 (KRT5/6; Invitrogen, 180267), Actin (Licor, 926-42212). Actin was used as loading control. Secondary antibodies were mouse or rabbit IRDey (Li-Cor

926-32213, 926-32212, respectively) used at 1:10.000 for 1 h at room temperature (RT) and detected and quantified using the Odyssey Infrared Imaging System (Li-Cor Fluorescent signal was detected by Odyssey image system (Li-Cor) and converted to gray scale.

Cell Migration Assay

Cell migration was examined by using trans-well Boyden chambers with 8 μm pore size (Corning, 353097). Briefly, 3×10^3 cells were resuspended in 250 μl H14 medium and seeded on the trans-well inserts in 24-well plate (Corning, 353047). H14 media with 10% FBS was added to the lower chamber, below filter. Cells were incubated for 48 h in 5% CO_2 at 37°C. Non-migratory cells from the upper part of the filter were removed with cotton swab and washed 3 times with 1× PBS. The filters were then fixed with methanol and stained with DAPI (Sigma, D9542-1MG). Cells were photographed in three random fields EVOS FL Auto 2 imaging system (ThermoFisher). Pictures were analyzed with ImageJ Software.

Low Attachment Assay

Anchorage independent growth was examined using 24-well ultra-low attachment plates (Corning, 3473). Briefly, D492 and D492M cells were single cell filtered and 500 cells/well were seeded into EGM5 media and cultured for 9 days. The growth of colonies was quantified under the microscope, counting all the colonies bigger than 40 μm.

Apoptosis Assay

Resistance to chemically induced apoptosis was examined by inducing the cells with 10 μM camptothecin [CPT, Sigma-Aldrich, C9911)] in 96-well plate format (Corning, 353072). and quantified using IncuCyte Caspase-3/7 Reagents (Essen Bioscience, 4440) on IncuCyte Zoom (Essen Bioscience) according to the manufacturer's instructions.

Lentivirus Packaging and Transfection

The packaging of lentiviral expression constructs into pseudoviral particles, was performed with the psPAX2 (Addgene, 12260) and PMDG.2 (Addgene, 12259) plasmids using Turbofect (Thermo Fisher Scientific, R05319) in HEK-293T cells. The supernatant was harvested after 48 and 72 h and filtered through 0,45 μm pore filter. For infection, cells were plated on T25 flasks, so they were 70–80% confluent following day and were infected with 1 ml of viral particles and 1 ml of fresh media in the presence of 8 μg/ml polybrene. Lentivirus-transduced cells were selected with antibiotics or sorted by FACS (Sony SH800), based on fluorescent dye to obtain stable pool of clones. The altered expression of *MEG3* was determined by qRT-PCR.

The list of lentiviral expression constructs (plasmids) used in the study and their selection marker (with final concentration in case of antibiotics): pLenti_sgRNA(MS2)_zeo (Zeocin Invitrogen 4 μl/ml), pLenti_dCas9-VP64_Blast (Blasticidin, 2 μg/ml), pLenti_dCas9-KRAB_mCherry (mCherry fluorescence), SAM MS2-P65-HSF1 Plasmids (Hygromycin 1 μl/ml).

CRISPRi/CRISPRa

To perform CRISPRi and CRISPRa, two vectors were used. First, vector with dCAS9 with effector domain KRAB (pLenti_dCas9-KRAB_mCherry, Genscript) and VP64 (pLenti_dCas9-VP64_Blast, Genscript) effector domain for CRISPRi and CRISPRa, respectively, was incorporated, using lentiviral transfection. Subsequently, vector with designed gRNA targeting specific site of our gene of interest *MEG3* was incorporated, in second round of lentiviral transfection. In case of gain of function studies with CRISPRa, one additional helper plasmid SAM (SAM MS2-P65-HSF1 Plasmids, Genscript) was used to further increase activation.

The sequence of gRNA for overexpression of *MEG3*: Guide 1: GCTCTCCGCCGTCTGCGCTA, the sequence of gRNA for downregulation of *MEG3*: Guide 2: GCGGGTGAGGGATCCTCTCGT, the sequence of gRNA for negative control: GCTTAGTTACGCGTGGACGA were cloned into pLenti_sgRNA(MS2)_zeo (Genscript).

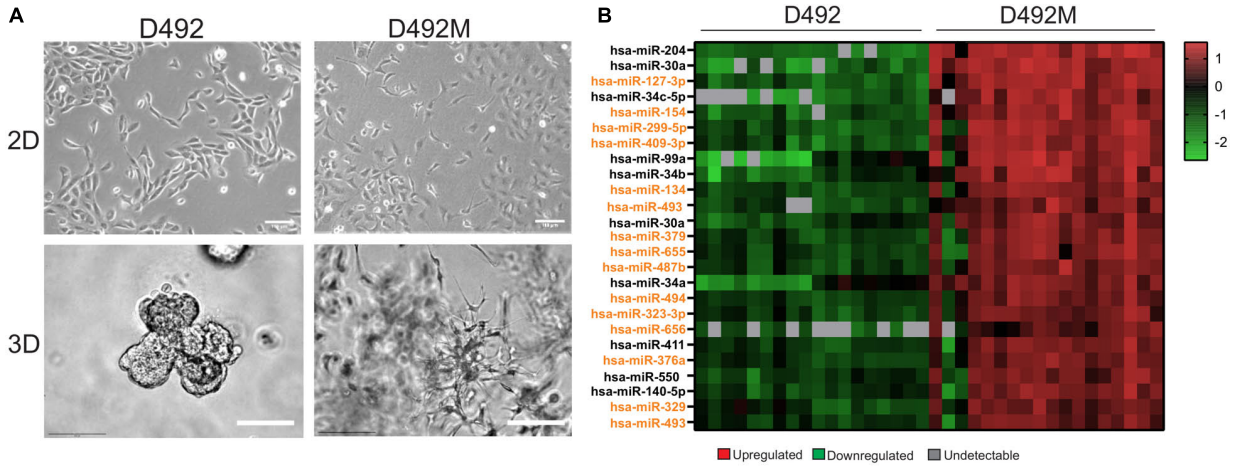
Statistical Analysis

Statistical differences of qRT-PCRs (**Figures 1E,F**, **Figures 5A,B**, and **Figures 7A–C**) and functional assay (**Figures 8B–E**) between samples were assessed with unpaired Student *t*-test. Statistical differences in **Figure 8A** was calculated using multiple unpaired Student *t*-test per row. Statistical differences of quantifications of western blots (**Figures 7B,C**) among samples were assessed using one-way ordinary ANOVA, followed by Tukey's multiple comparison test. Statistical differences in **Figure 4A** (left) was calculated using Kruskal Wallis Test (one-way ANOVA on ranks). Statistical analysis of qRT-PCRs in **Figure 2** were assessed with One-way ANOVA with Dunnett's multiple comparisons test. All statistical analyses were performed in GraphPad Prism. *P*-values below 0,05 were considered significant ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$).

RESULTS

MEG3 Is Highly Expressed in Cell Lines With a Mesenchymal Phenotype and in the Stromal Compartment of Breast Tissue

D492 and D492M are isogenic cell lines with stem cell and mesenchymal properties, respectively. D492 cells acquire cuboidal shape in 2D culture, and form branching structures in 3D culture, akin to terminal duct lobular units (TDLU) in the breast. In contrast, D492M is elongated and spindle-shaped in 2D culture and in 3D culture it forms irregular mesenchymal-like colonies (**Figure 1A**). We have previously shown that MIR203a and the MIR200 family are downregulated in D492M and their expression is essential for the epithelial phenotype (Hilmarsdottir et al., 2015; Briem et al., 2019a). Of miRNAs upregulated in D492M, the miRNAs at the DLK1-DIO3 locus are prominent. A microarray analysis of miRNA expression demonstrated that 15 of the 25 most highly expressed miRNAs in D492M compared to D492 belong to the DLK1-DIO3



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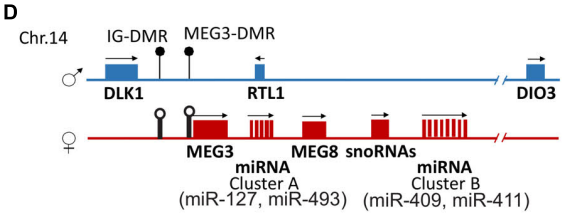
	lncRNA ID	D492M vs D492 - Fold change	D492M vs D492 FDR p-value correction	D492 - Normalized expression values	D492M - Normalized expression values
1	*HIF1A-AS2*	304.7	0.000	0.08	23.49
2	*MIR381HG*	217.3	0.077	0.04	8.38
3	*MIR155HG*	207.7	0.092	0.04	8.01
4	*RP11-175K6.1*	68.1	0.347	0.08	5.25
5	*LINC01551*	45.8	0.000	0.69	31.76
6	*RP11-102K13.5*	38.3	0.043	0.27	10.34
7	*MEG3*	17.9	0.000	10.64	190.67
8	*AC016831.7*	16.3	0.253	0.42	6.89
9	*MEG8*	12.0	0.000	5.05	60.60
10	*CASC19*	11.1	0.095	0.93	10.29



