# REPROGRAMMING STROMAL CELLS IN CHRONIC INFLAMMATION AND CANCER

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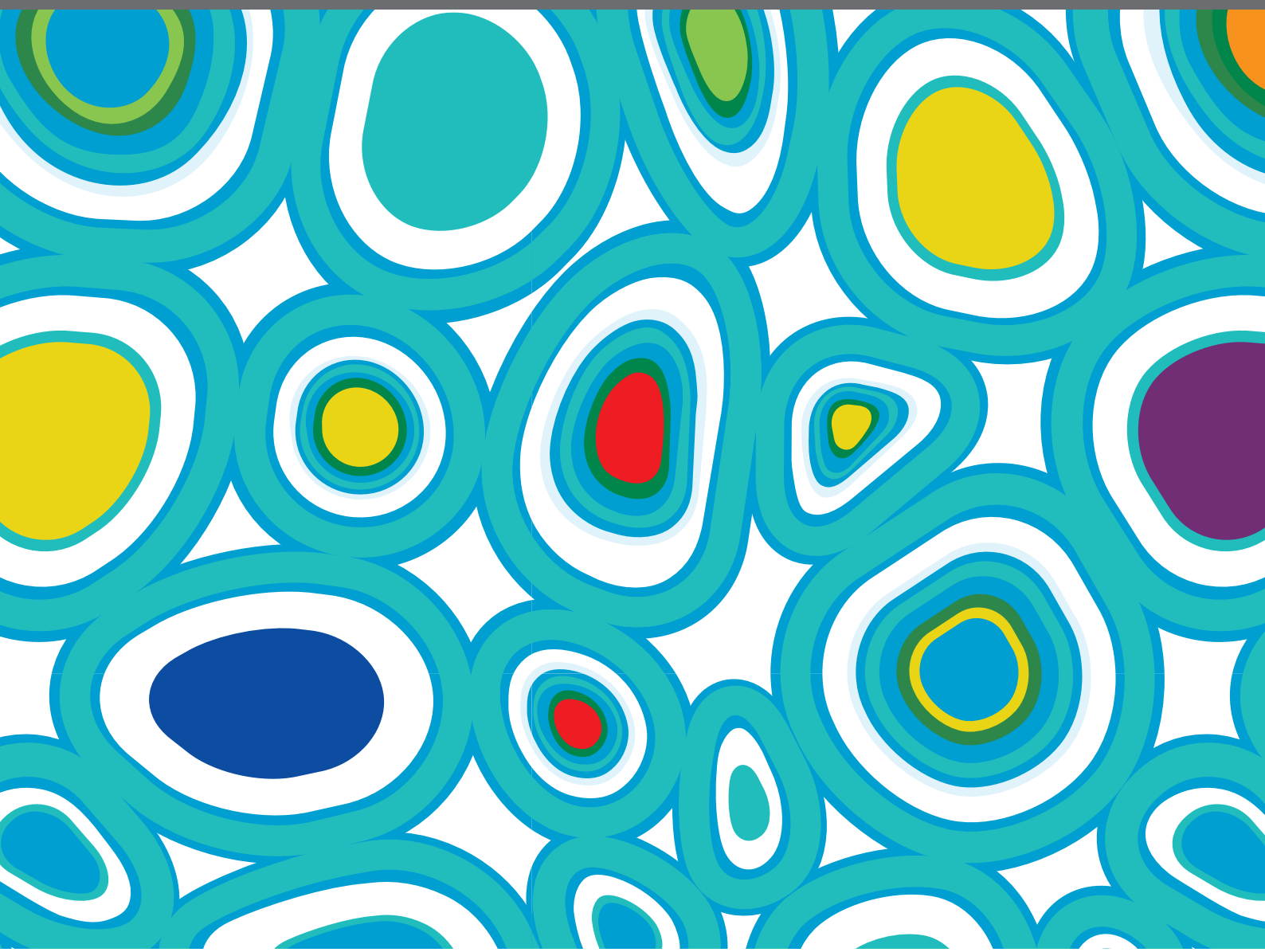

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# REPROGRAMMING STROMAL CELLS IN CHRONIC INFLAMMATION AND CANCER

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EDITORIAL
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# Editorial: Reprogramming Stromal Cells in Chronic Inflammation and Cancer

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**Keywords: inflammation, reprogramming, chronic inflammatory diseases, cancer, tumor microenvironment, 3D models, organoid, organotypic 3D culture**

## Editorial on the Research Topic

## Reprogramming Stromal Cells in Chronic Inflammation and Cancer

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Despite extensive research effort in recent decades, the underlying mechanisms involved in the reprogramming of stromal cells in chronic inflammatory conditions and cancer are still ill-defined. This research topic includes original research and reviews of important concepts on inflammatory microenvironment remodeling and 3D models.

Adipose progenitor cells constitute a small and heterogeneous group of cells that can be modulated by mature adipocytes as well as by resident immune cells to determine their function and their differentiation potential. Several pathologies can destabilize the relationships created and break the equilibrium. In their review, Pyrina and others summarize the current knowledge about various factors and different immune and stromal cell populations that fate decisions of the adipose progenitor cells in the context of obesity-related inflammation. They define how in the course of obesity the pro-inflammatory microenvironment through multiple contact- and paracrine-mediated interactions shape the differentiation of preadipocytes, towards a pro-fibrotic transcriptional mode that in turn promotes fibrosis.

The NR4A receptors are potent sensors of changes in cellular microenvironment allowing the control of physiological and pathological processes. In their review, Murphy and Crean illustrate how this subfamily of nuclear receptors can be considered as transcriptional regulators in mesenchymal stromal cells, cell cycle progression and cytokine production to control local immune response in physiological or in chronic inflammatory conditions. Authors made a focus on recent advances in the pharmacological control of these receptors.

Gastrointestinal cancers are among the most frequent cancer-types worldwide and have been strongly linked with chronic inflammation. For that reason, current studies and therapies focus on cancer cells but also on the cells present in the tumor microenvironment (TME). Melissari and others review the role of cancer associated fibroblasts (CAFs), a major component of the TME, in tumor initiation, promotion and metastasis. They focus on the mechanisms that drive fibroblast reprogramming in gastrointestinal cancers, as well as on recent advances in therapies and patient prognosis.

The role of tumor infiltrating neutrophils in cancer progression of gastric cancer is highly appreciated (Jailon et al., 2020). Zhang and others demonstrated that tumor-educated neutrophils secreted IL-17, IL-23, and TNFα, that induced the transition of mesenchymal stem cells to CAFs. CAFs in turn promoted gastric cancer growth revealing a pro-tumorigenic role of neutrophils in cancer microenvironment.

In an additional publication focusing on CAFs, the emerging grasp of the TME is consistently unfolding even in cell-endogenous and evolutionally conserved mechanisms. In their research article, the group led by Ruth Scherz-Shouval have worked on the synchronized cellular circadian clock which was previously reported to facilitate a plethora of processes including gene regulation and homeostasis in the cell. The authors were able to reveal a novel aspect of the circadian clock disrupted during tumorigenesis and metastasis as cancer cells were compared to normal fibroblasts. Out of many related genes, Period 2 (Per2) was identified as a cardinal hub associated with this disruption. When Per2 was eliminated or knocked-out, the authors were able to abolish the metastatic progression. Notably, the expression of Per2 was found mainly in CAFs, a critical component of the TME.

The role of fibroblasts is further described in cutaneous T-cell lymphoma (CTCL) which refer to a group of lymphoproliferative diseases characterized by the accumulation of malignant T cells in chronically inflamed skin lesions. In their review, Stoleranco and others describe the crosstalk between fibroblasts, keratinocytes and malignant T cells and how they contribute to CTCL progression. Among the complex signaling networks, authors focus on the activation of STAT proteins, the development of a Th2 inflammatory microenvironment, neovascularization of the tumor tissue and changes in the skin architecture.

Notably, there is growing evidence describing the role of the TME in the development and progression of hepatocellular carcinoma (HCC) (Santhakumar et al., 2020). HCC typically arises from fibrotic or cirrhotic liver, characterized by alterations in extracellular matrix (ECM) components. Upon various non-tumoral components, such as immune and endothelial cells, the hepatic stellate cells (HSCs) are the tissue-specific pericytes that are now reported to play a major role in HCC progression. The article from the group of Hien Dang reviewed the recent findings connecting the dots in the hepatic TME promoted by intercellular interaction involving HSCs. As fibrosis and cirrhosis are the fundamental preceding conditions for HCC, activation of HSCs is a key factor that contributes to these processes. HSCs were reported to secrete pro-fibrogenic cytokines, to interact with neighboring liver natural killer (NK) cells, to induce senescence and to undergo an increase in DNA methylation, all contributing to their participation in supporting the tumor cells and their prosperity. Notably, HSCs were found to share intercellular cross talk with T-regulatory cells, Myeloid Derived Suppressor Cells (MDSCs) and endothelial cells suggested by their ability to produce angiogenic factors. Furthermore, HSCs were reported to interact with non-cellular elements in the tissue, mostly evident by the ability to remodel the ECM directly. The conception that HSCs play a dramatic role in HCC development, can be translated into the clinics by using drugs such as metformin (targeting the JNK pathway) or anti-angiogenic agents.

Two studies in this special issue highlight the advantages of 3D models to study human pathology. Vergnolle and others characterized morphological and functional phenotypes of colonic organoids from Inflammatory Bowel Disease (IBD) and control patients. The authors demonstrated that IBD organoid cultures preserved an inflammatory phenotype and that clinically used treatments reduced some parameters associated with this inflammatory phenotype. This study highlights that IBD patient organoids constitute a reliable human pre-clinical model to study IBD pathology and investigate new strategies targeting epithelial repair. In the second study, Haykal and others, depict the importance of using and developing new models and tools taking into consideration tissue complexity. A focus is made on organotypic models allowing to study cancer progression in complex settings and offering potential benefits for personalized medicine.

Altogether, these topical articles highlight the complex interactions between parenchymal cells and the surrounding stroma in different contexts. Besides, the advantages of 3D models to study the human pathophysiology of chronic inflammatory diseases and cancer and for pharmacological drug testing are underlined. In the era of personalized medicine, a profound knowledge of tissue microenvironment could improve prognosis and therapy in clinical practice.

## AUTHOR CONTRIBUTIONS

AI, OM, TC, and IP conceived and wrote the editorial.

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ORIGINAL RESEARCH
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# Characterization of Human Colon Organoids From Inflammatory Bowel Disease Patients

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Inflammatory Bowel Diseases (IBD) are chronic inflammatory disorders, where epithelial defects drive, at least in part, some of the pathology. We reconstituted human intestinal epithelial organ, by using three-dimension culture of human colon organoids. Our aim was to characterize morphological and functional phenotypes of control (non-IBD) organoids, compared to inflamed organoids from IBD patients. The results generated describe the epithelial defects associated with IBD in primary organoid cultures, and evaluate the use of this model for pharmacological testing of anti-inflammatory approaches. Human colonic tissues were obtained from either surgical resections or biopsies, all harvested in non-inflammatory zones. Crypts were isolated from controls (non-IBD) and IBD patients and were cultured up to 12-days. Morphological (size, budding formation, polarization, luminal content), cell composition (proliferation, differentiation, immaturity markers expression), and functional (chemokine and tight junction protein expression) parameters were measured by immunohistochemistry, RT-qPCR or western-blot. The effects of inflammatory cocktail or anti-inflammatory treatments were studied in controls and IBD organoid cultures respectively. Organoid cultures from controls or IBD patients had the same cell composition after 10 to 12-days of culture, but IBD organoid cultures showed an inflammatory phenotype with decreased size and budding capacity, increased cell death, luminal debris, and inverted polarization. Tight junction proteins were also significantly decreased in IBD organoid cultures. Inflammatory cytokine cocktail reproduced this inflammatory phenotype in non-IBD organoids. Clinically used treatments (5-ASA, glucocorticoids, anti-TNF) reduced some, but not all parameters. Inflammatory phenotype is associated with IBD epithelium, and can be studied in organoid cultures. This model constitutes a reliable human pre-clinical model to investigate new strategies targeting epithelial repair.

**Keywords: inflammation, Crohn's disease, ulcerative colitis, organoid, intestine**

**Abbreviations:** CD, Crohn's disease; IBD, inflammatory bowel diseases; UC, ulcerative colitis.

## INTRODUCTION

Inflammatory Bowel Diseases (IBD) are chronic inflammatory disorders of the intestine that include Crohn's disease (CD) and ulcerative colitis (UC). IBD cause lifelong inflammatory disorders and severely impair the quality of life in patients (Panaccione et al., 2005). Current treatments target the inflammatory reaction *per se*, and in particular the recruitment of inflammatory cells (Neurath, 2017). However, the idea that a healed intestinal epithelium is necessary in addition to inhibition of immune cell recruitment has emerged. Mucosal healing is now considered as the next therapeutic challenge for IBD. This means that not only symptomatic improvement is expected with treatment, but also endoscopic remission, showing repaired tissues (absence of frailty, blood, erosion or ulcers) (Dave and Loftus, 2012). Indeed, a more favorable prognosis is associated with mucosal healing: patients having lower relapse and hospitalization rates (Peyrin-Biroulet et al., 2011; Burisch et al., 2013). Some of the current treatments used in the clinic are considered to favor, at least in part epithelial healing. This is the case for Mesalamine (5-ASA) (Baumgart et al., 2005), but also for anti-Tumor Necrosis Factor (TNF) (Fischer et al., 2013) and glucocorticoid treatments, which restore barrier function (Fischer et al., 2014). However, their effects on epithelial functions are considered very limited and the hope for future treatments is to strengthen the effects of therapies on epithelial healing.

The consensus to explain IBD's etiology is to consider that in genetically predisposed individuals, a combination of immune disorders with intrinsic (epithelial barrier function for instance) and extrinsic (microbiota) factors lead to the development of chronic inflammation. Genome-wide association studies have identified a number of epithelial mediators and epithelial signaling pathways associated with IBD (McCole, 2014). These studies brought the epithelium at the forefront for the development of new therapeutic strategies, although epithelial dysfunctions in IBD have not been exhaustively defined yet (Khor et al., 2011; Rivas et al., 2011; McCole, 2014). Novel models focusing on epithelial biology are therefore necessary in the context of IBD. Such models are important both to better characterize epithelial dysfunctions associated with IBD and to assess new therapeutic strategies aiming at promoting intestinal epithelium healing.

The technological breakthrough represented by the culture of organoids from isolated tissue stem cells now allows to re-create *in vitro* an intestinal epithelial organ (Sato and Clevers, 2013). This technology is based on the isolation of intestinal crypts, which are then cultured in three-dimensions. In the presence of appropriate growth factors, intestinal stem cells present in the isolated crypts proliferate and enter into differentiation processes, recreating a complex epithelium, which contains all cell types that compose the intestinal epithelium (paneth cells, enteroendocrine cells, goblet cells, enterocytes, tuft cells, etc.). The epithelium generated by three-dimension cultures of isolated crypts closes on itself, forming a sphere, in which epithelial cells are orientated with their apical side toward the lumen (Sébert et al., 2018). While a number of studies have employed culture organoids from intestinal crypts (Sugimoto et al., 2018; Yip et al., 2018; Ramesh et al., 2019), only very few studies have investigated the possibility to culture organoids from IBD patient-isolated intestinal crypts (Dotti et al., 2017; Noben et al., 2017; Howell et al., 2018). Importantly, they reported transcriptional or methylation differences between organoids from UC or CD patients compared to controls (Dotti et al., 2017; Howell et al., 2018). Both studies suggested that intestinal epithelial cells undergo changes during IBD development that could be involved in pathogenesis. However, none of these two studies has performed any characterization of the morphology, cell composition or functions of IBD organoid cultures. The potential for human IBD organoid cultures to be used as a model to test therapeutic options that could target the epithelium in IBD has not yet been addressed either. Here, we have characterized the morphological and functional phenotype of IBD patient's epithelium by using organoid cultures. Further, we have tried to establish whether organoid cultures from IBD patients could be used to test therapeutic approaches on epithelial healing.

## MATERIALS AND METHODS

### Human Tissue Materials

Biological samples were obtained from individuals treated at the Toulouse University Hospital who gave informed consent. The MICILIP research protocol was approved by the national ethics committee (#NCT01990716) and was financially supported by the Toulouse University Hospital (Denadai-Souza et al., 2018). The biocollection that included colonic resections was approved under the CODECOH national agreement: Colic collection: DC2015-2443). These samples were freshly collected from non-IBD controls (healthy zones of tissues resected from patients with colorectal cancer or endometriosis) and from IBD patients (in non-inflammatory zones). Tissues were collected from 26 patients with Crohn's disease, 8 patients with ulcerative colitis, and from 18 non-IBD patients. Characteristics and treatments for patients are provided in **Supplementary Table S1**.

### Isolation and Culture of Colon Crypts

Fresh colonic tissues or biopsies were harvested and colon crypts were isolated and cultured mostly as previously described (Jung et al., 2011; Sato et al., 2011), with some adaptation that are described in **Supplementary Material**.

### Treatments

Control organoids were treated by a pro-inflammatory cytokines cocktail [IL-1β, IL6, TNF-α (PeproTech)] at the concentration of 10 ng/ml (**Supplementary Figure S1**). IBD organoids were treated independently by three routinely used medications for the treatment of IBD patients: (anti-TNFα or 100 μM), methylprednisolone (10 or 100 μM), and 5-ASA (50 or 500 μM) (**Supplementary Figure S2**).

### Immunofluorescence Labeling

Organoids (in chamber slide) were fixed with 4% formaldehyde (FA, Sigma Aldrich) in Hank's Buffered Salt Solution (HBSS, Gibco) at 37°C for 40 min. Organoids were permeabilized with 0.5% triton X-100 (Sigma Aldrich) in HBSS at RT for 40 min and were incubated with a blocking buffer containing 3%

bovine serum albumin (BSA, Sigma Aldrich) in HBSS overnight at 4°C. Organoids were incubated 2 h at 37°C with primary antibodies. Next, organoids were incubated with secondary antibodies 1.5 h at 37°C and then stained for 20 min with DAPI (Invitrogen) and Phalloidin (see **Supplementary Table S2** for all antibodies) (Cenac et al., 2015). Finally, slides were mounted with Vectashield mounting medium (VectoLaboratories).

## Colonoid Imaging

The images of organoids were taken by confocal microscopy with a Leica SP8. Image J software was used to process and analyze images. Ten images at 20X and 10 images at 63X objectives were analyzed for each well, and an average of 2 wells were analyzed for each condition. To assess barrier function, organoids were incubated with 1 mg/ml of fluorescein isothiocyanate (FITC)-labeled dextran 4-kDa for 2-h at 37°C, and images were analyzed on a confocal microscope with a X63 lense.

## RNA and Protein Extraction

After an average of 10 days of culture, organoids (8–10 wells in 48 wells plates) were incubated in cell recovery solution (Corning) for 30 min at 4°C. After centrifugation, pellet-containing cells were resuspended in RP1 buffer (NucleoSpin® RNA/Protein kit, Macherey Nagel). Total RNA and protein were extracted according to the manufacturer's protocol.

## Reverse Transcription, and Real-Time Quantitative PCR Analysis

One μg of each purified RNA preparation was reverse-transcribed using Maxima First Strand cDNA Synthesis Kit (Fermentas Life sciences) and cDNAs were analyzed by qPCR using relevant primers as previously described (Rolland-Fourcade et al., 2017). qPCR analysis was performed on a LightCycler® 480 Real-time PCR system device (Roche Applied Science) using the LightCycler® 480 SYBR Green I Master (Roche Applied Science), according to the manufacturer's instructions, and specific primers (**Supplementary Table S3**). All experiments included a standard curve and all samples were analyzed in duplicate and expressed as a relative amount ($2^{-\Delta Ct}$). Relative expression of targeted genes was compared to GlycerAldehyde-3-Phosphate DesHydrogenase (GAPDH).

## Western Blot

Fifty microgram of total proteins were separated by 4–20% Mini-Protean Gel (Bio-Rad). Separated proteins were transferred onto a nitrocellulose membrane. Primary antibodies (**Supplementary Table S2**) as well as peroxidase conjugated secondary antibodies were used according to standard Western blot procedures. Peroxidase was detected by using the ECL™ Prime Western Blotting system (GE Healthcare). Quantification of signals was done using Image Lab software (Bio-Rad).

## Metabolic Activity Assay

Organoids were plated in 96-wells opaque plates after 6 or 9 days of culture. CellTiter-Glo® 3D reagent (Promega, France) was added to each well (volume 1:1), and incubated for 30-min at room temperature (Sébert et al., 2018). Triplicate measures were performed for each condition. Luminescence was measured with a Varioskan Flash (Thermo Scientific), and images of each well were taken with Apotome, in order to evaluate the surface occupied by all organoids (Fiji software). Results were normalized with the ATP standard curve and were expressed as ATP concentration per total surface of organoids.

## Statistical Analysis

Data are expressed as mean ± SEM. Analyses were performed using the GraphPad Prism 5 software. Statistical significance was determined by Student's *t*-test, Mann–Whitney, and Wilcoxon tests where appropriate. Significance was accepted for $p < 0.05$.

# RESULTS

## Characterization of Colonoid Cultures

Human colon crypts were purified from fresh tissue samples obtained from colectomies or biopsies harvested in healthy zones of non-IBD patient tissues. Crypts fragments (**Figure 1A** day 0 panel) were cultured in a three-dimensional Matrigel matrix for 10–12 days. CD45 immunostaining was negative in all crypt fragment samples, indicating that no inflammatory cells were present before the organoid culture began (not shown). Within the first 2 days, crypt architecture disappeared to form organoid structures composed of a monolayer of epithelial cells and a central lumen. At days 10–12 of culture, two types of structures were observed: colonospheres, which are composed of a monolayer of epithelial cells with a central lumen and colonoids exhibiting budding crypts (**Figure 1**). The epithelium of colonospheres and colonoids was polarized, with the basolateral side facing outwards (β-catenin labeling) and the apical side oriented toward the lumen (actin labeling) (**Figure 1B**).

At 10–12-days of culture, human colon organoids had positive staining for cytokeratin 20 (**Figure 2A**), chromogranin A (**Figure 2B**), and mucin 2 (**Figure 2C**), demonstrating that differentiated cells: colonocytes, enteroendocrine cells, and goblet cells respectively, were present in cultured colon organoids (**Figure 2**). In 12-days cultured organoids, epithelial turnover was present and characterized by Ki67 staining in proliferative zones and active caspase-3 staining in apoptotic zones (**Figures 2D,E**). Interestingly, apoptosis as revealed by caspase-3 staining was predominantly observed inside the lumen of organoids. Finally, 10–12-days cultured human colon organoids demonstrated functional barrier properties, as observed by cobblestone organization of epithelial cells highlighted by occludin staining (**Figure 2F**) and by the lack of passage of fluorescent dextran 4-kDa added to the medium (**Figure 2G**).

## Organoids From IBD Patients Have a Morphological Phenotype Different From Healthy Controls and Retain Some Inflammatory Features

Next, crypts isolated from tissues of both non-IBD and IBD patients (Crohn's and ulcerative colitis) were cultured. In IBD

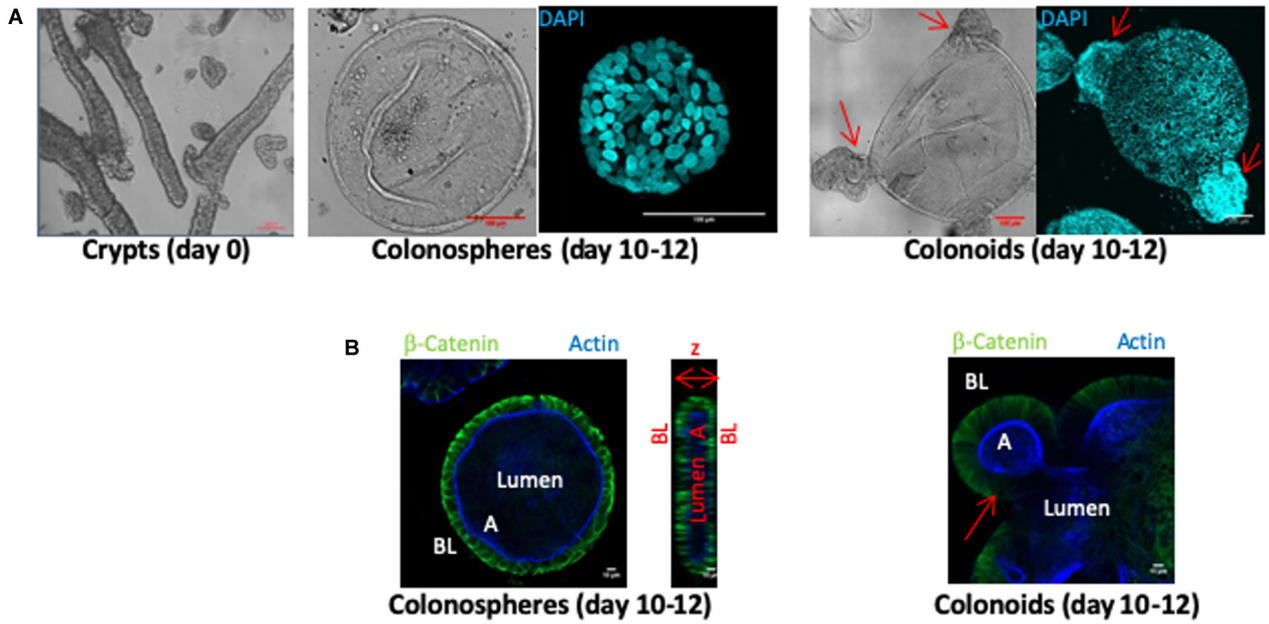


**FIGURE 1 |** *Ex vivo* culture of human colon crypts from control patients. All images were acquired with SP8 confocal microscope at 20X and 63X objectives and analyzed by Image J software. **(A)** Isolated colon crypts at day 0 (left panel), and after 10–12 days of culture (middle and right panels) (scale bar 100 μm). Colored confocal pictures shows DAPI nuclei labeling (cyan) in a 3D reconstruction. Ten to twelve-days cultures showed either perfectly rounded structures called colonospheres (middle panel), or budding structures called colonoids (right panels). **(B)** β-catenin labeling (green) stained basolateral side (BL) and actin labeling (blue) stained apical side **(A)** of organoid epithelial cell monolayers, defining a central lumen, in cross section of colonospheres (left panel) and colonoids (right panel) (scale bar 10 μm). Arrows indicated buds.

patients, tissues for crypt isolation were harvested at the margin of the inflamed zone, where the epithelium was still intact. Five categories of organoid structures were present in these cultures (**Figure 3A**). (1) Organoids with a simple columnar epithelium, (2) Organoids with a simple squamous epithelium, (3) Pseudostratified epithelium (4) Organoids with both squamous and columnar epithelium depending on the zones within the organoid and (5) Transitional structures, where the epithelium showed no organization. The proportion of simple columnar organoids was significantly decreased in IBD patient organoid cultures compared to controls (**Figure 3A**). In contrast, the proportion of pseudostratified structures was significantly increased in IBD patient organoid cultures compared to controls (**Figure 3A**). This last observation is consistent with the fact that in tissues from IBD patients, pseudostratified epithelium is often observed in healing zones (Feakins and British Society of Gastroenterology., 2013).

In addition, organoids from IBD patients had a significant smaller size than control organoids (**Figure 3B**). Similarly, the ratio of the number of colonoids (as opposed to colonospheres) over the total number of structures was significantly lower in organoids from IBD patients, as compared to control organoids (**Figure 3C**). As a matter of fact, mostly immature colonospheres were observed in cultures of organoids from IBD patients. As described above, in organoid cultures from control tissues, the organoids were polarized, with an apical side oriented toward the lumen. We called these organoids "orientated" (see **Figure 3D**). However, we noticed that in organoid cultures from IBD patients, a large number of organoids had an inverse orientation, with the cortical actin staining (representative of the apical side) facing outwards of the structures (photomicrograph in **Figure 3D**). We called these structures "inverted" and we quantified them. The proportion of inverted structures in organoid cultures from IBD patient was significantly higher than in cultures of control organoids, with nearly three times more inverted organoids (**Figure 3D**). Some organoids had debris inside their lumen, most likely due to excessive cell death, while others had clear lumen (**Figure 3E** microphotographs). We quantified the proportion of "clear" lumen structures and found it significantly lower in organoid cultures from IBD patients compared to controls (**Figure 3E**).

The expression of functional molecules characteristic of an inflammatory phenotype was studied in organoid cultures from IBD patients and controls. First, the mRNA expression of inflammatory cytokines such as MCP1 (**Figure 4A**), CXCL-8 (**Figure 4B**), and IP-10 (not shown) was similar in colon organoids from IBD patients or controls. Second, Western blot analysis of tight junction proteins revealed a significant decreased expression of ZO1, Occludin and Claudin-1 for colon organoids from IBD patients compared to controls (**Figures 4C–F**).

Metabolic status of organoids was investigated by dosing ATP in organoid cultures (**Figure 5**). Organoids from IBD patients demonstrated a significantly reduced viability/metabolic activity status, compared to cultured organoids from control non-IBD. This significant difference was observed both at day 6 and day 9 of culture (**Figure 5**).

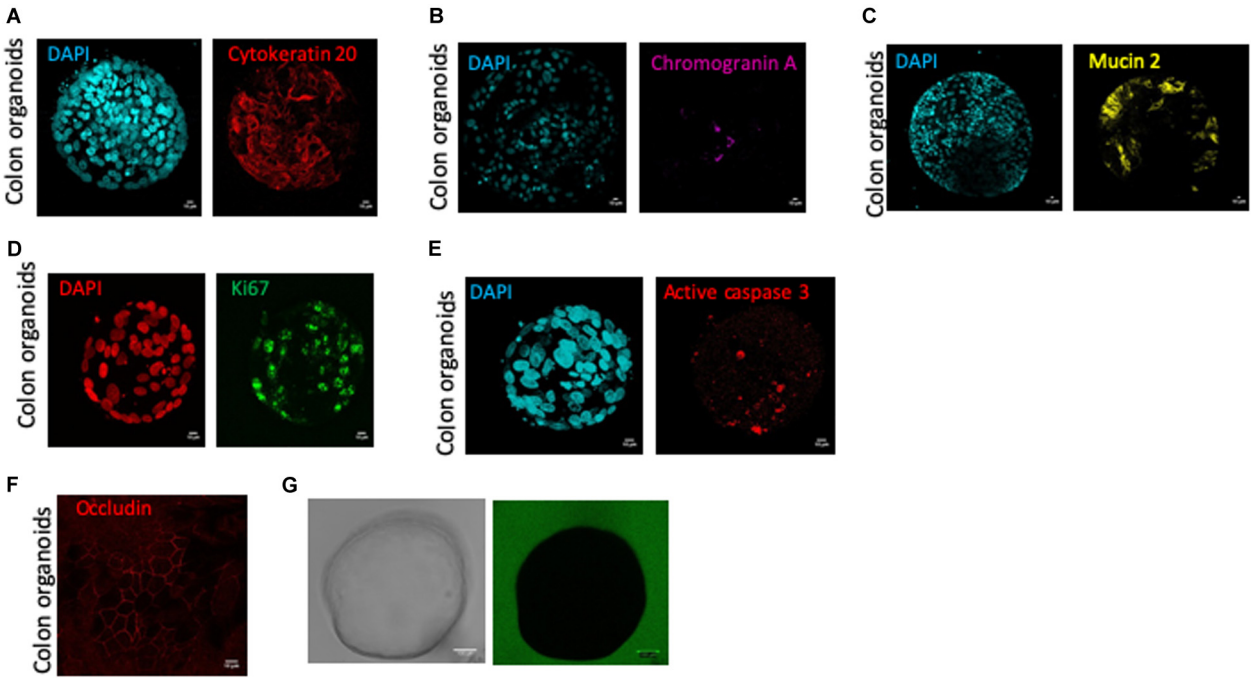


**FIGURE 2 |** Human colon organoid cell type composition and organoid sealing observation. Colonosphere cross sections are shown for each staining, and all images were acquired with SP8 confocal microscope at 63X objectives and analyzed by Image J software, after 10 days of culture of control organoids. Scale bar 10 μm **(A–G)**. **(A)** DAPI nucleus (cyan) and cytokeratin 20 (red) staining for colonocytes, **(B)** DAPI nucleus (cyan) and chromogranin A (pink) staining for enteroendocrine cells, **(C)** DAPI nucleus (cyan), mucin 2 (yellow) staining for goblet cells, **(D)** DAPI nucleus (red) and ki67 (green) staining for proliferative cells, **(E)** DAPI nucleus (cyan) and active caspase 3 (red) staining for apoptotic cells, **(F)** Occludin staining (red) for tight junction labeling **(G)** One colonosphere cultivated in the presence of FITC-4 KDa (green) in bright field (left) or fluorescence (right) microscopy.