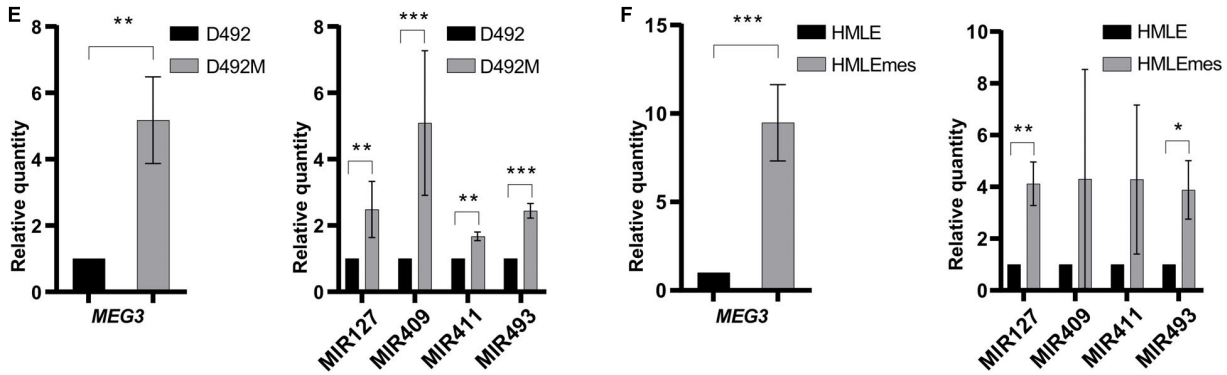


FIGURE 1 | The DLK1-DIO3 locus is upregulated in breast epithelial stem cells undergoing EMT. **(A)** D492 and D492M generate branching and mesenchymal structures in 3D culture, respectively. In 2D culture, D492 is cuboidal in shape and D492M is more spindle shaped. Scale bar = 100 μm. **(B)** Majority of the top upregulated miRNAs in D492M are from the DLK1-DIO3 locus. Microarray heat map showing top 25 upregulated miRNAs in D492M compared to D492. 15 of them are from the DLK1-DIO3 locus (highlighted in orange). **(C)** LncRNAs from the DLK1-DIO3 locus are among the most upregulated in D492M. RNAseq data showing top ten differentially expressed lncRNA, with lncRNAs from DLK1-DIO3 locus (*MEG3* and *MEG8*) highlighted in orange. **(D)** Schematic figure of the DLK1-DIO3 locus. The DLK1-DIO3 locus is located on chromosome 14 and is imprinted. It contains three paternally expressed protein coding genes (*DLK1*, *RTL1*, and *DIO3*) and many maternally expressed non-coding genes, among them lncRNAs (*MEG3* and *MEG8*) and over 50 miRNAs, among them MIR127 and MIR493 located in cluster A and MIR409 and MIR411 located in cluster B and numerous C/D-box-containing small nucleolar RNAs (snoRNAs). DMR – differentially methylated region, filled circles represent methylated DMRs, and unfilled represent unmethylated DMRs. **(E)** Upregulation of selected ncRNA from the DLK1-DIO3 locus verified with qRT-PCR. Graphs showing higher expression of *MEG3* in D492M compared to D492 (left) and higher expression of four representative miRNAs (MIR127, MIR493, MIR409, and MIR411) at the DLK1-DIO3 locus in D492M compared to D492 (right). Results shown as mean ± SD. Unpaired *t*-test was used to test significance: $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $n = 3$. **(F)** ncRNAs from the DLK1-DIO3 locus are upregulated in HMLEmes. qRT-PCR showing higher expression of *MEG3* and representative miRNAs (MIR127, MIR493, MIR409, and MIR411) from DLK1-DIO3 locus in HMLEmes compared to HMLE. Results shown as mean ± SD. Unpaired *t*-test was used to test significance: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $n = 3$.

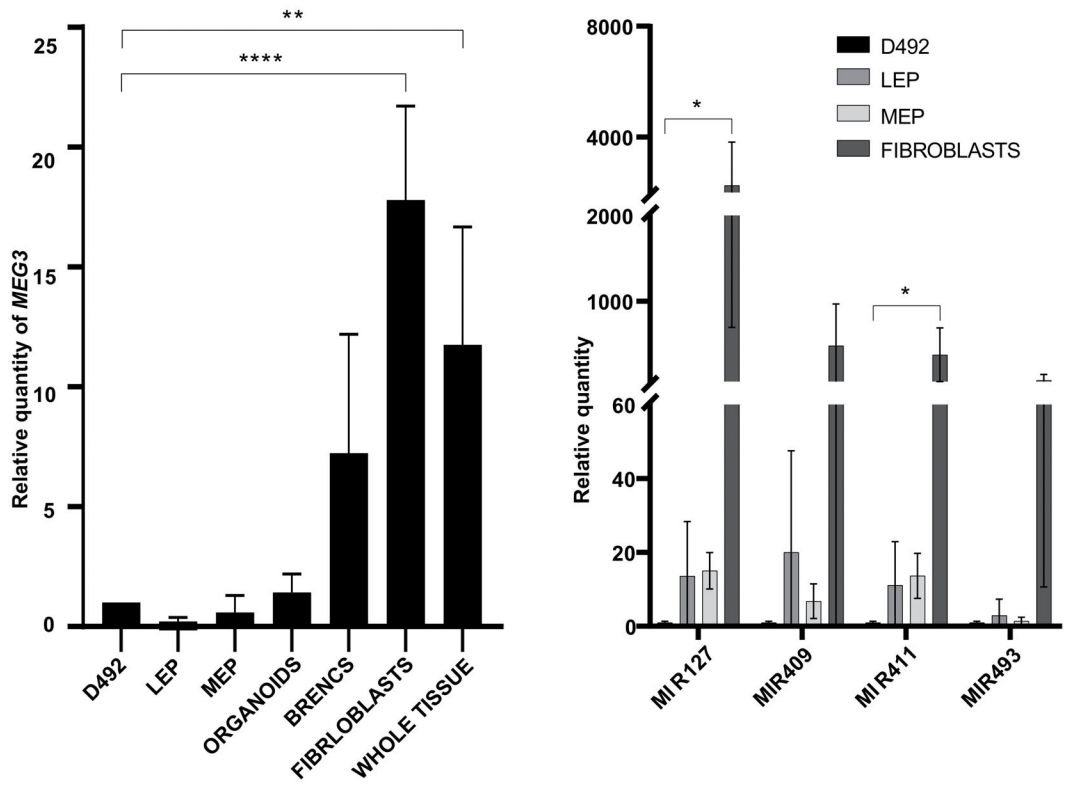


FIGURE 2 | The ncRNA from the DLK1-DIO3 locus are highly expressed in stromal cells and whole tissue compared to epithelial cells. qRT-PCR showing *MEG3* expression is higher in breast stromal cells (fibroblasts) and whole tissue than in D492 (left). (LEP – luminal epithelial cells, MEP – myoepithelial cells, BRENCs – breast endothelial cells). Results shown as mean ± SD. One-way ANOVA with Dunnett's multiple comparisons test was used to test significance: $^{**}p \leq 0.01$; $^{****}p \leq 0.0001$; $n = 3$. Expression of representative miRNAs MIR127 and MIR411 at the DLK1-DIO3 locus is higher in breast fibroblast than in D492 (right). LEP – luminal epithelial cells, MEP - myoepithelial cells. Results shown as mean ± SD. One-way ANOVA with Dunnett's multiple comparisons test was used to test significance: $^{*}p \leq 0.5$; $n = 3$.

locus (**Figure 1B**). Furthermore, small RNA sequencing revealed that 33 of the miRNAs belonging to the DLK1-DIO3 miRNA cluster have more than 1,5-fold increased expression in D492M compared to D492 (**Supplementary Figure 1**). Moreover, total RNA sequencing of D492 and D492M, revealed that *MEG3* and *MEG8* are amongst the most upregulated lncRNAs in D492M (**Figure 1C**). The non-coding part of the DLK1-DIO3 locus consists of maternally expressed lncRNAs *MEG3* and *MEG8* and miRNAs grouped into two clusters (**Figure 1D**). To confirm the sequencing results, we selected four representative miRNAs from the DLK1-DIO3 locus, two from each cluster (MIR127 and MIR493 from cluster A, MIR409 and MIR411 from cluster B). These miRNAs as well as the lncRNA *MEG3* had higher expression, as revealed by qRT-PCR, in D492M compared to D492 (**Figure 1E**). In another isogenic EMT cell model, HMLE (epithelial) and HMLEmes (mesenchymal variant) both *MEG3* and the representative miRNAs were more highly expressed in HMLEmes compared to HMLE (**Figure 1F**). Thus, our data suggests that increased *MEG3* expression is not a stochastic event but consistently associates with EMT induction in breast epithelial cell lines.

Next, we analyzed the expression of *MEG3* and miRNAs from the DLK1-DIO3 locus in primary cells from three healthy donors. We found that the expression of *MEG3* is higher in purified stromal cells (fibroblasts) than in epithelial cells (D492, luminal epithelial cells, myoepithelial cells and organoids; **Figure 2**, left). Interestingly, expression of *MEG3* in whole breast tissue lysates is closer to fibroblast expression levels than epithelial cells (**Figure 2**, left). This finding is most likely explained by the richness of stroma in normal breast tissue, whereas organoids contain only the epithelial cells. A similar pattern is seen with the four representative miRNAs, where MIR127 and MIR411 have higher expression in fibroblasts compared to their expression in D492 (**Figure 2**, right).

We next acquired a list of genes correlated the expression of MEG3 using the GOBO (Gene expression-based Outcome for Breast Cancer Online) dataset and submitted the list to DAVID (the database for annotation, visualization and integrated discovery, version 6.7) (Huang et al., 2009a,b) to identify pathways associated with *MEG3*. Herein, the expression of *MEG3* correlates with expression of extracellular matrix genes, which are in line with the observations of a high expression of *MEG3* in cells found in the stromal compartment (**Supplementary Figure 2A**). Using analysis of publicly available NGS data using MiPanda (Niknafs et al., 2018) we found positive correlation of *MEG3* with

common EMT markers in normal breast and breast cancer (**Supplementary Figure 2B**). Many of these have a correlation coefficient > 0.3 (Spearman correlation) which is considered a fair positive correlation (Chan, 2003). Interestingly, even more genes are positively correlated to *MEG3* expression in breast cancer as compared to normal breast tissue (**Supplementary Figure 2B**).

Collectively, the lncRNA *MEG3* and miRNAs from DLK1-DIO3 locus are highly expressed in the mesenchymal compartment compared to epithelial breast tissue and their expression positively correlate with numerous mesenchymal genes and EMT markers.