Taken together, these data demonstrated that cultured colon organoids from IBD patients have a different phenotype from non-IBD controls. After 10–12 days of culture, IBD epithelial organoids retained some inflammatory features, such as pseudostratification, slow growth, altered polarization and decreased expression of tight junction proteins. Interestingly, these inflammatory features appeared both in Crohn's disease and ulcerative colitis organoid cultures.

## Pro-inflammatory Cocktail Can Induce a Pro-inflammatory Phenotype in Control Organoids

We next investigated whether inflammatory stimulation of control non-IBD organoid cultures could induce an inflammatory phenotype in cultured organoids. We stimulated control colon organoid cultures at day 3 and day 6 of cultures, with an inflammatory cocktail composed of Interleukin-1, Tumor Necrosis Factor-alpha (TNFα) and Interleukin-6 (10 ng/ml each) (**Supplementary Figure S1**). Inflammatory cocktail stimulation did not significantly change the distribution of organoid categories (columnar, squamous, pseudostratified, transitional, etc.) (**Figure 3A**). However, incubation with the inflammatory cocktail significantly reduced the size of organoids in cultures (**Figure 3B**), the proportion of colonoids (**Figure 3C**) and increased the number of "inverted" organoid structures (**Figure 3D**), while having no effect on the proportion of "clear lumen" structures (**Figure 3E**). Incubation with the inflammatory cocktail was also able to induce a significant mRNA overexpression by colon organoids of the two inflammatory chemokines MCP-1 (**Figure 4A**) and CXCL-8 (**Figure 4B**), but did not change the expression of IP-10 (not shown). Inflammatory stimulation of control non-IBD organoid cultures significantly decreased the expression of tight junction proteins ZO1, and Occludin, but not Claudin-1, compared to control (unstimulated) condition (**Figures 4C–F**). Finally, cell viability as observed by measurement of ATP production was also significantly reduced in control organoids incubated with the inflammatory cocktail, but this reduction was observed right after the stimulation (day 6), and did not last at day 9 (**Figure 5**).

## Cell Composition Is Identical Between Controls and Inflamed Organoids

We wanted to determine whether the inflammatory phenotype observed for IBD organoids and for inflammatory cocktail-stimulated organoids could be explained by different organoid cell composition compared to controls. We therefore investigated the mRNA expression of proliferation genes (cyclin D1 and Ki67), the expression of intestinal stem cell markers (LGR5 and Ephrin B2), and the expression of differentiation markers (KRT20, Chromogranin A and MUC2). Cyclin D1 and Ki67, two proliferation markers were similarly expressed

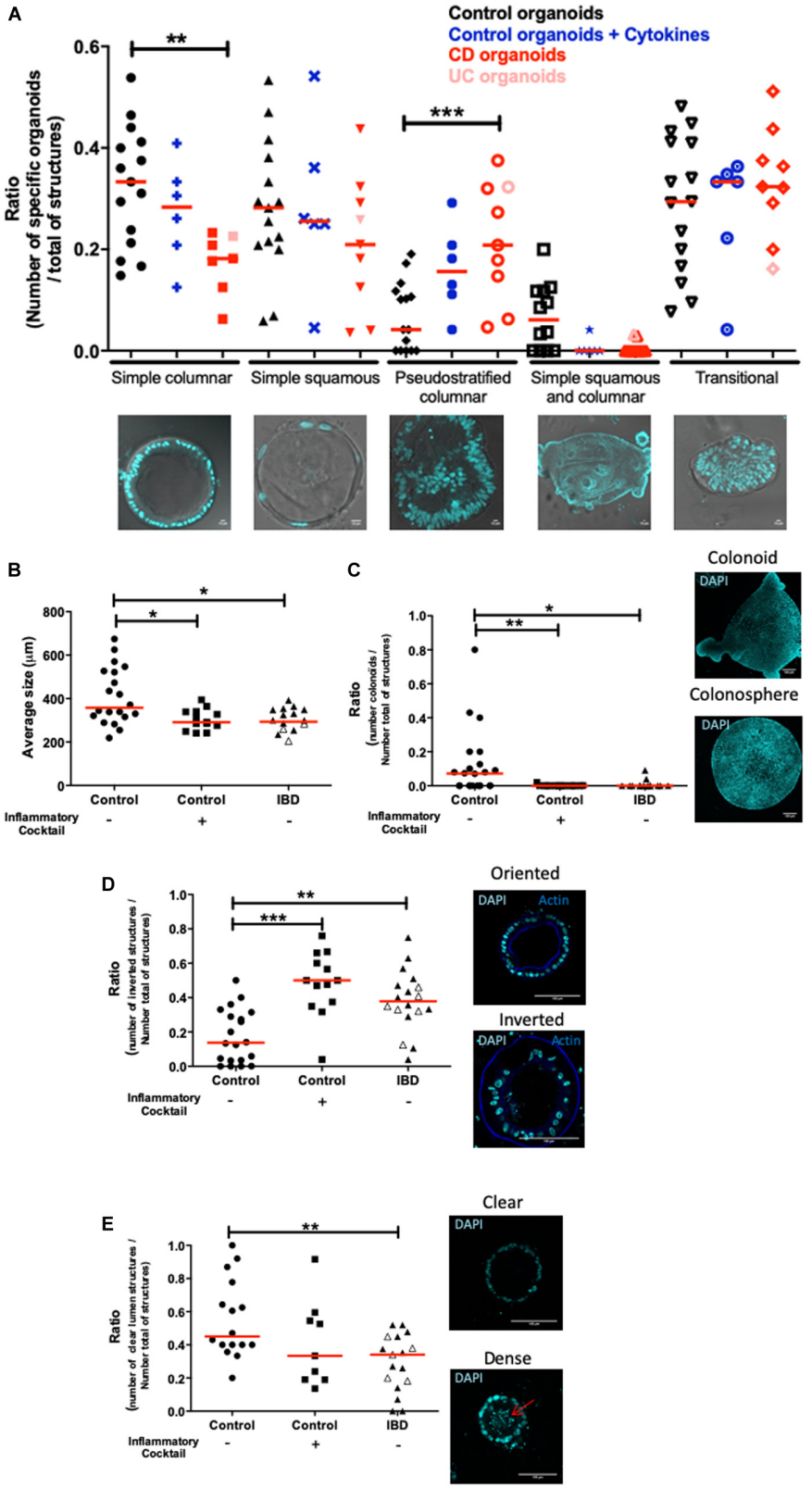


**FIGURE 3 |** Morphological differences between control and inflamed colon organoids. All images were acquired with SP8 confocal microscope at 20X and 63X objectives and analyzed by Image J software. Red lines on graphs represented medians. *P*-value < 0.05 for *, <0.01 for **, and <0.005 for *** (One-way Anova,

*(Continued)*

**FIGURE 3 |** Continued
Kruskal Wallis, and Dunns). **(A)** Morphological classification of cultured organoids in subgroups: simple columnar, simple squamous, pseudostratified columnar, simple squamous and columnar, and transitional. Control organoids derived from non-IBD patients were represented by black signs, blue signs represented control organoids treated with an inflammatory cocktail (IL-1β, TNF-α, and IL-6, at 10 ng/ml) from day 3 to day 10. Organoids derived from IBD patient tissues were represented in red: light red for Ulcerative Colitis, dark red for Crohn's disease. An average of 20 organoids per patient was imaged and analyzed. Medians were represented by red lines. *P*-value $< 0.05$ (One way anova, Kruskal–Wallis, and Dunns). For **(B–E)**, control organoids cultured from non-IBD patient tissues were represented by circles, square signs represented control organoids treated with an inflammatory cocktail (IL-1β, TNF-α, and IL-6, at 10 ng/ml) from day 3 to day 10, and organoids derived from IBD patient tissues were represented by triangles: open symbols for Ulcerative Colitis and closed symbols for Crohn's disease. **(B)** Average size of organoids (μm). **(C)** The number of colonoids (budding structures) per total number of organoids (colonospheres plus colonoids each represented on right panel). **(D)** DAPI nucleus (cyan) and actin (blue) labeling were used in cross sections of organoid cultures (right panels) to visualize oriented (actin labeling toward the lumen) (right upper panel) and inverted organoids (actin labeling outwards the lumen) (right bottom panel) (scale bar 100 μm). On the left panel, the number of inverted organoids per total number of organoids (oriented plus inverted) was represented. **(E)** Nuclei labeling in cross sections of organoids to visualize clear (right upper panel) and dense (right bottom panel) lumen, the arrow showed the presence of debris in the lumen. The number of organoids with "clear" empty lumen per total number of organoids (empty, dense and without lumen) was represented.

in control organoid cultures and in inflamed organoids (both inflammatory cocktail-stimulated and IBD organoids (**Figures 6A,B**). Intestinal stem cell markers LGR5 and Ephrin B2 were also similarly expressed in control organoids and inflamed organoids (**Figures 6C,D**). Finally, the differentiation markers KRT20, Chromogranin A and MUC2 were also similarly expressed in the three types of organoid cultures: controls non-IBD, controls non-IBD plus inflammatory cocktail, or IBD organoids (**Figures 6E–G**).

Taken together, these results suggested that inflamed organoids have similar proliferation, stem cell and differentiation capacities compared to controls. Differences in biological activities of inflamed organoids rather than their cell composition might explain their morphological and functional differences.

## Effects of Clinically Used Treatments on the Inflammatory Phenotype of Human Colon Organoids From IBD Patients

Human colon organoids from IBD patients were cultured in the presence or not of treatments currently used in the clinics: 5-ASA, methyl-prednisolone and anti-TNF (see **Supplementary Figure S2** for protocol). Four morphological parameters significantly altered in IBD organoid cultures compared to controls were followed: (1) the size of organoids (**Figure 7A**), (2) the proportion of colonoids (budding structures) (**Figure 7B**), (3) the proportion of structures with empty (clear) lumen (**Figure 7C**) and (4) the polarity of organoids (**Figure 7D**). The purpose of these experiments was to investigate whether clinically used treatments can modify the inflammatory phenotype of individual patient organoid cultures. Overall, considering the medians (shown by the line in each groups of **Figure 7**), all treatments had positive effects on some of the observed parameter, in a dose-dependent manner. However, not all treatments were efficient on all parameters.

Considering the lower size of IBD organoid structures compared to non-inflamed organoids, anti-TNF and 5-ASA, but not methyl prednisolone treatments had a significant effect, increasing the size of organoids compared to the no-treatment condition (**Figure 7A**). Both anti-TNF and 5-ASA treatments were able to bring the size of some IBD organoid cultures toward the level of controls (doted line) (**Figure 7A**). Only the highest dose of anti-TNF treatment and the two doses of 5-ASA treatment significantly increased the size of organoids compared to untreated cultures (**Figure 7A**). The positive effects of 5-ASA and anti-TNF treatments on the size of IBD organoids were observed both on Crohn's and ulcerative colitis organoid cultures (**Figure 7A**).

IBD organoid cultures showed a very low proportion of budding structures compared to controls. This is characterized by low counts of colonoids (**Figures 3C**, **7B**). All three treatments (anti-TNF, methyl prednisolone and 5-ASA) trended to bring the proportion of colonoids to control levels (doted line), but only the highest dose of prednisolone treatment was able to increase significantly the proportion of budding structures, compared to the untreated culture condition (**Figure 7B**). Positive effects of treatments on colonoid counts were observed both in Crohn's disease and ulcerative colitis organoid cultures (**Figure 7B**).

The number of empty (clear) lumen structures in organoid cultures was significantly decreased in IBD organoids compared to non-inflamed organoids (**Figure 3E**). This decreased proportion of organoids with clear lumen was unchanged in cultures containing anti-TNF or 5-ASA, but was significantly increased by the two doses of prednisolone added to the culture media (**Figure 7C**). For this parameter as well, the positive effect of prednisolone treatment was observed similarly in Crohn's disease and ulcerative colitis organoid cultures (**Figure 7C**).

Finally, it was observed that organoids from IBD patients had an increased inverted polarization compared to non-inflamed controls (**Figures 3D**, **7D**). Treatment with anti-TNF did not reduce significantly the proportion of inverted organoids compared to untreated conditions, but the median of highest dose of anti-TNF treatment was close to the uninflamed (non-IBD) level (doted line). The highest dose of ethyl prednisolone treatment and the two doses of 5-ASA treatments significantly reduced the number of inverted organoids compared to untreated culture (**Figure 7D**). The positive effect of treatment was observed similarly in Crohn's disease and ulcerative colitis organoid cultures (**Figure 7D**).

# DISCUSSION

The present study brings a number of new and important information on organoid cultures. It characterizes both morphological, and gene expression changes in primary organoid cultures from IBD patients, compared to primary organoid cultures from the colon of non-IBD individuals. Importantly,

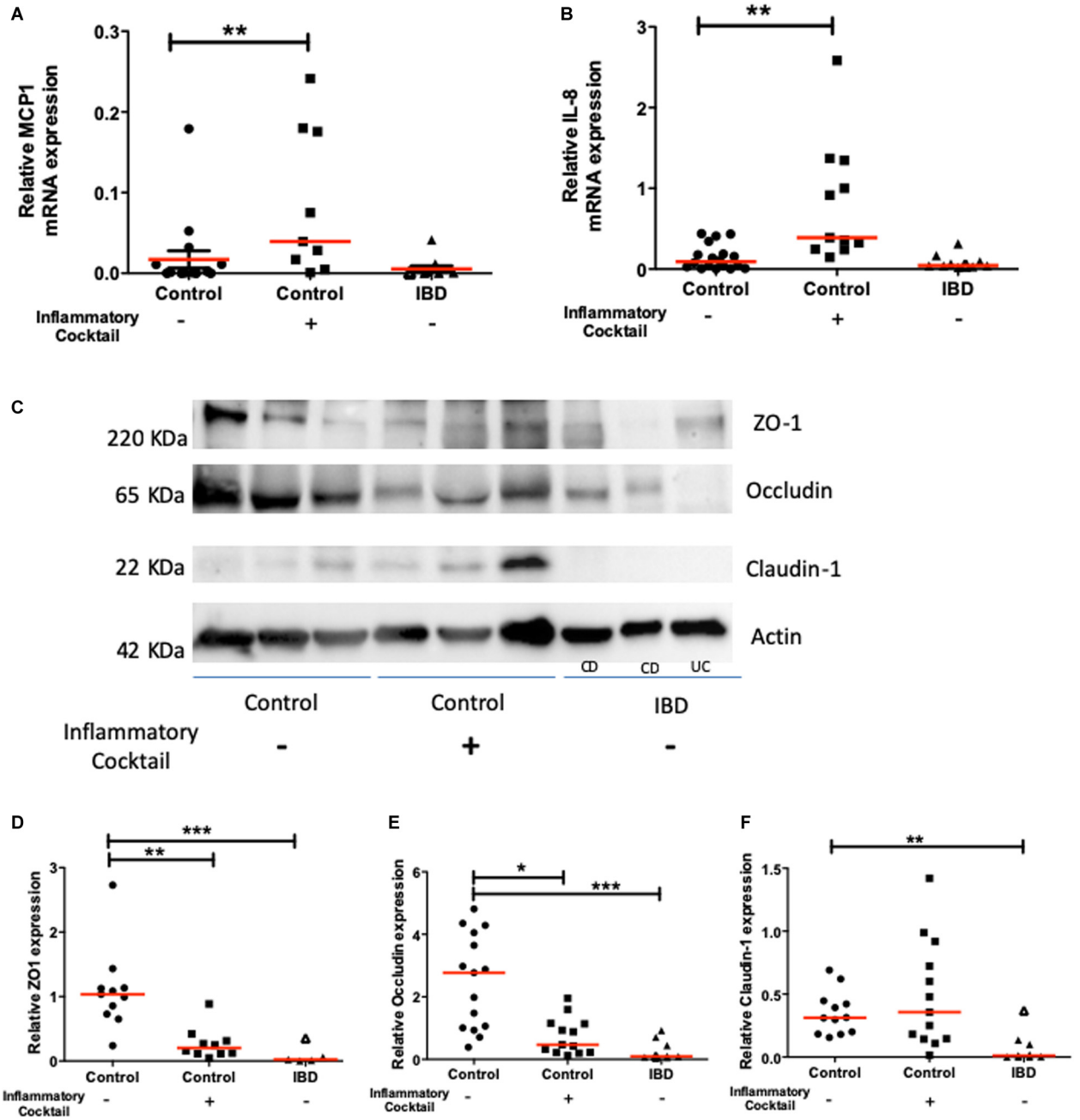


**FIGURE 4 |** Chemokine (MCP-1 and CXCL-8) and tight junction molecule (ZO-1, Occludin and Claudin-1) mRNA and protein expression respectively, in controls (circles), controls exposed to an inflammatory cocktail (IL-1β, TNF-α, and IL-6, at 10 ng/ml) from day 3 to day 10 (squares) and organoids from IBD patients (triangles): ulcerative colitis (UC) patients (opened triangles) and Crohn's disease (CD) patients (closed triangles). mRNA or proteins were extracted from organoids after 10 days of culture and data were normalized by GAPDH or actin expression respectively. Red lines represented medians. *P*-value $< 0.05$ for *, $<0.01$ for **, and $<0.005$ for ***(One-way Anova, Kruskal Wallis, and Dunns). **(A)** MCP1 mRNA expression. **(B)** IL-8 mRNA expression. **(C)** Representative Western-blot of the ZO-1, Occludin, Claudin-1 and Actin protein expression in tissues from 3 representative samples from controls (non-IBD), and IBD (Crohn's disease: CD, and ulcerative colitis: UC) patients. **(D)** Quantification of ZO-1 relative protein expression. **(E)** Quantification of Occludin relative protein expression. **(F)** Quantification of Claudin relative protein expression.