MEG3 Is Imprinted in Both D492 and D492M

The DLK1-DIO3 locus is imprinted and regulated by DNA methylation (Cui et al., 2018). Using pyrosequencing (Harrington et al., 2013) covering a heterozygous SNP (C/T) in *MEG3* (rs4906024) we confirmed monoallelic expression of *MEG3* in both D492 and D492M, with expression in both cell lines being from the T allele (**Figure 3**). As both cell lines are diploid at the *MEG3* locus on a DNA level a C/T ratio of 50% is expected which is consistent with the 48% C-allele prominence observed. On the mRNA deviation from expected monoallelic expression was not detected as results showed zero C allele expression in D492 and 2% in D492M. Hence, increased expression of *MEG3* in D492M is not caused by loss of imprinting. The expression remains monoallelic confirming that the increased expression originates from the non-imprinted allele.

Increased Expression of *MEG3* Is Negatively Correlated With Survival of Breast Cancer Patients

EMT has been suggested to promote metastatic behavior of epithelia-originating cancer (Felipe Lima et al., 2016) and, in addition, our data shows association of *MEG3* expression with the mesenchymal phenotype. We therefore investigated *MEG3* expression levels in different subtypes of breast cancer. We have evaluated the expression of *MEG3* in clinically well-defined breast tumors. Herein, normal like (NL) breast tumors had significantly higher expression of *MEG3* with a *p*-value of 0.0003 (**Figure 4A**, left). Survival analysis of all tumor samples showed reduced, but not significant overall survival in patients with high *MEG3* expression. However, as the normal-like tumors have in recent years been subjected to scrutiny as a possible misclassification due to low tumor cellularity and thus, high proportion of normal tissue. In light of our results showing high expression of *MEG3* in breast stromal tissue, and uncertainty that measured *MEG3* expression in the normal-like subgroup is representative of the primary tumor, we omitted NL breast tumors from the survival analysis (Elloumi et al., 2011; Prat and Perou, 2011; Yersal and Barutca, 2014). The results show significant worse overall survival of patients with high *MEG3* expression (**Figure 4A**, right). Corroborating our findings, using the GOBO database (Ringnér et al., 2011)[1], we found that high *MEG3* expression reduces distant metastasis free

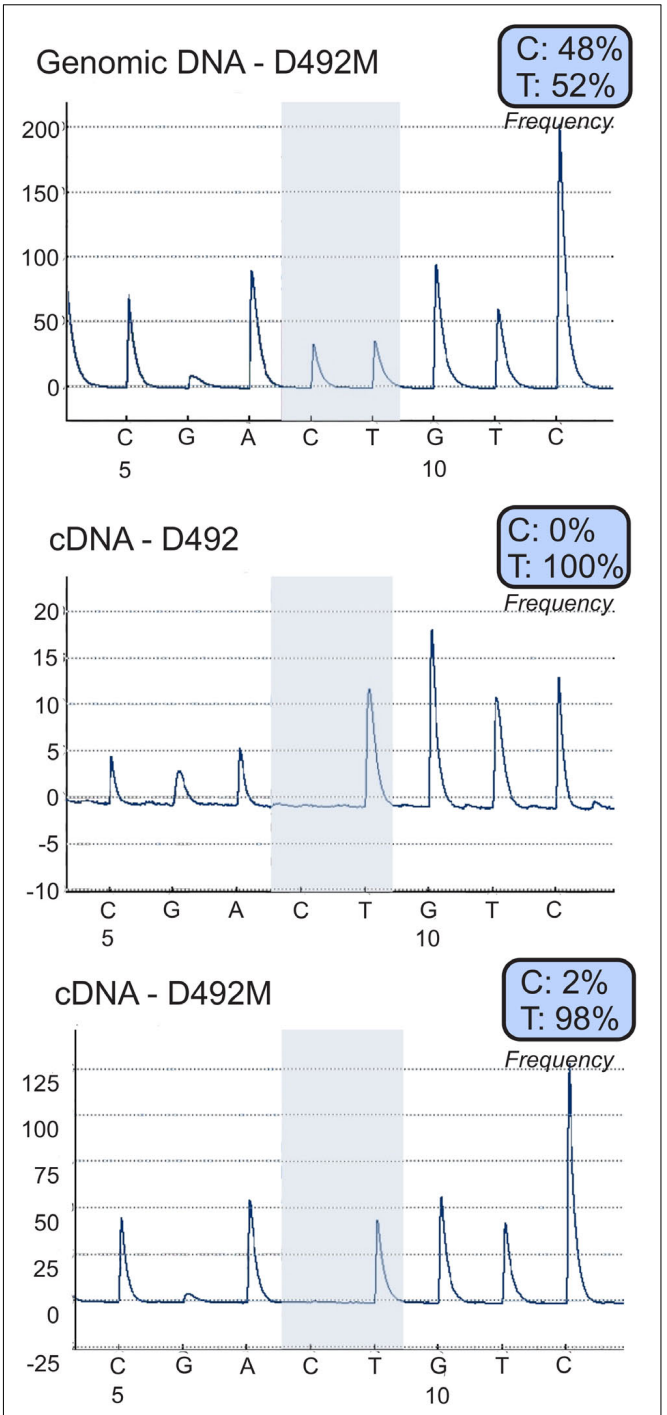


FIGURE 3 | Increased expression of *MEG3* in D492M is not caused by loss of imprinting. Allele specific expression analysis using pyro-sequencing shows that the increased expression of *MEG3* in D492M is not caused by loss of imprinting, but increased expression of the already expressed allele, the expression remains mono-allelic. Analyzed sequence: GAGCAC/TGTCCCA.

[1]http://co.bmc.lu.se/gobo/

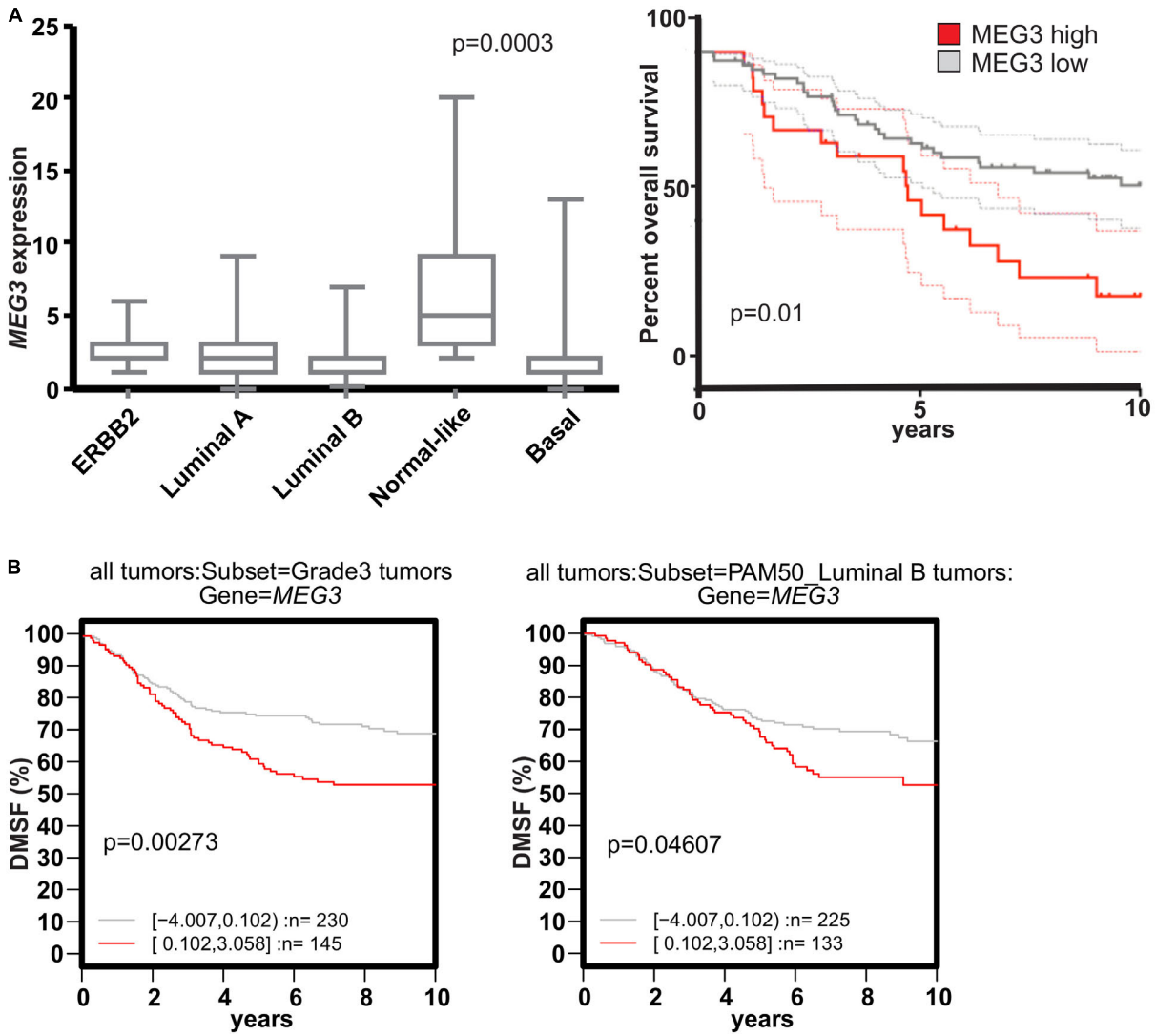


FIGURE 4 | *MEG3* expression negatively correlates with breast cancer prognosis. **(A)** High *MEG3* expression decreases overall survival in breast tumors. qRT-PCR shows that *MEG3* expression (Numbers of tumors per group: ErBB2 = 12, Luminal A = 33, Luminal B = 24, Normal = 10, Basal = 22) is significantly higher in normal-like (NL) breast cancer (left; $p = 0.0003$). High MEG3 expression is correlated with low overall patient survival (NL tumors omitted). Kruskal Wallis Test (or one-way ANOVA on ranks) was used to test the significance (right; $p = 0.01$). **(B)** High *MEG3* expression decrease distant metastasis free survival in grade three and Luminal B type tumors. Kaplan-Meier plot showing data from the online GOBO database: high expression of *MEG3* decrease DMSF (distant metastasis free survival), of poorly differentiated (grade 3) tumors (left; $p = 0.00373$) and luminal B type tumor (right; $p = 0.04607$).

survival (DMSF) of patients with poorly differentiated (grade 3) tumors (**Figure 4A**, left) and patients with luminal B tumors (**Figure 4B**, right).

Increased Expression at the DLK1-DIO3 Locus Contributes to the Basal and Mesenchymal Phenotype

To explore the functional role of *MEG3* in D492 and D492M, we established sublines with altered expression of *MEG3*. Using the CRISPRa approach (Cheng et al., 2013), we generated a D492 cell line with stable overexpression of *MEG3* ($D492^{MEG3}$). A control cell line was generated using a scrambled sgRNA ($D492^{CTRL}$). Furthermore, we used the CRISPRi approach (Gilbert et al., 2013; Qi et al., 2013), to generate knockdown of *MEG3* in D492M ($D492M^{KD-MEG3}$) and a control cell line was generated using scrambled sgRNA ($D492M^{KD-CTRL}$). The increase of *MEG3* expression was about seven-fold in $D492^{MEG3}$ compared to $D492^{CTRL}$ as determined by qRT-PCR (**Figure 5A**, left). Downregulation of *MEG3* in $D492M^{KD-MEG3}$ was more prominent, with about 20-fold reduced expression compared to $D492M^{KD-CTRL}$ (**Figure 5A**, right). Having established stable overexpression and downregulation of *MEG3* in D492 and D492M, we re-evaluated the epithelial/mesenchymal phenotypes of D492 and D492M, respectively. Based on phase contrast images, no obvious

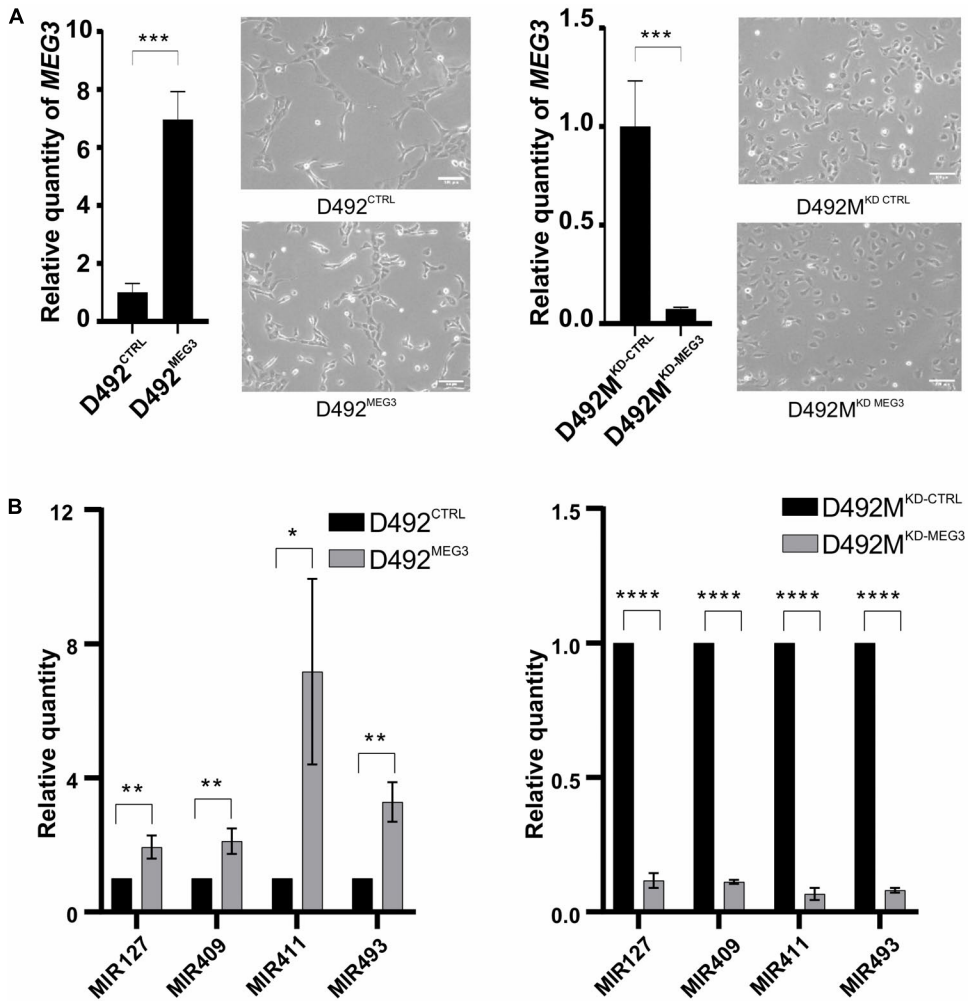


FIGURE 5 | Concomitant expression of non-coding RNAs from DLK-DIO3 locus with *MEG3*. **(A)** Overexpression and knock-down of *MEG3* in D492 and D492M, respectively. qRT-PCR confirming upregulation of *MEG3* in D492 ($D492^{MEG3}$) compared to D492 with scrambled control ($D492^{CTRL}$; left). Phase contrast pictures of $D492^{CTRL}$ and $D492^{MEG3}$ (below). qRT-PCR confirming knock-down of *MEG3* in D492M ($D492M^{KD-MEG3}$) compared to D492M with scrambled control ($D492M^{KD-CTRL}$; right). Results shown as mean ± SD. Unpaired *t*-test was used to test significance: $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$; $n = 3$. Phase contrast pictures of $D492M^{KD-CTRL}$ and $D492M^{KD-MEG3}$ (below). Scale bar = 100 μm. **(B)** miRNAs form the DLK1-DIO3 locus are upregulated with overexpression of *MEG3* and downregulated with knock-down of *MEG3*. qRT-PCR shows increased expression of four representative miRNAs from the DLK1-DIO3 locus in $D492^{MEG3}$ compared to $D492^{CTRL}$ (left) and their decreased expression in $D492M^{KD-MEG3}$ compared to $D492M^{KD-CTRL}$ (right). Results shown as mean ± SD. Unpaired *t*-test was used to test significance: $^{*}p \leq 0.05$ $^{**}p \leq 0.01$; $^{****}p \leq 0.0001$; $n = 3$.

difference in phenotype could be seen between $D492^{MEG3}$ and $D492^{CTRL}$ or $D492M^{KD-MEG3}$ and $D492M^{KD-CTRL}$ (**Figure 5A**, below). Interestingly, expression of the representative miRNAs located on the DLK1-DIO3 locus is increased in $D492^{MEG3}$ compared to $D492^{CTRL}$, to similar levels as seen in D492M (**Figure 5B**, left). Conversely, the expression of representative miRNAs is downregulated in $D492M^{KD-MEG3}$ compared to $D492M^{KD-CTRL}$ (**Figure 5B**, right). Thus, it appears, that the expression of miRNAs from the DLK1-DIO3 locus is concomitant with *MEG3* expression. To test, if that holds true, we used the cBioPortal and explored correlation of *MEG3* with miRNAs using data on invasive breast cancer from the Cancer Genome Atlas (TCGA) (Cancer Genome Atlas Network, 2012) we found that of 40 miRNAs that had positive correlation over 0,3 (Person score) with *MEG3,* 30 were located at the DLK1-DIO3 locus (with other miRNAs from the locus not being in the dataset; **Table 1**). This suggests that *MEG3* may be used as a marker for the expression of ncRNAs from the DLK1-DIO3 locus.

Next, we conducted RNA sequencing of our cell lines with stably altered expression levels of *MEG3* focusing on the analysis of $D492M^{KD-CTRL}$ vs $D492M^{KD-MEG3}$. There were 1235 significantly differentially expressed genes, with symmetric distribution over genes downregulated and upregulated in $D492M^{KD-MEG3}$, shown in the volcano plot ($p < 0.05$; **Supplementary Figure 3A**), with the list of top 30 up and down-regulated genes in $D492M^{KD-MEG3}$ (**Supplementary Figure 3B**). To identify unifying biological them from RNA-sequencing data, we performed Gene Set enrichment analysis (GSEA).

TABLE 1 | MiRNAs from the DLK1-DIO3 locus positively correlate with *MEG3* expression.