these data demonstrated that even after 10–12 days of culture, and in absence of any further immunological stimulation (no CD45 staining in isolated crypts), crypt stem cells isolated from IBD tissues regrow abnormal epithelium. This could be due to the long-term impregnation to inflammatory mediators of crypts isolated from IBD patient tissues. To grow organoids, tissues were harvested at the margin of inflamed tissues in non-inflamed zones. This choice was made in order to be certain that the

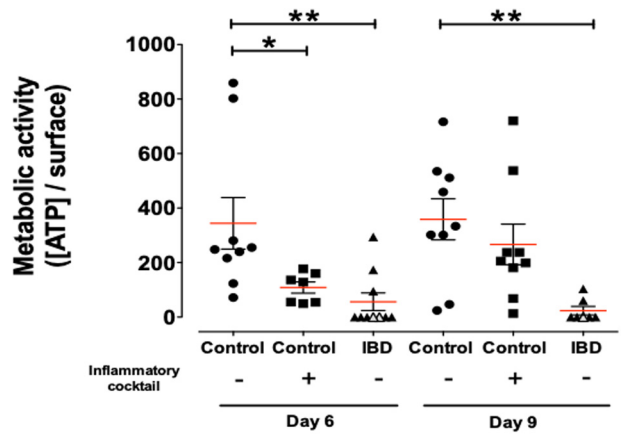


**FIGURE 5 |** Concentration of ATP release per surface of total organoids as a measure of metabolic activity/cell survival in organoids cultured for 6 and 9-days and harvested from control (non-IBD) patients (circles), control (non-IBD) organoids exposed to inflammatory cocktail (IL-1β, TNF-α, and IL-6, at 10 ng/ml) from day 3 to day 10 (squares) and organoids from IBD patients (triangles): ulcerative colitis (UC) patients (opened triangles) and Crohn's disease (CD) patients (closed triangles). Red lines represented medians. *P*-value $< 0.05$ for * and $<0.01$ for ** (One-way Anova, Kruskal Wallis, and Dunns).

isolated epithelium would be intact of ulcers and that sufficient and reproducible crypt numbers would be isolated. Therefore, isolated crypts might have been exposed chronically to adjacent inflammatory mediators. Such chronic exposure of intestinal epithelium to inflammatory mediators could induce epigenetic changes that are conserved in organoid cultures even in the absence of inflammatory mediators. Indeed, epigenetic variations have been identified in tissues from IBD patients (Kellermayer, 2012; McGovern et al., 2015). Interestingly, another recent study performed with organoid cultures from ulcerative colitis patients also suggests an imprinting of IBD stem cells (Dotti et al., 2017). In that study, authors identified a set of genes that exhibited sustained differential expression in cultured organoids from ulcerative colitis patients. These genes include LYZ, CLDN18, hZG16, hCLCA1, MUC12, and AQP8, which are involved in antimicrobial, barrier, mucus production or water transport functions. Another study recently confirmed that differential expression of mucus production gene (MUC2) is observed in organoids from IBD patients, and in particular from Crohn's disease patients, compared to controls (Noben et al., 2017). Taken together, such data implies that long-lasting epithelial defects are present in IBD patient epithelium, and may be acquired during the course of the disease thereby contributing to the perpetuation of the pathology. Another hypothesis to explain the IBD phenotype in organoid cultures, is that some genetic mutations that have been associated with IBD are involved in architectural organization of the colonic epithelium, conferring a permanent epithelial phenotype. This could be confirmed in future studies that would perform IBD organoid cultures after several cell passages, but it was not the purpose of the present study. Large-scale genome-wide association studies have identified more than 200 loci associated with IBD (Liu et al., 2015). Some of these genes are involved in innate immune response, impaired mechanisms of phagocytosis and autophagy, T cell signaling, but also epithelial barrier function (Prager et al., 2015). For instance, genes such as *ECM1, CDH1, LAMB1, ADAM17A, KIND1/FERMT1* or *HNF4α* are known to be involved in barrier function and single nucleotide polymorphism has been associated with IBD for these genes (Kern et al., 2007; Blaydon et al., 2011; Bianco et al., 2015). If such polymorphism is associated with a functional phenotype of impaired barrier function, then this could explain the decreased expression of tight junction proteins we observed in cultured organoids. The *OSMR* gene coding for the Oncostatin M receptor is also associated with IBD and is known to promote epithelial cell proliferation. Here again, such mutation could explain the smaller size of IBD organoids we observed (Beigel et al., 2014; Bianco et al., 2015). Mutations in the type VII collagen gene *COL7A1* are associated with IBD (Freeman et al., 2008; Uhlig, 2013). Considering the major role of type VII collagen in keratinocyte polarity (Dayal et al., 2014), a similar role could be proposed in intestinal epithelial cells, although no studies have yet investigated such role in this cell type. The inverted phenotype that we observed in 70% of IBD organoids could be explained by such mutation. Future studies will be needed to investigate the association of IBD gene polymorphism with specific organoid culture phenotype. However, our present study also highlights the possibility for pharmacological intervention to revert, at least in part, some of the inflammatory phenotype of organoid cultures. This could undermine the importance of genetic defects in IBD-associated epithelial dysfunctions. Altogether, the present model of primary organoid cultures could be considered as a most relevant model to recapitulate *in vitro*, the epithelial defects associated with IBD, and to potentially follow IBD epithelial features.

Our results generated with IBD patient organoid cultures suggested that inflamed organoids have similar proliferation, stem cell and differentiation capacities compared to controls. This was evidenced by the conserved expression of proliferation genes cyclin D1 and Ki67, of intestinal stem cell markers LGR5 and Ephrin B2, and of differentiation markers KRT20, Chromogranin A and MUC2. Similar results were reported by a recent study in which, organoid cultures from ulcerative colitis patients were compared to organoid cultures from non-inflamed controls, showing similar self-renewal capacity and comparable molecular features (Dotti et al., 2016). Importantly, the organoid structures that were followed in the present study expressed the cytokeratin-20 (KRT20) protein (**Figure 2A**), providing proofs of success in achieving human primary keratinocyte cultures as previously demonstrated (Torreggiani et al., 2019), and confirming the epithelial origin of those cells.

A second important point highlighted by the present study is that the phenotype of inflamed organoid can be recapitulated *in vitro*, by exposing control organoids to an inflammatory cocktail. We showed that a cytokine cocktail induced in control organoid cultures, the same size reduction, inverted phenotype, decreased proportion of colonoids, ZO-1 and Occludin decreased expression, decreased metabolic activity as it was observed in IBD patient organoid cultures. This suggests that *in vivo* as

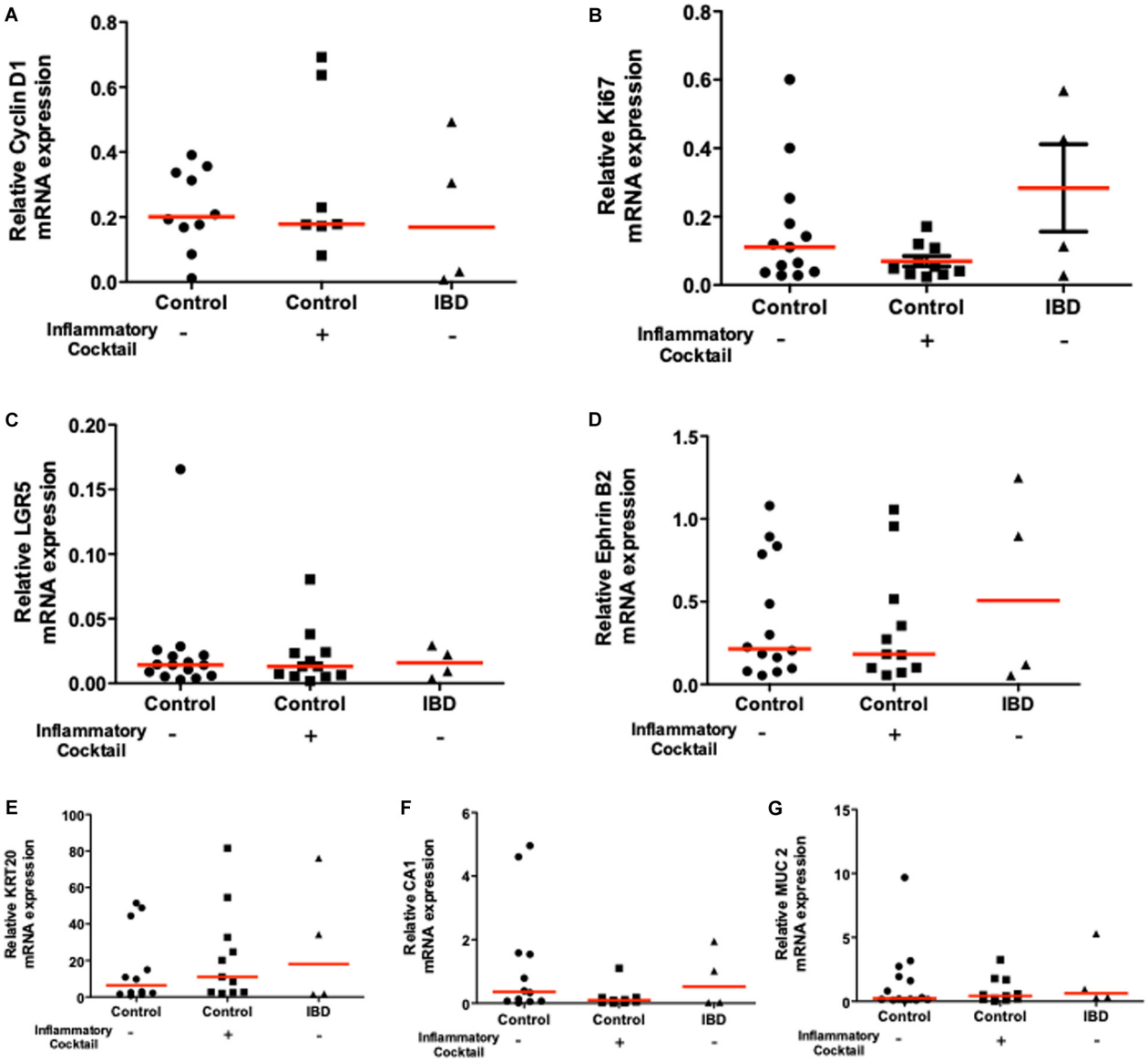


**FIGURE 6 |** mRNA expression of proliferation markers [Cyclin D1 and Ki67 **(A)** and **(B)** respectively], immaturity markers [LGR5 and Ephrin B2 **(C)** and **(D)** respectively], and differentiation markers [Carbonic Anhydrase-1: CA1, Cytokeratine-20: KRT20 **(E)**, Carbonic Anhydrase-1: CA1 **(F)**, and Mucin 2: MUC2 **(G)**] in 10 days cultured organoids from controls (circles), control organoids exposed to inflammatory cocktail (IL-1β, TNF-α, and IL-6, at 10 ng/ml) from day 3 to day 10 of cultures (squares) and organoids from IBD patients (triangles): ulcerative colitis (UC) patients (opened triangles) and Crohn's disease (CD) patients (closed triangles). Data were normalized to GAPDH. Red lines in graphs represented medians. No statistical significant difference was observed.

well, these parameters might be under the long-term control of inflammatory mediators. However, some other features of IBD organoids were not recapitulated in control organoids exposed to the inflammatory cocktail. This involves the increased presence of apoptotic debris in the lumen of IBD organoids, and their decreased expression of Claudin-1. This means that for these parameters, short-term exposure to inflammatory mediators might not be sufficient to induce this particular phenotype. Rather, genomic imprinting or mutation might be involved to induce these epithelial phenotypes. Indeed, a frequent occurrence of private variants of the X-linked inhibitor of apoptosis protein has been associated with Crohn's disease (Zeissig et al., 2015). Importantly, some inflammatory features such as chemokine (CXCL-8 and MCP-1) release are induced by transient exposure to inflammatory cocktail, but are not maintained in IBD organoid cultures (**Figures 4A,B**). This could mean that in the context of IBD, the regulation of those genes does not depend on long-term imprinting or gene mutation, but is a consequence of acute inflammatory response. Therefore, this model of stimulated organoid culture appears as a unique new tool that will help defining intestinal epithelium gene regulation by individual or combined mediators. In addition, it will clearly serve to screen