Correlated gene	Location	Pearson score	*P*-value	Spearman score	*P*-value
MIR-154/154*	Chr14	0.49	7.46E−20	0.44	1.27E−15
MIR-134/134	Chr14	0.48	2.09E−18	0.44	1.22E−15
MIR-199B/3P	Chr9	0.47	4.66E−18	0.47	8.03E−18
MIR-199A-1/3P; MIR-199A-2/3P#	Chr19; Chr1	0.47	4.66E−18	0.47	8.15E−18
MIR-127/3P	Chr14	0.46	4.18E−18	0.45	3.89E−16
MIR-136/136	Chr14	0.46	1.08E−16	0.41	2.15E−13
MIR-431/431*	Chr14	0.46	7.09E−17	0.39	5.23E−12
MIR-539/539	Chr14	0.44	1.66E−15	0.43	1.21E−14
MIR-199A-1/5P; MIR-199A-2/5P#	Chr19; Chr1	0.43	1.14E−14	0.39	2.18E−12
MIR-382/382	Chr14	0.42	1.53E−14	0.41	2.71E−13
MIR-199B/5P	Chr9	0.42	2.47E−14	0.40	1.06E−12
MIR-214/214*	Chr1	0.42	6.45E−14	0.39	4.35E−12
MIR-409/3P	Chr14	0.42	3.41E−14	0.37	3.34E−11
MIR-369/3P	Chr14	0.41	1.06E−13	0.37	5.93E−11
MIR-127/5P	Chr14	0.41	1.46E−13	0.36	9.70E−16
MIR-495/495	Chr14	0.4	1.07E−12	0.39	3.84E−12
MIR-758/758	Chr14	0.4	3.97E−13	0.36	1.09E−10
MIR-381/381	Chr14	0.39	3.10E−12	0.39	2.80E−12
MIR-485/3P	Chr14	0.39	2.12E−12	0.39	5.07E−12
MIR-125B-1/125B; MIR-125B-2/125B#	Chr11; Chr21	0.39	3.92E−12	0.37	6.52E−11
MIR-337/3P	Chr14	0.39	1.80E−12	0.37	7.17E−11
MIR-493/493*	Chr14	0.38	1.15E−11	0.37	2.83E−11
MIR-369/5P	Chr14	0.38	1.68E−11	0.33	2.98E−11
MIR-379/379	Chr14	0.37	5.82E−11	0.33	4.49E−09
MIR-370/370	Chr14	0.37	2.51E−11	0.32	1.29E−08
MIR-214/214	Chr1	0.36	1.88E−10	0.35	5.19E−10
MIR-708/708	Chr11	0.35	4.91E−10	0.35	3.60E−10
MIR-432/432	Chr14	0.35	5.39E−10	0.32	1.80E−08
MIR-409/5P	Chr14	0.35	3.48E−10	0.31	6.03E−08
MIR-323/3P	Chr14	0.35	6.01E−10	0.30	1.65E−07
MIR-376C/376C	Chr14	0.34	1.40E−09	0.35	7.42E−10
MIR-889/889	Chr14	0.34	1.57E−09	0.30	1.50E−07
MIR-493/493	Chr14	0.34	1.07E−09	0.29	5.38E−07
MIR-487B/487B	Chr14	0.33	7.98E−09	0.30	1.50E−09
MIR-655/655	Chr14	0.33	6.70E−09	0.30	1.90E−07
MIR-410/410	Chr14	0.33	4.36E−09	0.29	5.07E−07
MIR-184/184	Chr15	0.31	3.50E−08	0.34	2.68E−09
MIR-411/411	Chr14	0.31	2.83E−08	0.29	3.79E−07
MIR-654/3P	Chr14	0.31	3.64E−08	0.26	4.37E−06
MIR-22/22	Chr17	0.3	1.66E−07	0.29	2.72E−07

Out of 40 miRNAs that positively correlate with MEG3 in breast cancer (with correlation over 0.3), 30 are from the DLK1-DIO3 locus, highlighted in orange (TCGA, Nature 2012 data set). #Due to sequence similarities, these two miRNAs are indistinguishable in the sequencing data used.

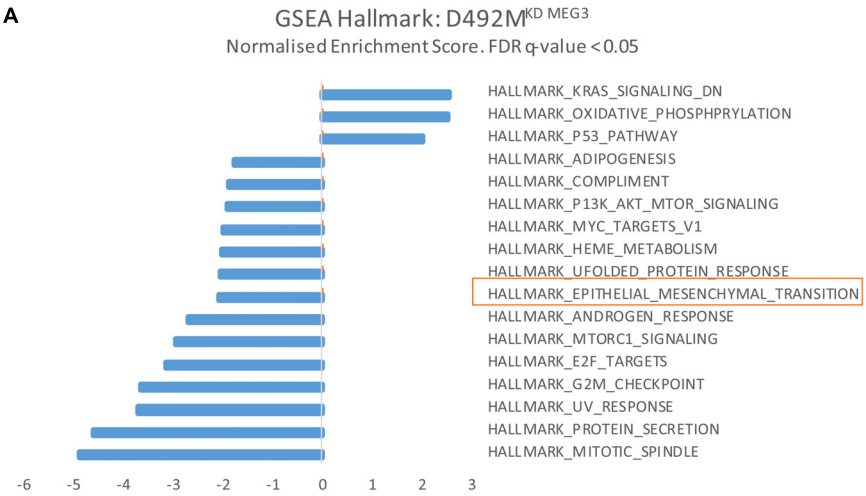
These gene sets consist of the defined gene lists, based on biological knowledge about biochemical pathways and co-expression data. Using the Hallmark dataset, one of the significantly, downregulated set of genes in $D492M^{KD-MEG3}$ was the epithelial-mesenchymal transition gene set, with normalized enrichment score (NES) of −2.03 and False discovery rate (FDR) q = 0.023 (**Figure 6A**). These genes define epithelial-mesenchymal transition, as in wound healing, fibrosis and metastasis. The genes belonging to this gene set are overrepresented toward the top of the ranked list, based on fold change of $D492M^{KD-CTRL}$ vs $D492M^{KD-MEG3}$ (**Figure 6B**, right). A manually curated list of mesenchymal genes from the Hallmark EMT dataset that are downregulated in $D492M^{KD-MEG3}$ is shown in **Figure 6B**, left. Further analysis of the RNA sequencing data of $D492M^{KD-MEG3}$ vs $D492M^{KD-CTRL}$, using common literature-based markers of breast tissue has showed that luminal epithelial markers *GATA3* and *MUC1* are upregulated, while myoepithelial *KRT14*, mesenchymal *VIM*, *ZEB2*, *SNAI2*, *LAMA1*, *CDH2*, and stem cell *MME*, *CTNNB1* are downregulated with knock down of *MEG3* (**Figure 6C**).

Expression of mesenchymal and basal markers was additionally confirmed on RNA level by qRT-PCR and on protein level western blot. Most of the core EMT-related transcription factors (EMT-TF) were affected by *MEG3*. $D492^{MEG3}$ has increased expression of *SNAI2* compared to $D492^{CTRL}$ (**Figure 7A**, left). On the other hand, $D492M^{KD-MEG3}$ has decreased expression of *SNAI2*, *ZEB1*, *ZEB2* and *TWIST1* compared to $D492M^{KD-CTRL}$ (**Figure 7A**, right). Luminal cytokeratin 19 (*KRT19*) and basal/myoepithelial cytokeratin 14 (*KRT14*) are also affected by manipulation of *MEG3* expression levels. Thus, $D492^{MEG3}$ shows increased *KRT14* and decreased *KRT19* expression compared to $D492^{CTRL}$ on both mRNA (**Figure 7B**, left) and protein level (**Figure 7C**, left). $D492M^{KD-MEG3}$ shows decreased *KRT14* expression compared to $D492M^{KD-CTRL}$ (**Figure 7B**, right). Furthermore, $D492^{MEG3}$ shows increased expression of other myoepithelial markers such as *CDH3* (P-cad), *TP63* or *KRT5* compared to $D492^{CTRL}$ as determined both at mRNA (**Figure 7D**, left) and protein level (**Figure 7E**). Also, $D492M^{KD-MEG3}$ shows decreased expression of myoepithelial markers *KRT5* on mRNA level (**Figure 7D**, right) and of TP63 on protein level (**Figure 7E**, middle) compared to $D492M^{KD-CTRL}$. This suggests that *MEG3* expression induces a shift toward a basal/myoepithelial phenotype. However, our cell lines with stably altered expression of *MEG3* do not show a significant switch in E-cadherin (CDH1) to N-cadherin (CDH2) expression (**Supplementary Figure 4**), which may explain why there are no clear changes in morphology.

MEG3 Induces Mesenchymal Properties and Stemness

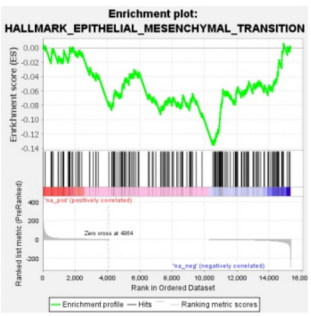
As *MEG3* has previously been ascribed to have a role in pluripotency and stemness (Stadtfeld et al., 2010b; Kaneko et al., 2014), we asked how *MEG3* manipulation affects mesenchymal and stem cell properties of D492 and D492M. The expression of both aldehyde dehydrogenase (*ALDH1A3*) and integrin alpha 6 (*ITGA6*; **Supplementary Figure 6**), markers of stemness, is increased in $D492^{MEG3}$ compared to $D492^{CTRL}$. Next, we employed several functional assays to assess the effect of *MEG3* levels in D492 and D492M on mesenchymal and stem cell properties. $D492^{MEG3}$ is more resistant to chemically induced apoptosis than $D492^{CTRL}$ (**Figure 8A**). Migration can be assessed *in vitro* using the wound healing assay or by trans-well migration

A



B

Gene	Name	Log2 FC	p-value
POSTN	Periostin	-2.45	$3.69x10^{-10}$
DST	Dystonin	-1.57	$2.21x10^{-9}$
IGFBP3	Insulin like grow th factor binding protein 3	-1.28	$4.99x10^{-19}$
ITGAV	Integrin alpha-V	-1.25	$1.62x10^{-42}$
ITGB1	Integrin subunit beta 1	-1.23	$1.48x10^{-56}$
VCAN	Versican	-1.19	$1.44x10^{-5}$
FERMT2	Fermitin family homolog 2	-1.07	$1.16x10^{-5}$
LAMA1	Laminin subunit alpha 1	-1.01	$2.82x10^{-26}$
COL12A1	Collagen type XII alpha 1 chain	-0.98	$9.17x10^{-19}$
CDH2	N-cadherin	-0.97	$1.21x10^{-24}$
SERPINE2	Serpine 2	-0.97	$2.42x10^{-18}$
FN1	Fibronectin 1	-0.95	$3.13x10^{-19}$
LAMC1	Laminin subunit gamma 1	-0.92	$2.61x10^{-25}$
LAMA3	Laminin subunit alpha 3	-0.92	$6.25x10^{-28}$
SERPINE1	Serpine 1	-0.9	$3.10x10^{-15}$
FBN2	Fibronectin 2	-0.89	$6.38x10^{-18}$
LAMC2	Laminin subunit gamma 2	-0.88	$1.66x10^{-22}$
VEGFC	Vascular endothelial grow th factor C	-0.74	$1.86x10^{-17}$
FGF2	Fibroblast grow th factor 2	-0.65	$3.47x10^{-11}$
SNAI2	Snail family transcriptional repressor 2	-0.63	$3.32x10^{-8}$
VIM	Vimentin	-0.49	$3.04x10^{-7}$
COL4A1	Collagen type IV alpha 1 chain	-0.48	$1.19x10^{-4}$



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Gene	Name	Log2 FC	Marker	p-value
GATA3	GATA3	0.42	Luminal epithelial	$8.19x10^{-4}$
MUC1	Mucin 1	0.83	Luminal epithelial	$1.97x10^{-6}$
CDH2	N-cadherin	-0.97	Mesenchymal	$1.21x10^{-24}$
LAMA1	Laminin subunit alpha 1	-1.01	Mesenchymal	$2.82x10^{-26}$
SNAI2	Snail family transcriptional repressor 2	-0.63	Mesenchymal	$3.32x10^{-8}$
ZEB2	Zinc finger E-box binding homeobox 2	-0.43	Mesenchymal	$3.76x10^{-6}$
VIM	Vimentin	-0.49	Mesenchymal	$3.04x10^{-7}$
CTNNB1	Catenin beta-1	-0.3	Stem cell	$4.1x10^{-4}$
MME	Membrane metallo-endopeptidase	-2.97	Stem cell	$1.19x10^{-72}$

FIGURE 6 | Knock down of *MEG3* in D492M decrease mesenchymal markers. **(A)** Epithelial-mesenchymal transition gene set is enriched pathway in $D492M^{KD-MEG3}$ (highlighted in orange). Bar plot of Gene Set Enrichment Analysis (GSEA) with Hallmark dataset showing all significantly (False discovery rate-FDR $q \leq 0.05$) enriched pathways in $D492M^{KD-MEG3}$. Gene set Epithelial-mesenchymal transition has normalized enrichment score (NES) of –2.06 and FDR $q = 0.014$. **(B)** Knock-down of *MEG3* correlates with downregulation of mesenchymal genes relevant for breast cells. Enrichment plot showing the Enrichment Score (ES) of the genes in the Hallmark gene set Epithelial-mesenchymal transition in $D492M^{KD-MEG3}$. The genes are overrepresented toward the top the ranked list of $D492M^{KD-MEG3}$ (right). Table with relevant genes for breast cells, significantly ($p \leq 0.05$) deregulated, from the Hallmark gene list epithelial-mesenchymal transition showing Log2 fold change (FC; left). **(C)** Luminal epithelial markers (*GATA3* and *MUC1*) are upregulated, while myoepithelial (*KRT14*), mesenchymal (*CDH2*, *LAMA1*, *SNAI2*, *VIM*, and *ZEB2*) and stem cell (*CTNNB1* and *MME*) are downregulated with knock down of *MEG3*. Genes from literature-based list of markers significantly ($p \leq 0.05$) differentially expressed in $D492M^{KD-MEG3}$ vs $D492M^{KD-CTRL}$.

where the cells migrate toward a chemo-attractant. In the wound healing assay, $D492^{MEG3}$ has slightly increased migration rate compared to $D492^{CTRL}$, while $D492M^{KD-MEG3}$ has decreased migration rate compared to $D492M^{KD-CTRL}$ (**Supplementary Figure 5A**). In the trans-well migration assay, $D492^{MEG3}$ has about two-fold increased migration rate compared to $D492^{CTRL}$ and $D492M^{KD-MEG3}$ has reduced migration rate compared to $D492M^{KD-CTRL}$ (**Figure 8B**). *MEG3* manipulation, however, did not affect invasion in a transwell invasion assay (**Supplementary Figure 5B**). We performed

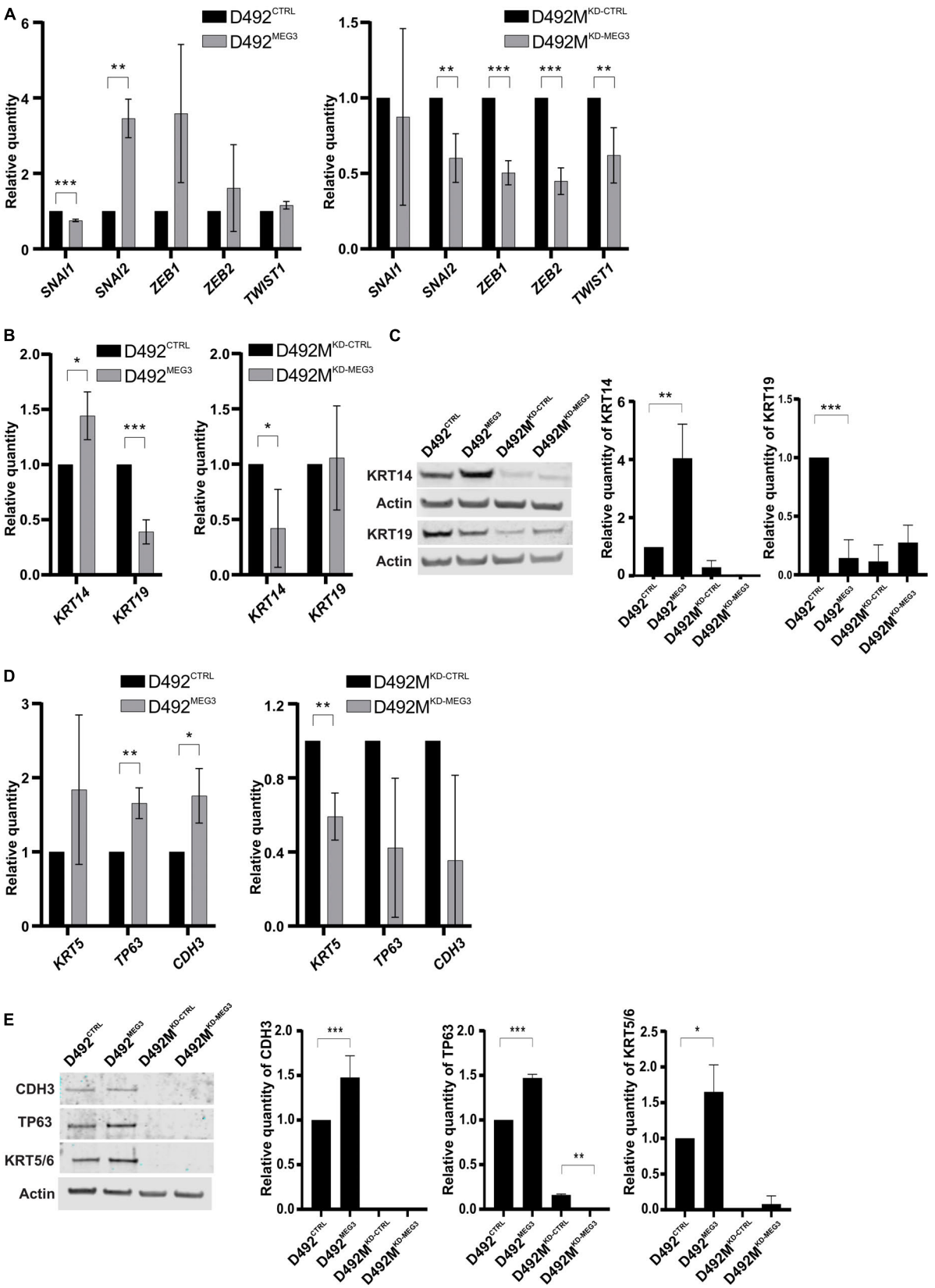

FIGURE 7 | Continued

FIGURE 7 | *MEG3* induce partial EMT. **(A)** *MEG3* increases expression of EMT transcription factors in D492 and the knock down of *MEG3* decrease expression of TF-EMT in D492M. qRT-PCR showing $D492^{MEG3}$ increased expression of transcription factors (TF) *SNAI2* compared to $D492^{CTRL}$ (left) and decreased expression of EMT related TF *SNAI2*, *ZEB1*, *ZEB2* and *TWIST1* in $D492M^{KD-MEG3}$ compared to $D492M^{KD-CTRL}$ (right). Results shown as mean ± SD. Unpaired *t*-test was used to test significance: $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $n = 3$. **(B)** *MEG3* increases expression of myoepithelial marker *KRT14* and decrease expression of luminal epithelial marker *KRT19* on mRNA level. qRT-PCR showing $D492^{MEG3}$ has increased expression of *KRT14* and decrease expression of *KRT19* compared to $D492^{CTRL}$ (left). $D492M^{KD-MEG3}$ has decreased expression of *KRT14* compared to $D492M^{KD-CTRL}$ (right). qRT-PCR results shown as mean ± SD. Unpaired *t*-test was used to test significance: $^{*}p \leq 0.05$; $^{***}p \leq 0.001$; $n = 3$. **(C)** qRT-PCR results confirmed on protein level. Representative pictures of western blot (WB) with its quantification (below). $D492^{MEG3}$ has increased protein level of KRT14 and decreased protein level of KRT19 compared to $D492^{CTRL}$. WB results shown as mean ± SD. One-way ordinary ANOVA, followed by Tukey's multiple comparison test was used to test significance: $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $n = 3$. **(D)** *MEG3* increase expression of myoepithelial markers *TP63* and *CDH3* and knock-down of *MEG3* decrease expression of myoepithelial marker *KRT5*, on mRNA level. qRT-PCR showing $D492^{MEG3}$ has increased expression of *TP63* and *CDH3* compared to $D492^{CTRL}$ (left). $D492M^{KD-MEG3}$ has decreased expression of *KRT5* compared to $D492M^{KD-CTRL}$ (right). qRT-PCR results shown as mean ± SD. Unpaired *t*-test was used to test significance: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $n = 3$. **(E)** qRT-PCR results confirmed on protein level. Representative pictures of western blot (WB) with its quantification (below). $D492^{MEG3}$ has increased protein level of CDH3 (P-cad), TP63 (p63) and KRT5 compared to $D492^{CTRL}$. $D492M^{KD-MEG3}$ has decreased protein level of TP63 compared to $D492M^{KD-CTRL}$. WB results shown as mean ± SD. One-way ordinary ANOVA, followed by Tukey's multiple comparison test was used to test significance: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $n = 3$.

mammosphere assays in rBM (reconstituted basement membrane, Matrigel) (**Figure 8C**) and in low attachment plates (**Figure 8D**), with comparable results. $D492^{MEG3}$ increases the formation of colonies compared to $D492^{CTRL}$ while $D492M^{KD-MEG3}$ decreases the formation of colonies compared to $D492M^{KD-CTRL}$. In addition, we co-cultured $D492^{MEG3}$ with endothelial cells (HUVECs) and observed increased size of colonies and less branching compared to $D492^{CTRL}$ (**Figure 8E**). Finally, manipulation of *MEG3* levels slightly affected proliferation rate of $D492M^{KD-MEG3}$ compared to $D492M^{KD-CTRL}$ (**Supplementary Figure 5C**).