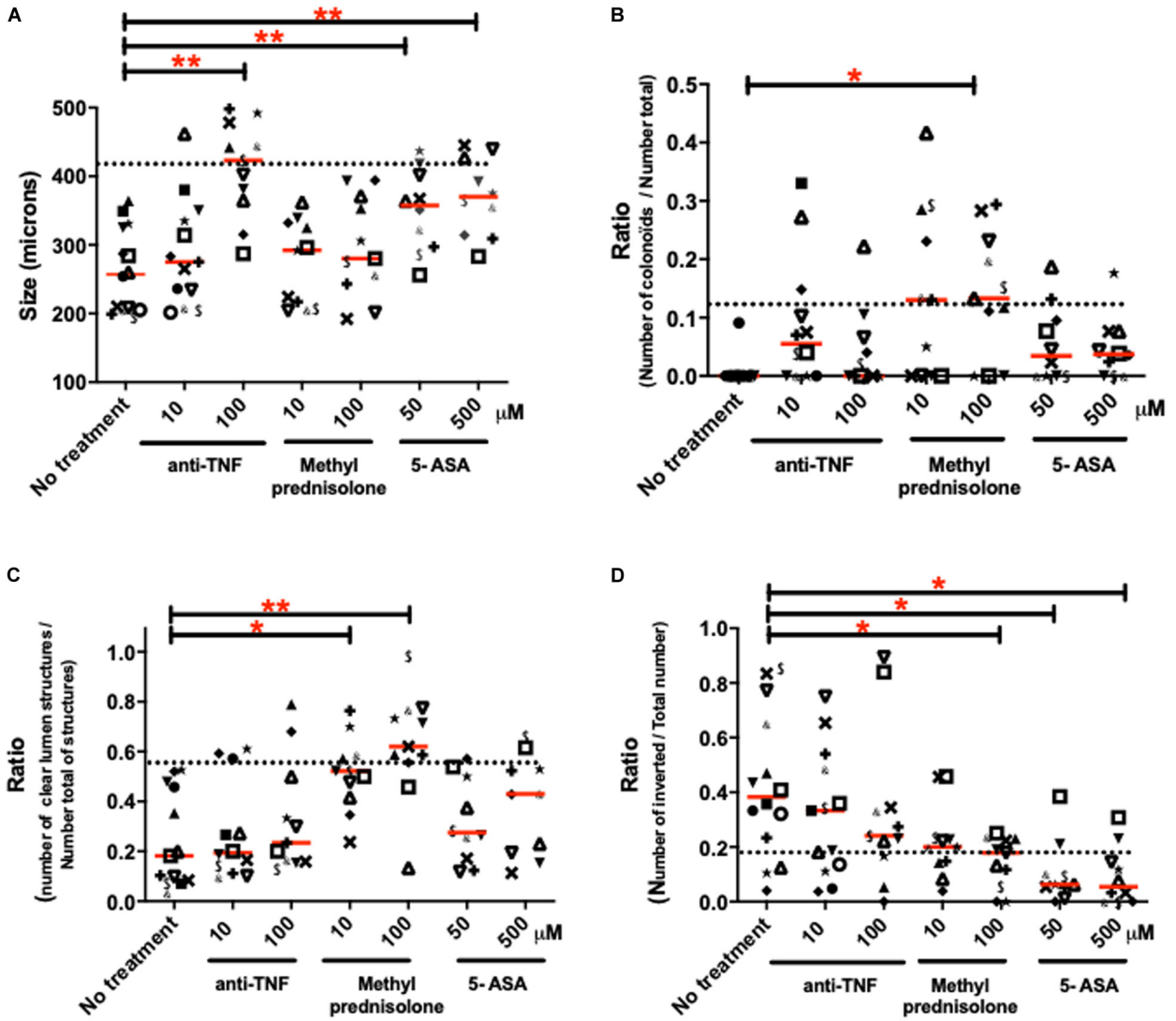


**FIGURE 7 |** Effects of different treatments used in clinics on the morphological characteristics of IBD organoids. IBD organoids were derived from IBD patients. Crohn's disease (CD) patient organoids were represented with closed symbols and keyboard symbols, while ulcerative colitis (UC) patient organoids were represented by opened symbols. One patient was represented by the same symbol in all figure panels. IBD organoids were treated from day 0 to day 10 with anti-TNF (Remicade) at 1, 10, and 100 µM, or methyl-prednisolone (Solupred) at 10 and 100 µM, or 5-ASA (Pentasa) at 50 and 500 µM. Where possible, an average of 20 organoids was studied per condition per patient. Medians were represented by red lines on graphs. Doted lines represented for each criteria, the median measured for this criteria in control (non-IBD), non-treated organoid cultures. Statistics were not performed on these graphs due to the low n number in some groups. **(A)** Average size of organoids (µm) **(B)** Number of colonoids (budding structures) per total number of organoids (colonospheres plus colonoïds) **(C)** Number of organoids with empty lumen per total number of organoids (empty, dense and without lumen). **(D)** Number of inverted organoids per total number of organoids (oriented plus inverted).

the efficacy of new therapeutic approaches targeting the effects of inflammatory mediators on epithelial repair.

A third major point raised by the present study is the demonstration that IBD organoid cultures could be used to test the effects of therapeutic options on epithelial biology. We demonstrated that overall, all treatments (anti-TNF, corticosteroids and 5-ASA) had positive impact on the observed parameters in a dose-dependent manner. This is important as it means that such treatments could participate at least to some extent, to epithelial regeneration and repair. The fact that these treatments positively impacted inflamed IBD organoid cultures seems surprising as these treatments were originally developed to target the immune response. It was interesting to note that not all treatments were efficient on the same parameters, and also that some treatments could be more efficient than others in some patients. This first result paves the way to a more extensive study, including large numbers of patients that could aim at defining whether the response of culture organoids could be predictive of the patient's response to treatment. Similarly, the effects of new therapeutic options focusing on epithelial repair could therefore be tested directly in tissues from patients, and their efficiency could be compared to that of clinically used treatments.

## CONCLUSION

In conclusion, the extensive characterization of colon organoid cultures from IBD patients compared to non-IBD controls that we have performed in the present study brings important new knowledge. First, it demonstrates the possible use of such model to further characterize epithelial dysfunctions associated with IBD. Second, we showed that this model constitutes a reliable human preclinical model to investigate new therapeutic approaches targeting epithelial repair.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by either the MICILIP research protocol (National Ethics Committee #NCT01990716), or the biocollection for human samples that included colonic resections (CODECOH national agreement: Colic collection: DC2015-2443). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

EA, MQ, and MS have performed the experiments, analyzed the data, and contributed to the manuscript drafting. DB, SK, GP, J-PD, SA, CD, LA, and EM have provided technical and/or material support, and contributed to manuscript editing. SC, PL, CD, CR-S, AF, EM, and NV have analyzed and critically reviewed the data, obtained funding, and contributed to the manuscript editing. EA and NV performed the study design, concept, supervision, and manuscript writing.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00363/full#supplementary-material

**FIGURE S1 |** Protocol for the culture of organoids from IBD and non-IBD patients. Inflamed organoids were obtained from colon biopsies or resection of IBD patients. But inflamed organoids could be obtained from control patients. When control organoids were formed (at day 3) a chronic inflammatory stress (IL-1β, TNF-α, and IL-6, at 10 ng/ml) was applied at each change of medium. After 10 days of culture morphological and functional study was performed as well as expression of pro-inflammatory mediators or expression of tight junction proteins.

**FIGURE S2 |** Inflamed organoids obtained from colon biopsies or resection of IBD patients were subjected or not to a clinical treatment [anti-TNF (10 or 100 μM); methyl-prednisolone (10 or 100 μM); 5-ASA (50 or 500 μM)]. Treatments were added at day 0 and at each medium change. After 10 days of culture morphological study was performed.

**TABLE S1 |** Characteristics and outcomes of control and IBD patients.

**TABLE S2 |** Antibodies used for immunofluorescence labeling (IF) and western blot (WB) studies.

**TABLE S3 |** Primers used for quantitative RT-PCR studies (from 5′ to 3′).

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MINI REVIEW
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# Fate of Adipose Progenitor Cells in Obesity-Related Chronic Inflammation

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Adipose progenitor cells, or preadipocytes, constitute a small population of immature cells within the adipose tissue. They are a heterogeneous group of cells, in which different subtypes have a varying degree of commitment toward diverse cell fates, contributing to white and beige adipogenesis, fibrosis or maintenance of an immature cell phenotype with proliferation capacity. Mature adipocytes as well as cells of the immune system residing in the adipose tissue can modulate the function and differentiation potential of preadipocytes in a contact- and/or paracrine-dependent manner. In the course of obesity, the accumulation of immune cells within the adipose tissue contributes to the development of a pro-inflammatory microenvironment in the tissue. Under such circumstances, the crosstalk between preadipocytes and immune or parenchymal cells of the adipose tissue may critically regulate the differentiation of preadipocytes into white adipocytes, beige adipocytes, or myofibroblasts, thereby influencing adipose tissue expansion and adipose tissue dysfunction, including downregulation of beige adipogenesis and development of fibrosis. The present review will outline the current knowledge about factors shaping cell fate decisions of adipose progenitor cells in the context of obesity-related inflammation.

**Keywords: preadipocyte, adipose progenitor, inflammation, adipogenesis, beiging, obesity, fibrosis**

## INTRODUCTION

In the past two decades, adipose tissue (AT) has been extensively studied in both rodents and humans, especially regarding mechanisms involved in obesity-related metabolic dysregulation. There are two morphologically and functionally distinct types of AT: white AT (WAT) and brown AT (BAT). WAT is predominantly responsible for energy storage in the form of triglycerides and secretes hormonal regulators, namely adipokines, such as leptin and adiponectin, which can regulate whole-body's metabolic homeostasis. BAT, in contrast, has a non-shivering heat production capacity, due to expression of uncoupling protein-1 (UCP-1) (Kershaw and Flier, 2004; Peirce et al., 2014; Rosen and Spiegelman, 2014). Beige, or brite adipocytes represent a type of adipocytes that morphologically resemble white rather than brown fat cells and reside within WAT, but express

UCP-1 and exert brown-like properties (Rosen and Spiegelman, 2014; Alexaki and Chavakis, 2016; Cinti and Giordano, 2020).

In addition to adipocytes, the stromal vascular cell fraction (SVF) of the AT contains further cell types, such as endothelial cells, various immune cells and adipocyte progenitors (preadipocytes or adipose progenitors cells, APs) (Ailhaud et al., 1992; Church et al., 2014). APs reside in perivascular regions of the AT, and can differentiate into mature adipocytes (Tang et al., 2008; Rosen and Spiegelman, 2014; Vishvanath et al., 2016). A recent study defined a developmental hierarchy of APs starting from Dipeptidyl peptidase-4-postive ($DPP4^+$) cells that give rise to committed $ICAM1^+$ and $CD142^+$ preadipocytes capable of adipogenic differentiation (Merrick et al., 2019). Commonly, preadipocytes are described as $CD45^-$, $CD31^-$, $Lin^-$, $CD29^+$, and $Sca\text{-}1^+$ cells (Rodeheffer et al., 2008; Tang et al., 2008; Berry and Rodeheffer, 2013). Platelet-derived growth factor-receptor β positive ($PDGFR\beta^+$) APs were described as predisposed to a white adipogenic (Gao et al., 2018), while $PDGFR\alpha^+$ progenitors to a beige adipogenic or a fibrogenic phenotype (Lee et al., 2012; Marcelin et al., 2017). A recent study provides an insight in the heterogeneity of APs in the mouse VAT based on scRNAseq analysis, demonstrating the existence of two distinct populations of AT-derived stem cells and three populations of preadipocytes (Cho et al., 2019).

During obesity, the expansion of the AT is driven by two processes: hypertrophy (increased adipocyte size) and hyperplasia (increased adipocyte numbers). Hyperplastic growth is considered more metabolically favorable (Shao et al., 2018; Vishvanath and Gupta, 2019), while AT hypertrophy is associated with the development of hypoxia and release of pro-inflammatory cytokines and chemokines by the adipocytes, leading to the recruitment of immune cells and the formation of a pro-inflammatory microenvironment in the AT (Arner et al., 2010; Chatzigeorgiou et al., 2014; García-Martín et al., 2015; Choe et al., 2016; Chung et al., 2018; Michailidou, 2019). Macrophages play a crucial role in the development of AT inflammation. They shift from an anti-inflammatory M2-like (M2-MΦ) to a pro-inflammatory M1-like (M1-MΦ) phenotype and form "crown-like" structures surrounding dead adipocytes (Cinti et al., 2005; Lumeng et al., 2007a; Chawla et al., 2011; Cinti and Giordano, 2020). M1-MΦ secrete pro-inflammatory mediators such as tumor necrosis factor (TNF), interleukin 1 beta (IL-1β), and IL-6 (Chawla et al., 2011; Chmelar et al., 2013; Shapouri-Moghaddam et al., 2018). AT inflammation is also featured by the accumulation within the obese AT of several innate and adaptive immune cells including natural killer cells, neutrophils, $CD8^+$ cytotoxic- and type l T helper-lymphocytes, which also produce pro-inflammatory factors (Cildir et al., 2013; Chung et al., 2018; Kane and Lynch, 2019).

Studies suggest that various stromal cells, including AT fibroblasts and endothelial cells, create an adipose niche for APs (Jiang et al., 2017; Zhang et al., 2018), while resident and infiltrating immune cells also contribute to the niche formation, especially in the context of obesity-related inflammation (Nawaz et al., 2017). Considering that preadipocytes are plastic cells that respond to both niche and systemic signals (Jeffery et al., 2016), the pro-inflammatory microenvironment of the obese AT might play a critical role in determining the fate of APs. The present review focuses on the current knowledge about AP fate driven by both intracellular and extracellular factors in the context of obesity-related chronic inflammation.

# ADIPOGENESIS

## White Adipogenesis and AT Expansion

The hyperplastic growth of the AT occurs through the process of adipogenesis, namely the highly dynamic transformation of immature fibroblast-like precursor cells into mature lipid-loaded adipocytes (Rosen and MacDougald, 2006). Peroxisome proliferator-activated receptor-γ (PPARγ) and members of the CCAAT-enhancer-binding proteins (C/EBP) family are master-regulators of this process. However, a considerable number of signaling pathways, including Wnt, Notch, Hedgehog, MAPK, and pro- and anti-adipogenic mediators (KLF and GATA transcription factors, cell cycle proteins) regulate the adipogenic conversion (Farmer, 2006; Rosen and MacDougald, 2006; Ghaben and Scherer, 2019). Of note, APs from different AT depots display qualitative and quantitative heterogeneity. For instance, in both mice and humans, APs from subcutaneous fat depots have shown *in vitro* higher growth rates and adipogenic potential compared to those from visceral AT (Permana et al., 2004; Tchkonia et al., 2005; Macotela et al., 2012). Nevertheless, *in vivo* evidence from adult C57BL/6 mice, supports that following HFD-feeding, visceral AT expands through both adipocyte hypertrophy and hyperplasia, while subcutaneous AT almost exclusively via cellular hypertrophy (Vishvanath and Gupta, 2019). Several markers, such as CD36 and Zfp423 have been suggested to define preadipocyte populations with pronounced adipogenic capacity (Gupta et al., 2012; Gao et al., 2017).

A crosstalk between APs and immune cells could orchestrate adipogenesis in both lean and obese state (Bing, 2015; Chung et al., 2017). For instance, macrophages constitute 5–10% of the SVF in lean mice and their numbers increase in obesity (Weisberg et al., 2003; Weinstock et al., 2019; Daemen and Schilling, 2020). Both proliferation of tissue-resident macrophages and monocyte infiltration contribute to the expansion of this population in the obese AT (Weisberg et al., 2003; Amano et al., 2014; Zheng et al., 2016). Several studies have shown that pro-inflammatory macrophage-conditioned medium inhibits AP differentiation and leads to insulin resistance in mouse and human preadipocytes *in vitro* (Constant et al., 2006; Lacasa et al., 2007; Lumeng et al., 2007b). The potential contribution of individual components of the macrophage secretome to this process has gained strong attention. TNF is a major factor contributing to the anti-adipogenic effect of macrophages, possibly via an epigenetic reprogramming-dependent mechanism (Isakson et al., 2009; Andersen et al., 2019). IL-6 exerts an inhibitory effect on IRS-1, Glut4, and PPARγ, thereby contributing to the decreased adipogenic capacity of human preadipocytes (Rotter et al., 2003; Gustafson and Smith, 2006; Almuraikhy et al., 2016). IL-1β from pro-inflammatory macrophages inhibits insulin sensitivity in both

APs and mature adipocytes of mice and humans (Lagathu et al., 2006; Gao et al., 2014). Moreover, the aforementioned cytokines participate in a positive-feedback loop induction of pro-inflammatory gene expression (IL-6, MCP-1, IL-1β, TNF, and IL-8) in APs (Gustafson and Smith, 2006; Isakson et al., 2009; Gao et al., 2014). Priming of human preadipocytes toward a pro-inflammatory phenotype is also mediated by elevated extracellular glucose levels, which accompany obesity and insulin resistance (Rønningen et al., 2015). Pro-inflammatory priming of murine preadipocytes is also mediated by leptin, the levels of which are elevated during obesity (Ouchi et al., 2011; Palhinha et al., 2019). Contrastingly, adiponectin, which is decreased in the obese AT, promotes the differentiation of 3T3-L1 preadipocytes and insulin sensitivity (Fu et al., 2005).

Besides macrophages that contribute to obese AT remodeling, other immune cells within the AT can affect preadipocyte differentiation as well. For instance, murine monocyte-derived dendritic cells (DCs), which accumulate in the obese AT, display anti-adipogenic properties *in vitro* (Pamir et al., 2015; Macdougall and Longhi, 2019). The inhibition of AP differentiation during obesity and AT inflammation promotes the hypertrophic, rather than hyperplastic expansion of the AT, due to storage of the supplied energy in the form of lipids by mature adipocytes and not by differentiating APs (Hammarstedt et al., 2018; Gustafson et al., 2019).

Apart from immune cells, the interaction of APs with the highly abundant endothelial cells of the AT may shape the adipogenic potential of preadipocytes, likely via vasculature-derived factors (Cao, 2007). Indeed, vascular endothelial growth factor (VEGF) is considered a key factor coupling adipo- and angiogenesis in the mouse AT and may favor adipogenesis within adipogenic/angiogenic cell clusters (Nishimura et al., 2007; Sun et al., 2012; Breier et al., 2017). Along this line, a recent study suggested a possible role of endothelial cells in the regulation of fatty acid transport and PPARγ activation in human preadipocytes, due to secretion of PPARγ ligands by endothelial cells (Gogg et al., 2019). Moreover, a spatial and functional overlap of $CD34^+$ APs with pericytes has been described, which plays a role in the stabilization of the AT vasculature (Traktuev et al., 2008).