DISCUSSION

In this study, we show that ncRNAs from the DLK1-DIO3 locus are highly expressed in stromal/mesenchymal cells in the breast and positively correlate with the expression of EMT genes in breast tissue. *MEG3* expression was monoallelic in both D492 and D492M and gain and loss of function studies have shown concomitant expression of *MEG3* with miRNAs from the DLK1-DIO3 locus, indicating that *MEG3* could be used as a marker for the expression of the non-coding RNAs from the locus. *MEG3* expression was shown to be negatively correlated with survival of breast cancer patients, particularly with the luminal B subtype. Furthermore, we demonstrate that enhanced *MEG3* expression accompanied by increased expression of the ncRNAs at the DLK1-DIO3 locus, contributes to partial EMT more correctly referred to as epithelial plasticity, seen by increased expression of EMT related TFs, increase of basal/mesenchymal markers and enhanced properties such as migration, resistance to apoptosis and clonogenic capacity.

We used an isogenic breast cell line model to study the expression pattern and functional role of ncRNAs, both miRNAs and lncRNAs, in EMT. Of interest was the largest miRNA locus in the human genome and the lncRNA *MEG3*, both within the DLK1-DIO3 imprinted region on chromosome 14. The non-coding part of the DLK1-DIO3 locus has higher expression in cells with mesenchymal phenotype (D492M) compared to cells with epithelial phenotype (D492). These results were validated in primary breast tissue and in another cellular model of EMT. Furthermore, we have shown that *MEG3* expression correlates with expression of extracellular matrix proteins, which are secreted by cells with a mesenchymal phenotype, and with mesenchymal genes in breast tissue. Data from pyrosequencing demonstrate that the expression of *MEG3* is monoallelic in both D492 and D492M indicating that the increased expression of *MEG3* in D492M is not due to loss of imprinting. We have shown that *MEG3* negatively correlates with survival of luminal B breast cancer patients and patients with grade 3 breast cancer. This is in line with a recent study where high expression of *MEG3* was identified to be a negative prognostic marker for breast cancer (Yao et al., 2019).

Many studies suggest *MEG3* as a tumor suppressor, largely due to the observation that *MEG3* expression is lower in tumor tissue compared to normal tissue (Sheng et al., 2014; Sun et al., 2014, 2016; Yin et al., 2015; Chak et al., 2017; Molina-Pinelo et al., 2018). Our data demonstrates that *MEG3* expression levels are comparable in whole normal breast tissue and in stroma (fibroblasts), however, the expression of *MEG3* in epithelial cells is much lower. There was considerable variation of the *MEG3* expression in breast tissue samples that could be partially due to different proportions of subset of fibroblasts associated with ducts vs TDLUs. There are studies confirming existence of, for instance, two distinct functionally specialized lineages of lobular vs ductal fibroblast (Morsing et al., 2016) or myoepithelial cells (Fridriksdottir et al., 2017), which could be identified by specific marker expression. Importantly, relative proportions of stromal and epithelial compartment are different in normal and cancerous human breast tissue. Breast cancers arise in vast majority from epithelial cells, with TDLUs being the predominant site of breast tumor occurrence (Tabar et al., 2014). Therefore, it would be expected that expression of *MEG3* is higher in normal breast tissue, as it comprises relatively more stromal cells compared to breast cancer tissue. In line with this, expression of *MEG3* from whole breast tissue is distorted as proportions of stroma vs epithelia in normal/cancer tissue are different, resulting in misleading interpretations. Using RNA only from unsorted normal tissue will mainly represents expression of stromal cells. Therefore, it is crucial to use a proper control when comparing expression of genes in normal vs tumor tissue. Single-cell RNA-sequencing or sorted stromal and epithelial cells would give more informative results as it would enable distinctions between epithelial and stromal tissue

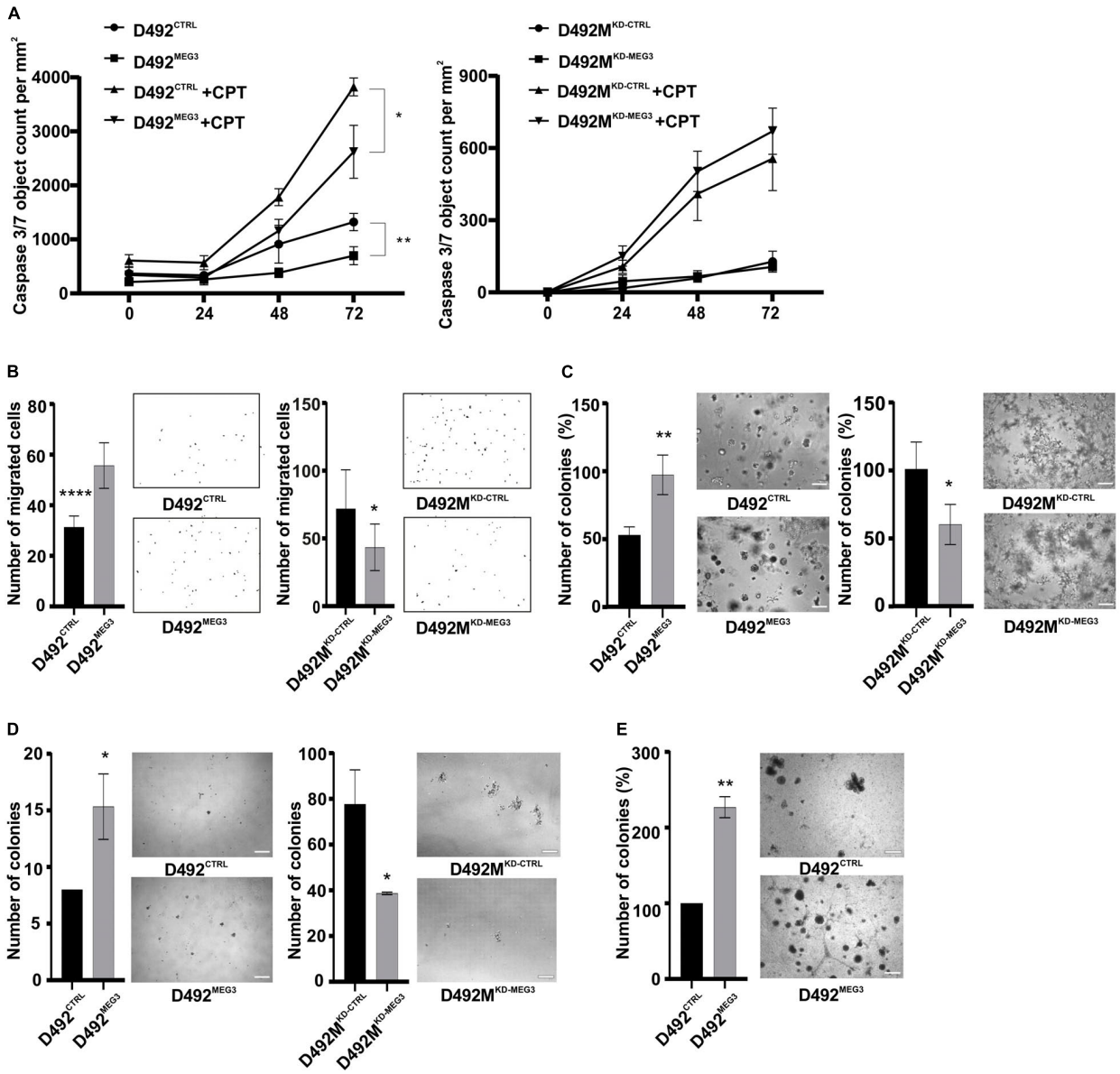

FIGURE 8 | *MEG3* increases stem cell properties. **(A)** *MEG3* increase resistance to chemically induced apoptosis. Apoptosis assay: D492^{MEG3} is more resistant to chemically induced apoptosis compared to D492^{CTRL} (left). Data is analyzed on Incucyte Zoom and displayed as Caspase 3/7 object count/mm^2. Results are shown as mean ± SD. Multiple unpaired Student *t*-test per row was used to test significance at 72-h time-point: *$p \leq 0.05$; **$p \leq 0.01$; $n = 6$. **(B)** *MEG3* increases migration and knock down of *MEG3* decreases migration through trans-well filters. Migration assay: D492^{MEG3} has increased migration rate compared to D492^{CTRL} (left), with representative pictures on side. D492M$^{KD-MEG3}$ migrates less compared to D492M$^{KD-CTRL}$ (right), with representative pictures on side. Quantification of number of migratory cells, analyzed using ImageJ software, results shown as mean ± SD. Unpaired *t*-test was used to test significance: *$p \leq 0.05$; ****$p \leq 0.0001$; $n = 6$. **(C)** *MEG3* increases clonogenic capacity in 3D culture in rBM (reconstitute basement membrane). Mammosphere assay: D492^{MEG3} has higher clonogenic capacity compared to D492^{CTRL} (left). D492M$^{KD-MEG3}$ has lower clonogenic capacity compared to D492M$^{KD-CTRL}$ (right). Data shown as % mean ± SD, *$p \leq 0.05$; **$p \leq 0.01$; $n = 3$. Scale bar = 500 μm. **(D)** *MEG3* increases clonogenic capacity in low attachment assay. D492^{MEG3} increases the formation of colonies compared to D492^{CTRL} (left), with representative pictures on side. D492M$^{KD-MEG3}$ decreases the formation of colonies compared to D492M$^{KD-CTRL}$ (right), with representative pictures on side. Results shown as % of mean ± SD. Unpaired *t*-test was used to test significance: *$p \leq 0.05$; **$p \leq 0.01$; $n = 3$. Scale bars = 200 μm. **(D)** Co-culture of D492^{MEG3} with endothelial cells (HUVECs) increases number and size of colonies and forms less branching compared to D492^{CTRL}. Results shown as % of mean ± SD. Unpaired *t*-test was used to test significance: **$p \leq 0.01$; $n = 3$. Representative pictures scale bars = 200 μm.

compartments. In this paper we show that *MEG3* expression negatively correlates with survival in breast cancer, particularly in grade three tumors and the luminal B subtype. However, our study does not determine if the high *MEG3* expression represents increased stromal infiltration in the tumors or elevated expression in cancer cells.