Of interest, a subpopulation of $CD142^+$ adipogenesis-regulatory cells (Aregs) was recently identified among the stromal cell population of the mouse AT. This unique subtype of precursor cells is increased in the obese AT and can suppress the differentiation of preadipocytes in a paracrine-dependent manner (Schwalie et al., 2018). However, as described above, a later study did not support this finding (Merrick et al., 2019).

## Beige Adipogenesis

Beige adipogenesis is integral to the metabolic homeostasis of WAT. It contributes to insulin sensitivity by upregulating molecular thermogenic signatures of WAT. Specifically, upon adrenergic stimulation or exposure to cold, beige or brite adipocytes can upregulate UCP-1 expression and adopt a thermogenic phenotype, resembling brown adipocytes (Wu et al., 2012; Alexaki and Chavakis, 2016; Wang and Seale, 2016; Shao et al., 2019). However, a non-canonical UCP-1 mechanism can also contribute to the formation of this phenotype (Bertholet et al., 2017; Ikeda et al., 2018). Lineage-tracing studies allowed to distinguish between $Myf5^+$ progenitors that can differentiate into brown adipocytes or myocytes, and $Myf5^-$ precursors that are committed to white and beige adipocyte differentiation (Gesta et al., 2007; Obregon, 2014; Peirce et al., 2014). Furthermore, apart from the common white-beige progenitor, beige adipocytes can also derive from a transdifferentiation of mature white adipocytes into beige ones (Lee et al., 2012; Rosenwald et al., 2013).

Until now, "beiging" or "browning" of the WAT is considered as metabolically more favorable than "whitening," and is essentially a feature of the lean WAT, while it is diminished in the course of obesity and the development of AT inflammation (Alexaki and Chavakis, 2016). Indeed, several innate and adaptive immune cell types have been implicated in the regulation of beige adipogenesis in a positive or negative fashion (Lee et al., 2015; Chung et al., 2017). Multiple studies have suggested the beneficial role of cells of type 2 immunity, such as eosinophils and M2-MΦ in the induction and maintenance of beige adipogenesis in mice, especially in the lean WAT (Lee et al., 2013; Qiu et al., 2014; Alexaki and Chavakis, 2016). For instance, the release of IL-4 by eosinophils is required for the maintenance of the alternatively activated M2-MΦ population, which promote induction of beige adipogenesis in mice (Wu et al., 2011; Qiu et al., 2014; Hui et al., 2015). Nevertheless, contradictory data exist pertinent to the mechanism by which M2-MΦ exert their beige adipogenesis-promoting effect on APs and mature adipocytes and whether the latter may depend on the release of catecholamines by the M2-MΦ or not (Qiu et al., 2014; Fischer et al., 2017). In addition, type 2 innate lymphoid cells (ILC2) promote beige adipogenesis in mice, predominantly by propagating the maintenance of eosinophils and M2-MΦ in a IL-5- and IL-13-related manner (Molofsky et al., 2013). Along this line, type 2 cytokines secreted by ILC2s and eosinophils can stimulate beige adipogenesis in murine $PDGFR\alpha^+$ APs, thereby triggering signaling via the IL-4Rα present on the latter (Lee et al., 2015). Another mechanism suggests that mouse AT beiging is stimulated by IL-33-mediated activation of ILC2 and the release by them of a methionine-enkephalin peptide (Brestoff et al., 2014). Interestingly, a recent study showed that murine PRDM16-expressing adipocytes, favor a fibrogenic-to-adipogenic transition of APs, thus promoting beige adipogenesis by secreting β-hydroxybutyrate (Wang W. et al., 2019).

In contrast, the development of a pro-inflammatory microenvironment in the obese WAT restricts the potential of APs toward beige adipogenesis. The increased numbers of M1-MΦ and the concomitant release of pro-inflammatory mediators, such as IL-1β and TNF, contribute to the impaired browning of the obese murine WAT and the suppression of UCP-1 expression, likely in a Toll-like receptor 4 (TLR4) and Nod-like receptor 3 (NLRP3) inflammasome-dependent way (Sakamoto et al., 2016; Okla et al., 2018). Additionally, signaling via the IL-18/IL-18R1 system has been reported to impede energy expenditure and mouse AT beiging *in vivo* (Pazos et al., 2015). Interestingly, a major mechanism involved in the diminished beige adipogenesis in mice and humans during obesity-related

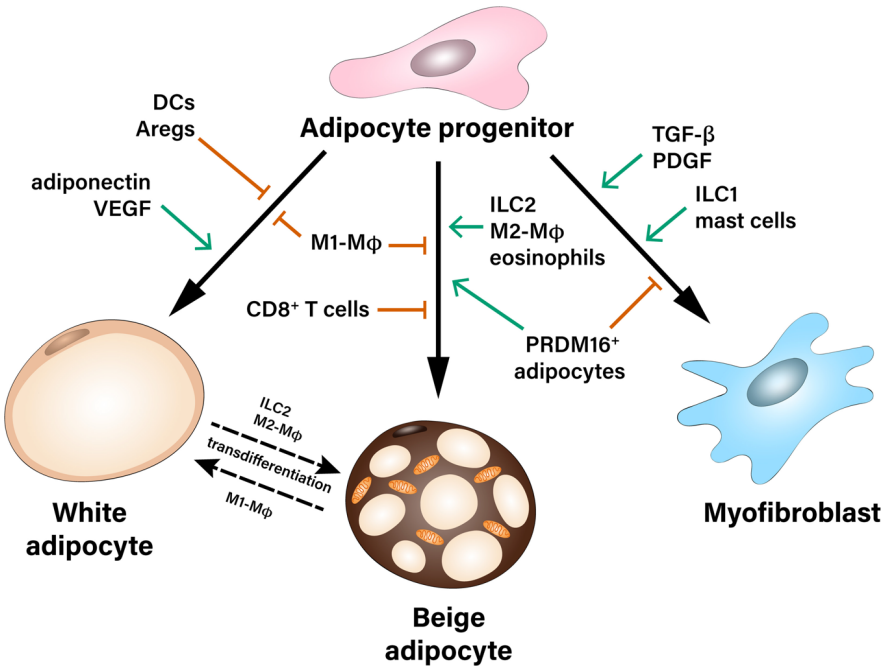


**FIGURE 1 |** Multiple contact- and paracrine-mediated interactions shape differentiation of adipocyte progenitors toward white or beige adipocytes or myofibroblasts. Pro-inflammatory M1-like macrophages (M1-MΦ) inhibit both white and beige differentiation of preadipocytes. Dendritic cells (DCs), as well as adipogenesis-regulatory cells (Aregs) contribute to the inhibition of white adipogenic differentiation. On the other hand, paracrine factors like adiponectin and VEGF stimulate precursor's commitment toward white fat cells. Beige adipocytes can derive either from common white/beige adipocyte progenitors or from transdifferentiation of mature white adipocytes. Pro-inflammatory M1-MΦ and $CD8^+$ T cells inhibit beige adipogenesis during obesity, while Type 2 innate lymphoid cells (ILC2), M2-like macrophages (M2-MΦ), eosinophils as well as a subpopulation of $PRDM16^+$ adipocytes were described as positive regulators of beige adipogenesis. Adipocyte progenitors can differentiate into myofibroblasts and therefore, contribute to the development of fibrosis. This process is stimulated by pro-fibrotic factors (TGF-β, PDGF) as well as type 1 innate lymphoid cells (ILC1) and mast cells.

WAT inflammation relies on the direct integrin-mediated interaction between M1-like macrophages and APs as well as mature adipocytes (Chung et al., 2017). Furthermore, $CD8^+$ T cells were also shown to inhibit beiging of the obese murine WAT in an IFN-γ-dependent manner (Moysidou et al., 2018).

## FIBROSIS

Fibrosis is considered a pathophysiological consequence of the persistent low-grade inflammation in the WAT in obesity. Myofibroblasts are the major cell type contributing to the extracellular matrix (ECM) deposition in fibrosis of various organs. They can originate from different precursor cells under the effect of transforming growth factor beta 1 (TGF-β1) and platelet-derived growth factor (PDGF) deriving from inflammatory cells (Wynn, 2008; Sun et al., 2013; Marcelin et al., 2019).

TGF-β inhibits human preadipocyte differentiation into mature adipocytes, while it promotes collagen production and cell proliferation, a process that can be controlled by Jak-Stat signaling (Weiner et al., 1989; Keophiphath et al., 2009; Babaei et al., 2018). TGF-β is involved in the generation of $Sca\text{-}1^-SMA^+ITGA5^+$ fibrogenic progenitor cells in the murine WAT. This process depends on myocardin-related transcription factor A (MRTFA) and results in the shift of the fate of perivascular progenitors from APs with adipogenic potential toward pro-fibrotic cells (Lin et al., 2018).

PDGFRα has been recognized as an anti-adipogenic factor that favors the generation of profibrotic cells in mice (Iwayama et al., 2015; Sun et al., 2017). Marcelin et al. showed the existence of two subsets of $PDGFR\alpha^+$ adipocyte progenitors based on the level of their CD9 expression. In both humans and mice, AT $CD9^{high}$ cells were described as pro-fibrotic progenitors, while $CD9^{low}$ precursors were rather committed to adipogenesis. The $CD9^{low}$ subpopulation was almost lost in the fibrotic obese WAT, while $CD9^{high}$ progenitors' frequency positively correlated with the degree of WAT fibrosis (Marcelin et al., 2017). Similar characterizations of profibrotic vs. adipogenic cells were identified in humans and mice based on Ly6C and CD34 expression. Specifically, Hepler et al. (2018) described in the mouse AT the coexistence of $Ly6C^+$ $PDGFR\beta^+$ fibro-inflammatory progenitors along with the highly adipogenic $Ly6C^-CD9^-PDGFR\beta^+$ cells. Similarly, $CD34^{high}$ APs were described as pro-fibrotic cells in the human visceral AT according to their secretome profile (Buffolo et al., 2019). On the contrary, another study did not find significant differences in proliferative, adipogenic and fibrogenic potential between $CD34^-$, $CD34^{low}$, and $CD34^{high}$ cells (Raajendiran et al., 2019). Subpopulations of fibro-inflammatory progenitors increase in

numbers following AT expansion, and exert an anti-adipogenic effect on other adipocyte precursor cells via secretion of soluble factors (Marcelin et al., 2017; Hepler et al., 2018; Buffolo et al., 2019).

Importantly, the extent of AT fibrosis positively correlates with the number of crown-like structures in the obese AT (Cinti et al., 2005; Buechler et al., 2015), implying that AT fibrosis may be triggered by the pro-inflammatory microenvironment. Transcriptomic analysis of human preadipocytes cultured with conditioned medium from pro-inflammatory macrophages revealed an upregulation in the expression of ECM components (Henegar et al., 2008). Moreover, macrophage-derived IL-1β promotes the expression of ECM remodeling enzymes, such as metalloproteinases 1 (MMP1) and 3 (MMP3), in human APs (Gao and Bing, 2011). Not only cells of the monocytic lineage contribute to the stimulation of ECM production by preadipocytes. Mast cells accumulate in the mouse and human obese AT preferentially in depots with progressed fibrosis and provoke the secretion of collagen V by AT fibroblasts, which can contribute to the suppression of the adipogenic differentiation of APs. Of note, the secretion of collagen V in the obese AT is triggered by the release of mast cell protease 6 (MCP-6) by mature mast cells (Divoux et al., 2012; Hirai et al., 2014). Other ECM components of the AT, like collagen VI and its derivative endotrophin, can trigger fibrosis in the AT and contribute to preadipocytes' myofibroblastic transformation (Khan et al., 2009; Sun et al., 2014; Jones et al., 2020). Additionally, while ILC2 drive beige adipogenesis, type 1 innate lymphoid cells (ILC1) promote AT fibrogenesis in human and mice in an IFN-γ dependent manner (Wang H. et al., 2019).

In conclusion, in the lean WAT, APs represent a highly heterogeneous cell population; yet with intrinsic white or beige differentiation potential rather than a pro-fibrotic one. Contrastingly, in obesity, interactions of APs with cells of both the innate and adaptive immunity that accumulate in the obese WAT can trigger fibrosis by inducing a pro-fibrotic transcriptional program in APs.

## CONCLUSION AND FUTURE PERSPECTIVE

APs are a highly heterogeneous population of stromal AT cells. Different subtypes of APs can have a varying degree of commitment toward white, beige adipocyte or fibroblast differentiation. Along this line, extensive *in vivo* and *in vitro* studies report the identification of numerous AP subpopulations. However, in several of these studies the characterization of the multiple AP subtypes is based on different experimental approaches (Burl et al., 2018; Cho et al., 2019; Min et al., 2019). This issue is further complicated by the regional variation of APs within the different fat depots and the distinct abundance of different progenitor subtypes therein. Thus, the identification of reliable and broadly acceptable molecular and surface markers to distinguish the various AP subtypes is imperative. It is recognized, that the number of adipocytes is set during childhood and adolescence and stays nearly constant in adulthood with a 10% turnover rate in lean and obese individuals (Spalding et al., 2008; Rodríguez et al., 2015; Meln et al., 2019). Consequently, a deeper insight into AP subtypes and crosstalk mechanisms with other cells could shed the light on how the fate of preadipocytes can be predetermined early in life and lead to the development of obesity and accompanying metabolic complications later.

Importantly, the crosstalk between APs and immune cells in the AT orchestrates AP fate in both lean and obese state. For instance, obesity-related AT inflammation leads to reduced beige adipogenic and increased pro-fibrotic potential of APs. So far, the majority of studies have focused on the interaction between macrophages and APs, while less information exists pertinent to the role of cells of the adaptive immunity as well as less abundant stromal cell types, which may also shape the differentiation potential of APs (**Figure 1**). Identification of the specific contribution of different immune and stromal cell populations, which may affect fate decisions of APs, as well as better understanding of the molecular mechanisms implicated in this crosstalk is needed for the development of new therapeutic strategies against obesity-related AT dysfunction.

## AUTHOR CONTRIBUTIONS

IP, TC, and AC wrote the manuscript, while ZM, K-JC, and MK edited the manuscript. All authors contributed to the article and approved the submitted version.

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# Fibroblast Reprogramming in Gastrointestinal Cancer

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Gastrointestinal cancers are a significant cause of cancer mortality worldwide and have been strongly linked with chronic inflammation. Current therapies focus on epithelial/cancer cells; however, the importance of the tumor microenvironment in the development and treatment of the disease is also now well established. Cancer-associated fibroblasts (CAFs) are a major component of the tumor microenvironment, and are actively participating in tumor initiation, promotion and metastasis. They structurally and functionally affect cancer cell proliferation, tumor immunity, angiogenesis, extracellular matrix remodeling and metastasis through a variety of signaling pathways. CAFs originate predominantly from resident mesenchymal cells, which are activated and reprogrammed in response to cues from cancer cells. In recent years, chronic inflammation of the gastrointestinal tract has also proven an important driver of mesenchymal cell activation and subsequent CAF development, which in turn are capable of regulating the transition from acute to chronic inflammation and cancer. In this review, we will provide a concise overview of the mechanisms that drive fibroblast reprogramming in cancer and the recent advances on the downstream signaling pathways that regulate the functional properties of the activated mesenchyme. This new mechanistic insight could pave the way for new therapeutic strategies and better prognosis for cancer patients.

**Keywords: cancer-associated fibroblasts, tumor microenvironment, synthetic activation, epigenetic reprogramming, metabolic reprogramming**

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## INTRODUCTION

CAFs are an essential component of the tumor microenvironment and accumulating evidence supports their substantial contribution to cancer development and progression (Kalluri, 2016; Kobayashi et al., 2019; Sahai et al., 2020). They originate predominantly from tissue-resident fibroblasts that are activated in response to signals from cancer cells and the tumor microenvironment. Additional cellular sources, such as bone marrow-derived mesenchymal stromal cells (BM-MSCs), fibrocytes, as well as epithelial and endothelial cells have also been reported. Fibroblast activation includes increased proliferation, changes in their physicochemical properties, such as shape alteration and increased contractility, and the production of a variety of effector molecules. These include cytokines and chemokines, extracellular matrix (ECM) components and remodeling enzymes, growth factors, metabolites and signaling molecules that mediate CAF functions to support cancer growth, metastasis and resistance to therapy (Kalluri, 2016; Kobayashi et al., 2019; Sahai et al., 2020). The increased insight into CAF functions and their association with poor prognosis in cancer patients has brought into focus the potential of CAF

targeting for cancer treatment. It is thus interesting to consider the possibility of the reversal of CAF reprogramming as a promising therapeutic strategy in cancer, although potential anti-tumor CAF properties should also be taken into account (Gieniec et al., 2019).