Another reason for classifying *MEG3* as tumor suppressor is its action on stabilization of p53 (Ghafouri-Fard and Taheri, 2019). However, inactivation of p53 is a frequent event in cancer, estimated to have about 50 % occurrence (Gasco et al., 2002; Marine et al., 2006; Haupt and Haupt, 2017). The percentage is even higher, when the inactivation in p53's regulatory pathways is considered (Joerger and Fersht, 2016). Therefore, the use of cell lines which lack active p53, such as D492 and D492M, offers a different approach, more relevant for studying breast cancer signaling pathways, to study the role of DLK1-DIO3. The role of p53 in the cell is that of a tumor suppressor, impacting acts in proliferation, cell cycle and genomic stability (Mercer, 1992). In D492 cell lines, as could be expected, we did not observe effect on cell proliferation. Recently, Uroda and colleagues' stated, that cell cycle arrest by *MEG3* is exclusively p53-dependent, (Uroda et al., 2019), in line with our suggestions that *MEG3* can have a different role in cells lacking p53. Collectively, these observations could explain the conflicting results about role of *MEG3* in tumors.

Many imprinted genes are located in clusters regulated by a differentially methylated regions (DMRs) (Bartolomei and Ferguson-Smith, 2011). In our study targeting the *MEG3* promoter, we have observed concomitant expression of *MEG3* with other miRNAs from the DLK1-DIO3 locus. Our data may support previous studies showing that the *MEG3* promoter controls expression of all maternally expressed genes from the DLK1-DIO3 locus (Tierling et al., 2006; Ioannides et al., 2014; Sanli et al., 2018). Zhu et al. (2019) have shown that the MEG3-DMR overlaps with the *MEG3* gene promoter and any deletion in this region inactivates both MEG3-DMR and the *MEG3* gene. Their data shows, that it is the MEG3-DMR, not the *MEG3* gene, which regulates imprinting (and expression). Therefore, by targeting the *MEG3* promoter at the MEG3-DMR all the non-coding RNAs at the DLK1-DIO3 locus are inactivated. *MEG3* expression can be considered as a marker for the expression of other ncRNAs at the locus.

Cellular plasticity, an important contributor to heterogeneity and drug resistance in breast cancer can be conveyed through EMT/MET (Liu et al., 2014). Partial EMT (p-EMT) may reflect cellular plasticity better than full-EMT and consequently, cells possessing this state adapt more easily to a new environment, which is necessary for cancer cell invasion and metastasis (Thiery, 2002; Tam and Weinberg, 2013; Lambert et al., 2017). Notably, a recent report highlights the importance of the intermediate stages of EMT for the intravasation of tumor cells and for metastasis formation in experimental breast or skin tumors (Pastushenko et al., 2018). Similarly, another study showed that cancer cells might only reach an intermediate EMT stage allowing for increased motility, while keeping its cellular plasticity (Brabletz et al., 2018). It has also been observed that full mesenchymal phenotype (EMT), has a low capacity to form metastasis compared to p-EMT (Schmidt et al., 2015). The essential criteria for aggressive behavior does not need to be a particular phenotype, but rather enhanced cellular plasticity, as is also observed for hybrid E/M cells (Grosse-Wilde et al., 2015). Thus, EMT may be viewed as a trans-differentiation process where epithelial and mesenchymal cells interconvert by passing through an intermediate "stem-like" state (Grosse-Wilde et al., 2018).

EMT is a complex process and meta-analysis indicates that there are possibly different types of EMT (Liang et al., 2016). We have shown, that by manipulating *MEG3* expression, and thus changing the expression of the non-coding genes at the DLK1-DIO3 locus, the majority of these EMT related TFs are affected, indicating an important role of the ncRNAs the DLK1-DIO3 locus in the EMT process. One of the most typical hallmarks of EMT is downregulation of *CDH1* (E-cadherin) and epithelial-specific keratins (Peinado et al., 2007). Altered expression of *MEG3* does not lead to change of E-cadherin expression and therefore *MEG3* may have induced only a partial EMT phenotype. However, it has been shown, that cells with p-EMT phenotype display concomitant expression of epithelial and mesenchymal markers (Armstrong et al., 2011) and loss of E-cadherin is not a prerequisite for EMT (Hollestelle et al., 2013). Cells undergoing collective migration have hybrid EMT phenotype characterized by E-cadherin expression, which helps to maintain cell–cell contacts (Friedl et al., 2012; Aceto et al., 2015). Furthermore, we have shown that altered expression of *MEG3* revealed distinct luminal and myoepithelial marker expression. Increased expression of *KRT14* and decreased expression *KRT19* indicate increased myoepithelial differentiation, which has been connected to a partial EMT phenotype (Petersen et al., 2001). Study on collective migration revealed *KRT14* as a key regulator of metastasis (Cheung et al., 2016) and the same applied for collective invasion, which was facilitated by subpopulation of cells expressing *KRT14* (Cheung et al., 2013). The observed increase of myoepithelial/basal differentiation in cells with higher expression of *MEG3* was supported with altered expression of other markers such as *KRT5*, *TP63*, and *CDH3*.

A key characteristic defining breast stem cells is the ability to form of mammospheres (Dontu et al., 2003; Grosse-Wilde et al., 2015). Morel and colleagues confirmed that human mammary epithelial cells undergoing EMT exhibited better mammosphere-forming capabilities (Morel et al., 2008) and Shimono et al. have shown that mammosphere-forming activity is abrogated in both normal and malignant mammary stem cells when the EMT program is shut down (Shimono et al., 2009). In this study phenotypic differences upon altered MEG3 expression were more prominent in 3D than in 2D cell culture, where *MEG3* increased mammosphere formation ability and slightly decreases branching potential in 3D culture. Furthermore, we have shown increased expression of *ALDH1A3* and *ITGA6*, in cells with overexpression of *MEG3*, supporting role of *MEG3* in stemness.

We propose that increased expression of *MEG3*, and thus increased expression of the ncRNAs at the DLK1-DIO3 locus, in D492 leads to partial EMT phenotype/enhanced plasticity, seen by molecular changes with increased mesenchymal and myoepithelial/basal genes and increased migration and resistance to apoptosis. In contrast, the repression of *MEG3*, and the maternally imprinted ncRNAs, in D492M leads to decreased mesenchymal and basal gene expression and decreased migration and resistance to apoptosis. Nguyen-Ngoc et al.

also demonstrated, that motility can occur in cells that retain an epithelial molecular signature (Nguyen-Ngoc et al., 2012). This supports our observation, that manipulation of *MEG3* expression did not affect the morphological phenotype, but rather affected the functional phenotype. These characteristic properties of cells undergoing EMT were originally proposed to occur in breast cancer by Mani and colleagues (Mani et al., 2008), showing that stem-like and p-EMT properties share many characteristics, such as increased migration, resistance and survival (Creighton et al., 2009; Armstrong et al., 2011; Hanahan and Weinberg, 2011).

Increased understanding of branching morphogenesis in the breast and the regulation of EMT and MET may hold the key for future development of methods and drugs that neutralize the invading properties of cancer cells. Currently, there is need for biomarkers to accurately monitor the EMT/MET process that may improve treatment. Prognostic value of *MEG3* in human malignancies remains controversial and requires further investigation. Our results and conflicting data from the literature suggest that *MEG3* has a complex role in breast tissue.

DATA AVAILABILITY STATEMENT

The RNAseq data for this article has been submitted to GEO, with the GEO accession number GSE142268, see here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142268

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Icelandic National Bioethics Committee VSN-13-057 and VSN-11-105-V2. The Icelandic Data Protection Commission (2001/523 and 2002/463) Landspitali Ethical Committee No. 35/2013. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MM, TG, JB, ZB, EB, GT, and BH: conceptualization and design of the study. ZB, EB, JB, AS, and BH: data acquisition. ZB, AS, EB, GT, SS, and BH: data analysis. ZB, GT, TG, and BH: drafting the manuscript. All authors participated in data interpretation, revision of the manuscript and approved the final version to be published.

FUNDING

This work was supported by Grants from Landspitali University Hospital Science Fund, University of Iceland Research Fund, Icelandic Science and Technology Policy Council Research Fund no. 1103010061, Icelandic Science and Technology Policy – Grant of Excellence: 52144051, "Vísindasjóður Krabbameinsfélagsins" (Icelandic Cancer Society Science Fund) 2017 and 'Göngum saman', a supporting group for breast cancer research in Iceland (www.gongumsaman.is). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank Rosa B. Barkardottir, Adalgeir Arason, Bjarni A. Agnarsson, and Oskar Thor Johannsson for their contribution to this work by providing RNA samples from breast cancer patients and pathological and clinical information. We would also like to thank Gudrun Johannesdottir for excellent technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00461/full#supplementary-material

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