In this review, we summarize current knowledge on how fibroblasts are converted to CAFs, particularly focusing on gastrointestinal cancers, including colorectal cancer (CRC), pancreatic ductal adenocarcinoma (PDAC), hepatocellular carcinoma (HCC), and gastric cancer (GC). We specifically analyze the three major types of CAF reprogramming, namely synthetic, epigenetic and metabolic, and focus on the signals and downstream molecular pathways that regulate this transition (**Figure 1**).

# FIBROBLAST REPROGRAMMING: TYPES AND UNDERLYING MOLECULAR MECHANISMS

## Synthetic Activation

A variety of signals, such as growth factors, cytokines, chemokines, Toll-like receptor (TLR) and Hedgehog ligands, mechanical forces and ECM components activate fibroblasts to produce effector molecules that are responsible for CAF functions (**Figure 1A**; Kalluri, 2016; Kobayashi et al., 2019; Sahai et al., 2020). TGFβ and IL-1 are probably the most ubiquitous and well-characterized such stimuli.

TGFβ is a dominant effector in all gastrointestinal cancers and mediates the conversion of fibroblasts to CAFs. Its importance is highlighted by the association of a CAF-specific TGFβ signature with poor prognosis, immune cell exclusion and resistance to immunotherapy in CRC (Calon et al., 2015; Tauriello et al., 2018). The pleiotropic effects of TGFβ encompass changes in fibroblast adhesion through the production of collagens and fibronectin, changes in cell shape through αSMA overexpression, increased proliferation and increased synthetic activity, including the production of ECM enzymes, growth factors, chemokines, and cytokines (Calon et al., 2012; Hawinkels et al., 2014; Ishimoto et al., 2017; Biffi et al., 2019). TGFβ functions predominantly by downstream activation of SMADs and JAK/ STAT signaling pathways, as shown in CRC and liver fibrosis (Calon et al., 2012; Tang et al., 2017). In contrast, it downregulates IL-1R1 and blocks the JAK/STAT cascade in PDAC fibroblasts, favoring thus the generation of CAFs with a myofibroblastic phenotype (Biffi et al., 2019). Notably, it also promotes epithelial-to-mesenchymal and endothelial-to-mesenchymal transition, as well as the recruitment of bone marrow-derived mesenchymal cells (BM-MSCs) and fibrocytes (Polyak and Weinberg, 2009; Quante et al., 2011). Other growth factors can also regulate CAF reprogramming either alone or in combination with TGFβ. For example, PDGF has been shown to drive recruitment and activation of fibroblasts in CRC and GC and its blockade leads to reduced tumor growth and metastasis (Ostman, 2004; Kitadai et al., 2006; Kodama et al., 2010; Sumida et al., 2011; Manzat Saplacan et al., 2017). It also complements the function of TGFβ on both pancreatic (PSC) and hepatic (HSC) stellate cells to promote their proliferation and migration (Dong et al., 2004; Cadamuro et al., 2013).

Inflammation is a significant predisposition factor for the initiation and progression of gastrointestinal cancers. The abundance of inflammatory signals mediates reciprocal interactions between cancer cells, stroma and immune cells to accelerate the development of an inflammatory TME and the phenotypic switch of CAFs (Greten and Grivennikov, 2019). Recently, Biffi and Tuveson (2020) highlighted the importance of IL-1 signaling in shaping CAF functions in PDAC, by showing that tumor-derived IL-1α antagonizes TGFβ signaling and stimulates the production of a cytokine cascade, including LIF, IL-6 and CXCL8 (Biffi et al., 2019). This acts in an autocrine manner to activate the JAK/STAT3 pathway in CAFs, resulting in a positive feedback loop that leads to high IL-1R1 expression and inflammatory CAF formation (Biffi et al., 2019). IL-1β also drives tumor fibrosis and cancer cell proliferation, survival and chemoresistance in PDAC through the IRAK4-NFκB pathway (Zhang et al., 2018). The IL-1-NFκB axis plays also an important role in the activation or survival of HSCs, at least in liver fibrosis (Gieling et al., 2009; Pradere et al., 2013). Both IL-1α and IL-1β increase CAF motility in GC through the regulation of Rhomboid 5 homolog 2 (RHBDF2), which promotes TGFβR1 cleavage by ADAM17 (Ishimoto et al., 2017). In the intestine, they induce cytokine and prostaglandin production by intestinal mesenchymal cells, which promote inflammation and can support the establishment of a cancer stem cell niche (Li et al., 2012; Scarpa et al., 2015). Interestingly, IL-1β was recently shown to drive the activation of subsets of PDGFRα$^{+}$ fibroblasts, contributing thus to EGF-dependent serrated polyp formation in the mouse cecum (He et al., 2019). In contrast, in vivo deletion of IL-1R1 in *ColVI*$^{Cre+}$ mesenchymal cells had no effect in either Apc-driven spontaneous or inflammation-induced intestinal carcinogenesis (Koliaraki et al., 2019). These studies highlight both the diverse and opposing roles of IL-1 agonists in cancer and their potential distinct functions in different fibroblast or CAF subpopulations (Voronov et al., 2013).

TNF and members of the IL-6 family are also important inflammatory inducers of fibroblast activation. TNF has been shown to act synergistically with IL-1α to promote proinflammatory gene expression in PSCs through NFκB activation (Biffi et al., 2019). Accordingly, in vivo inhibition of TNF was able to reduce desmoplasia in mice, which was associated with decreased PSC viability (Zhao et al., 2016). In addition, in vitro studies ascribe a pro-fibrogenic role for TNF on HSCs, as it promotes myofibroblast survival and activation through NFκB activation, while iRhom2-mediated inhibition of TNFR signaling protects against liver fibrosis (Tarrats et al., 2011; Pradere et al., 2013; Bonnardel et al., 2019; Sundaram et al., 2019). IL-6 and IL-11 were recently also shown to play a role in fibroblast activation in CRC through STAT3 activation and subsequent expansion of activated fibroblasts and the induction of a proangiogenic profile, which drove colorectal carcinogenesis in vivo (Heichler et al., 2019). Similarly, IL-6 was sufficient to induce the trans-differentiation of normal fibroblasts to CAFs

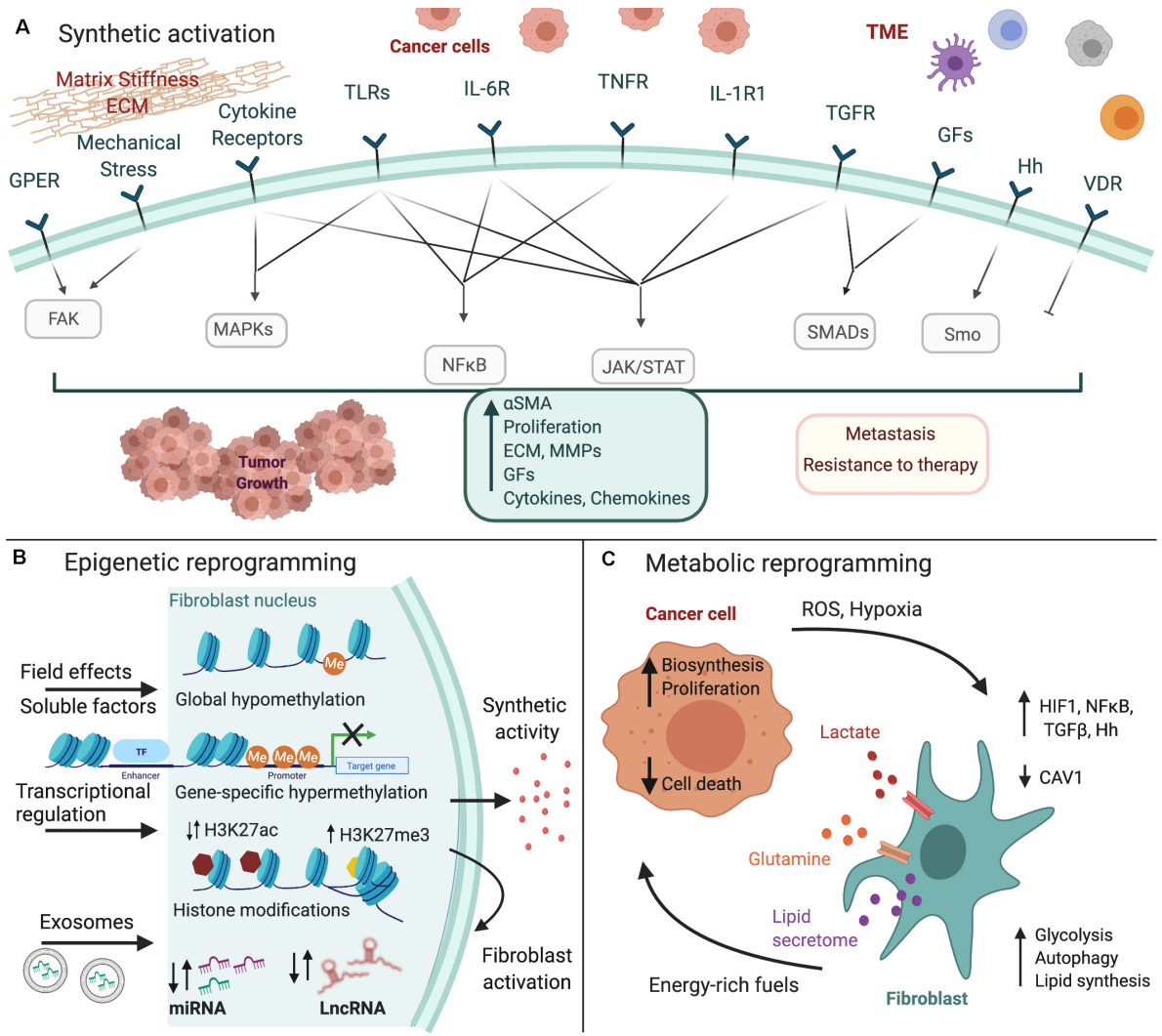


**FIGURE 1 |** Fibroblast reprogramming in gastrointestinal cancer. Cancer cells and the tumor microenvironment (TME) induce the **(A)** synthetic activation of fibroblasts to CAFs, as well as their **(B)** epigenetic and **(C)** metabolic reprogramming. Major inducing signals and downstream pathways that regulate these processes are shown. The figure was created with BioRender.com.

via STAT3 phosphorylation and downstream activation of a Twist1/CXCL12 axis (Lee et al., 2015).

Besides these major cytokine signals, other cytokines and chemokines, such as IL-33 and CXCL12 also contribute to fibroblast activation. In humans and mouse models of intestinal cancer, cancer cell-derived IL-33 activates fibroblasts and promotes the expression of ECM components and growth factors associated with intestinal tumor progression (Maywald et al., 2015). Accordingly, IL-33 drives hepatic fibrosis through activation of HSCs via MAPK signaling (Tan et al., 2018). CXCL12 can promote epithelial-to-mesenchymal transition (EMT), recruit BM-MSCs in gastric cancer and drive the expansion of $\alpha SMA^{+}$ myofibroblasts and Gremlin 1-expressing mesenchymal stem cells (Quante et al., 2011; Shibata et al., 2013). Other immune cell-derived inflammatory cytokines, such as IL-17, IL-22, IL-31, IL-4 and IL-13, have also been shown to activate quiescent tissue-resident fibroblasts during inflammation or fibrosis, although their role in CAF reprogramming is not yet clear (Andoh et al., 2007; Tsuchida and Friedman, 2017).

Beyond cytokines and chemokines, innate immune signals have emerged as an additional stimulus for CAF reprogramming, linking further the inflammatory TME with fibroblast activation and cancer progression (Koliaraki et al., 2020). CAFs express innate immune receptors and respond to produce effector molecules that affect tumourigenesis in gastrointestinal tumors. This is mediated through downstream activation of MAPK and NFκB signaling pathways, which independently of the stimuli have been shown to play an important role in the synthetic reprogramming of CAFs in colorectal cancer (Koliaraki et al., 2012, 2015, 2019; Henriques et al., 2018). We recently showed that innate activation of intestinal mesenchymal cells through TLR4/MyD88 pathway in the $Apc^{min/+}$ mouse model resulted

in the production of pro-tumourigenic and immunomodulatory effector molecules, while it also resembled the gene expression profile of human CRC CAFs (Koliaraki et al., 2019). Similarly, TLR9 activation has been shown to lead to PSC reprogramming and the production of CCL11 to support PDAC tumor growth (Zambirinis et al., 2015).

The Hedgehog (Hh)/Smoothened (Smo) signaling pathway has also been shown to regulate CAF activation in gastrointestinal cancers, although it seems to exert opposite functions depending on the organ affected. Several studies have shown that genetic deletion or pharmacological inhibition of Hh signaling leads to depletion of the PDAC-associated stroma and enhanced drug delivery, xenograft growth inhibition, as well as reduction of HSC activation and concomitant liver fibrosis (Yauch et al., 2008; Olive et al., 2009; Michelotti et al., 2013; Swiderska-Syn et al., 2014). In contrast, Hh activity was found reduced in human CRC and stroma-specific Hh activation markedly decreased tumor load and progression in a mouse model of CRC partly via the modulation of BMP signaling (Gerling et al., 2016). Notably, clinical trials using Hh inhibitors in patients with CRC or PDAC have so far failed, indicating that stromal heterogeneity, compensatory mechanisms and therapy resistance could interfere with CAF reprogramming approaches in solid tumors (Berlin et al., 2013; Kim et al., 2014; Catenacci et al., 2015).

Besides driving CAF activation, other pathways have been shown to suppress or reverse it and could be promising candidates for therapeutic interventions. A prominent example is the vitamin D receptor (VDR), which acts as a modulator of CAF reprogramming in gastrointestinal cancers (Ferrer-Mayorga et al., 2017). Mechanistically, it has been shown to suppress PSC activation, resulting in stromal remodeling and increased intratumoral gemcitabine effects in PDAC, while interaction between VDR and p62/SQSTM1 suppressed HSC activation and liver cancer (Sherman et al., 2014; Duran et al., 2016). Another such example is the activation of p53 by Nutlin-3a, which can reprogram activated PSCs to quiescence (Saison-Ridinger et al., 2017).

Finally, beyond soluble mediators, the biophysical properties of the TME have been also implicated in CAF activation and the induction of a synthetic phenotype. Mechanical stress induces collagen overexpression and crosslinking, fiber rearrangement, ECM deposition and degradation by fibroblasts, which can thus mediate the remodeling of the ECM and increase matrix stiffness (Mohammadi and Sahai, 2018). Matrix stiffness and the resulting mechanical stress further activates fibroblasts in a continuous self-promoting loop resulting in cancer cell proliferation and migration. Several studies in gastrointestinal inflammation and cancer propose that these stimuli activate fibroblasts through FAK, MRTF-SRF, and YAP-TEAD signaling pathways, leading to increased αSMA expression and the regulation of cytoskeletal dynamics (Johnson et al., 2013, 2014; Foster et al., 2017). Many of these effects are also dependent on TGFβ, Rho and ROCK signaling (Zhao et al., 2007; Johnson et al., 2014). Accordingly, HSCs sense mechanical stress through integrins, GPCRs and DDRs, activating Rho, YAP, PAK1, and JAK2/PI3K/AKT-MYOCD, respectively (Martin et al., 2016; Kang, 2020). Interestingly, another mechanosensor, the G protein-coupled estrogen receptor (GPER) shows tumor-restricting capacity, as it acts through Rho/myosin axis and YAP deactivation to inhibit the ability of PSCs and HSCs to remodel the ECM (Cortes et al., 2019a,b).

## Epigenetic Reprogramming

Epigenetic abnormalities, including changes in DNA methylation, abnormal patterns of histone modifications and post-transcriptional regulation through micro and long non-coding RNAs support genetic changes in cancer cells to drive tumor initiation and progression. Similar genetic mutations driving CAF differentiation are rare (Qiu et al., 2008; Bianchi-Frias et al., 2016). However, CAFs maintain their properties in vitro, indicating that they could also be epigenetically modified to a stably activated cell state (**Figure 1B**; Kalluri, 2016).

Both global and gene-specific changes in DNA methylation patterns have been detected in stromal cells and shown to affect tumor growth and progression. GC CAFs display global hypomethylation of DNA along with hypermethylation at a subset of genes, such as *HOXB6* (Jiang et al., 2008). Global alterations in 5-methylcytosine and 5-hydroxylmethylcytosine play an important role in HSC activation and concomitant liver fibrosis (Page et al., 2016). PDAC CAF differentiation is accompanied by decreased cytosine methylation and increased hydroxymethylation in response to cancer cell-derived lactate and subsequent CAF metabolic alterations that drive activation of the demethylase TET (Bhagat et al., 2019). An example of gene-specific methylation change includes the cancer cell-induced methylation and concomitant downregulation of the SOCS1 gene in PDAC CAFs, which enhanced cancer cell growth through the STAT3/IGF1 axis (Xiao et al., 2016). Increased PGE2 production in response to *H. pylori* infection was shown to drive hypermethylation of miR-149, increased IL-6 secretion and CAF activation in GC (Li et al., 2015). Interestingly, CRC stromal cells showed hypermethylation of the *SEPT9* gene, which was temporally subsequent to epithelial cells, suggesting that DNA methylation in CAFs could be attributed to field effects from cancer cells (Wu et al., 2007). These examples highlight the role of cancer cells in the epigenetic reprogramming of fibroblasts, although more research is necessary to define the molecular mechanisms involved in this process.

Evidence on the regulation of histone modifications in stromal cells and their role in fibroblast activation during carcinogenesis is still limited. Nevertheless, genome-wide H3K27me3 analyses of primary GC CAFs revealed loss of H3K27me3 in genes involved in the maintenance of the stem cell niche, including WNT5A, the inhibition of which was shown to suppress cancer cell growth and migration (Maeda et al., 2020). Similarly, the histone acetyltransferase P300 was shown to mediate stiffness-induced activation of HSCs by altering the acetylation status of H3K27Ac, at least on the CXCL12 gene promoter (Dou et al., 2018). Additional genome-wide screenings in combination with functional in vivo studies on gastrointestinal cancer are necessary to delineate the role of the "histone-code" on the activation of stromal cells.

Changes at the levels of multiple miRNAs have also been implicated in the reprogramming of fibroblasts in gastrointestinal

cancers. For example, activated PSCs differentially express 84 miRNAs (Masamune et al., 2014), while culture-induced HSC activation resulted in the deregulation of 259 miRNAs (Coll et al., 2015). Among deregulated mi-RNAs, miR-21 is of particular interest, as it is involved in the activation of fibroblasts in colorectal and pancreatic cancer, as well as oesophageal squamous cell carcinoma through mechanisms that include activation of the TGFβ pathway and the promotion of the metabolic reprogramming of CAFs (Bullock et al., 2013; Li et al., 2013; Nouraee et al., 2013; Chen et al., 2018). These changes can be either the result of complex deregulated transcriptional and post-transcriptional networks in differentiating fibroblasts or the consequence of miRNA transfer from cancer cells mainly through exosomes. Concerning the latter, several studies have indeed confirmed this mechanism also for gastrointestinal cancers. For example, pancreatic cancer cells can reprogram normal fibroblasts to CAFs through secreted microvesicles containing miR-155, miR-1246, and miR-1290 (Pang et al., 2015; Masamune et al., 2018). In addition, metastatic hepatocellular carcinoma cells secrete exosomal miR-1247-3p that targets B4GALT3, which in turn activates β1 integrin-mediated NFκB signaling in fibroblasts (Fang et al., 2018). Finally, long noncoding RNAs have been described as regulators of stromal activation in HSCs (Zhou et al., 2016). These can act through a circuit comprising also miRNAs, such as GAS5 that restrains hepatic fibrosis by targeting miR-23a through the PTEN/PI3K/Akt signaling pathway (Dong et al., 2019) and HOTTIP which promotes the activation of HSCs via the downregulation of miR-148a (Li et al., 2018).

## Metabolic Reprogramming

Alterations in cancer cell energetics are now considered a hallmark of cancer (Hanahan and Weinberg, 2011; Faubert et al., 2020). The most prominent such change is a shift of glucose metabolism towards aerobic glycolysis versus mitochondrial oxidative phosphorylation (OXPHOS), a phenomenon that is known as the "Warburg effect". This allows cancer cells to take advantage of glycolytic intermediates and activate the biosynthesis of macromolecules and organelles that support rapid growth and proliferation (Vander Heiden et al., 2009; Faubert et al., 2020). Fibroblasts in the surrounding tumor microenvironment also exhibit a similarly altered metabolism towards aerobic glycolysis, which leads to the release of energy-rich fuels, mainly lactate, but also pyruvate, glutamine and ketone bodies (Wu et al., 2017). These are transferred from CAFs to cancer cells through MCT4 and MCT1 transporters, respectively, where they are used to replenish the TCA cycle, to support OXPHOS and biosynthesis pathways maximizing proliferation and reducing cell death. This phenomenon is termed the "Reverse Warburg effect" (Wilde et al., 2017). In line with this, MCT1 and MCT4 levels were found upregulated in CRC and were associated with low survival in patients with CRC and gastrointestinal stromal tumors (GISTs) (Lehuede et al., 2016; Martins et al., 2016). Similarly, PDAC CAFs displayed diverse expression of the hypoxic marker carbonic anhydrase X and MCT4 and altered metabolic properties, which supported the invasiveness of cancer cells and were correlated with shorter patient survival (Knudsen et al., 2016).

Besides glucose metabolism, CAFs also display a lipid metabolic shift. For example, activated PSCs showed a remodeled and increased lipid secretome and produced lysophospatidylcholines, which support membrane lipid synthesis, while they were further converted to LPA via autotaxin enzymatic activity to facilitate tumor growth (Auciello et al., 2019). Similarly, CAFs in CRC accumulated fatty acids and phospholipids via an increase in fatty acid synthase (FASN), and were then transferred to cancer cells to induce their migration (Gong et al., 2020).

CAFs are also characterized by increased autophagy, which generates recycled nutrients from broken down organelles that in turn are used by cancer cells to cover their needs. For example, in PDAC, cancer cell-induced autophagy in CAFs leads to the secretion of non-essential amino acids, and specifically alanine, which in turn fuels the TCA cycle and lipid biosynthesis in the cancer cells (Sousa et al., 2016). In CRC, co-culture of fibroblasts and cancer cells resulted in an upregulation of oxidative stress-related enzymes and autophagy genes and the downregulation of CAV1 in fibroblasts that in turn promoted cancer cell proliferation (Zhou et al., 2017).

Mechanistically, CAF metabolic reprogramming and autophagy are induced mainly by reactive oxygen species (ROS) and hypoxia, which through downstream activation of HIF1 and NFκB promote the metabolism of glucose to lactate and glutamate and mediate the loss of caveolin-1 (CAV1) (**Figure 1C**) (Martinez-Outschoorn et al., 2010). Inflammatory mediators can also induce autophagy in fibroblasts through NFκB signaling, providing evidence for immune regulation of metabolic reprogramming, similarly to their synthetic activation (Martinez-Outschoorn et al., 2011). TGFβ also mediates CAFs' metabolic reprogramming either through the downregulation of CAV1 or the upregulation of autophagy/mitophagy inducers. It acts by downregulating isocitrate dehydrogenase 1 (IDH1), leading thus to an increase of glutamine metabolism and α-ketoglutarate (α-KG) concentration, which in turn suppresses CAV1 expression (Hou et al., 2017). CAV1 is a crucial regulator of CAFs' metabolic switch and its inhibition is sufficient to activate fibroblasts, impair mitochondrial function and induce a glycolytic switch in fibroblasts through the upregulation of glycolytic enzymes (Sotgia et al., 2012). Finally, the Hh pathway has been shown to play a significant role in the reprogramming of quiescent HSC to myofibroblasts during liver fibrosis, and potentially CAF differentiation, through the activation of aerobic glycolysis and lactate accumulation (Chen et al., 2012). Additionally, Hh signaling together with YAP were found to induce glutaminolysis, concomitant activation of HSC and fibrosis progression (Du et al., 2018).

## Reprogramming and Heterogeneity of CAFs

Differences in reported CAF functions, including tumor-promoting and restraining roles have long led to the hypothesis that distinct CAF subpopulations could exist within tumors (Kalluri, 2016; Gieniec et al., 2019; Biffi and Tuveson, 2020). Representative such examples include the tumor-promoting

effects of CAF depletion or suppression in PDAC mouse models (Rhim et al., 2014; Özdemir et al., 2014). However, low cell abundancy and lack of analytical techniques have until recently hindered the functional characterization of these potentially different subtypes. Advances in single-cell analysis technologies have increased our understanding of tumor heterogeneity, including that of the microenvironment and specifically CAFs. Concerning gastrointestinal cancers, a recent single-cell transcriptomic analysis of mouse and human PDAC identified three CAF subsets, namely inflammatory (iCAFs), myofibroblastic (myCAFs) and antigen-presenting CAFs (apCAFs) (Elyada et al., 2019). Mechanistic studies using organoids and mouse models showed that iCAFs express inflammatory markers and are located within the desmoplastic stroma, while myCAFs are αSMA positive and adjacent to tumor cells. Importantly, they are activated by different stimuli, IL-1α and TGFβ, respectively, the spatial distribution of which can regulate the swift from one CAF state to the other (Ohlund et al., 2017; Biffi et al., 2019). apCAFs express MHC class II and CD74 and can induce T-cell receptor (TCR) ligation in $CD4^+$ T cells in an antigen-dependent manner, while they can be also converted to myofibroblasts (Elyada et al., 2019). These studies offer compelling evidence to support the idea that CAF reprogramming depends on the availability of stimuli in the surrounding microenvironment and may thus define CAF phenotypic and functional heterogeneity, although the CAFs' diverse cellular sources could also contribute, as shown for other types of cancer (Raz et al., 2018).

Single-cell RNA transcriptomic analyses have also been performed for colorectal and head and neck cancer. These revealed the presence of normal fibroblasts along with two CAF subsets, a myofibroblastic $\alpha SMA^+$ and an ECM-expressing $FAP^+$ population, although further functional characterisation of these is still missing (Li et al., 2017; Puram et al., 2017). Nevertheless, these studies indicate common diversity and potentially similar subpopulations in gastrointestinal tumors.

## DISCUSSION

In this review, we have provided a concise overview of the molecular mechanisms underlying fibroblast reprogramming in gastrointestinal cancers. This is especially important as CAF phenotype reversal has been proposed as a potential therapeutic strategy in cancer (Valkenburg et al., 2018; Chen and Song, 2019). Both soluble factors and mechanical cues drive the reprogramming of fibroblasts through the activation of downstream signaling pathways in fibroblasts, indicating the impact of fibroblast localization in the growing tumor. Multiple inducers and mechanisms underlying the synthetic activation of CAFs have already been identified, but similar research on the epigenetic and metabolic reprogramming of CAFs is still limited. Additional mechanistic insights into these processes could help identify novel targets for therapeutic approaches, as well as diagnosis and patient stratification. Notably, new targets should be in the future assessed under the prism of the emerging concepts of CAF heterogeneity that is defined by potential different cell sources, location, and available stimuli.

## AUTHOR CONTRIBUTIONS

M-TM, NC, and MS wrote sections of the manuscript and prepared the figure. VK critically revised the manuscript. All authors read and approved the submitted version.

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REVIEW
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# Hepatic Stellate Cells and Hepatocarcinogenesis

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Hepatic stellate cells (HSCs) are a significant component of the hepatocellular carcinoma (HCC) tumor microenvironment (TME). Activated HSCs transform into myofibroblast-like cells to promote fibrosis in response to liver injury or chronic inflammation, leading to cirrhosis and HCC. The hepatic TME is comprised of cellular components, including activated HSCs, tumor-associated macrophages, endothelial cells, immune cells, and non-cellular components, such as growth factors, proteolytic enzymes and their inhibitors, and other extracellular matrix (ECM) proteins. Interactions between HCC cells and their microenvironment have become topics under active investigation. These interactions within the hepatic TME have the potential to drive carcinogenesis and create challenges in generating effective therapies. Current studies reveal potential mechanisms through which activated HSCs drive hepatocarcinogenesis utilizing matricellular proteins and paracrine crosstalk within the TME. Since activated HSCs are primary secretors of ECM proteins during liver injury and inflammation, they help promote fibrogenesis, infiltrate the HCC stroma, and contribute to HCC development. In this review, we examine several recent studies revealing the roles of HSCs and their clinical implications in the development of fibrosis and cirrhosis within the hepatic TME.

**Keywords: hepatic stellate cells, hepatocytes, hepatocellular carcinoma, tumor microenvironment, inflammation, fibrosis**

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth leading cause of cancer related deaths in western countries (Choo et al., 2016; Bray et al., 2018), accounting for up to 90% of all primary liver cancers (Lozano et al., 2012). Although the percentage of HCC cases is dramatically higher in eastern Asia and most African countries, HCC is on the rise in the United States (U.S.) (Rawla et al., 2018). This trend is primarily due to increases in the incidences of chronic hepatic inflammation including fatty liver disease (FLD) (Nordenstedt et al., 2010) and chronic hepatitis C (HCV) infection (Kanwal et al., 2011). Although the distribution of HCC etiology varies between geographic regions, the most common etiology worldwide is viral hepatitis. In developing areas, such as Sub-Saharan Africa and Eastern Asia, hepatitis B virus (HBV) passed through vertical transmission at birth is the most common etiology (El-Serag, 2012). In developed worlds such as North America, HCV infection acquired later in life is endemic (Armstrong et al., 2006; Barazani et al., 2007; Wasley et al., 2008). In addition to viral hepatitis and FLD, alcoholic cirrhosis is a major contributing etiology of HCC; all of which can induce fibrosis, cirrhosis, and ultimately lead to development of HCC. This pathway

to malignancy, driven largely through fibrosis, is supported by the fact that 90% of HCC patients have cirrhosis (Parkin et al., 2005; Ananthakrishnan et al., 2006).

One of the main components in the development of fibrosis, cirrhosis and HCC (Wynn, 2008; Sokolovic et al., 2010) are the liver-specific pericytes, known as hepatic stellate cells (HSCs), which are located in the perisinusoidal space of the liver (Tsuchida and Friedman, 2017). Under normal conditions, HSCs exist in a quiescent state containing abundant lipid droplets of vitamin A (Blaner et al., 2009), and are highly sensitive to extracellular signals from fibrotic stimuli (Yin et al., 2013) including hepatitis, inflammation, or tissue injury (Wynn, 2008). In the presence of liver injury, HSCs are activated, transitioning from a quiescent to a myofibroblast phenotype with proliferative, migratory and invasive capabilities (Coulouarn et al., 2012; Carloni et al., 2014; Novikova et al., 2017). The well-known sequence of HSC activation can be split into two pathways. The first includes 'initiation,' which describes changes in HSC gene expression and phenotype rendering them more sensitive to paracrine stimuli. The second pathway includes 'perpetuation,' which amplifies the HSC-initiated phenotype via enhanced proliferation and proinflammatory signaling (i.e., damaged associated molecular patterns, DAMPs), interleukins, complimentary and growth factors from nearby damaged hepatocytes, endothelial cells and immune cells, resulting in promotion of fibrogenesis (Friedman, 2000) (**Figure 1**). Extracellular matrix (ECM) molecules (ex, type I and III collagen) are secreted by activated HSCs and accumulate to form scar tissue in the space of Disse (Friedman, 2008a) (**Figure 1**). This scar tissue functions to protect the liver from further damage; however, sustained activation of HSCs leads to chronic fibrosis and cirrhosis (Lee and Friedman, 2011; Friedman et al., 2013).

Activated HSCs play an essential role in fibrosis and hepatocarcinogenesis (Tsuchida and Friedman, 2017). Mediators in the activation of HSCs and the hepatic tumor microenvironment (TME) consist of transforming growth factor beta (TGFB), platelet derived growth factor (PDGF), connective tissue growth factor (CCN2, previously CTGF), vascular endothelial growth factor (VEGF), viral infection, focal adhesion kinase-matrix metalloproteinase 9 (FAK-MMP9) signaling, p53/21, phosphatidylinositol 3-kinase/protein kinase B (AKT) (PI3K/AKT), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), and interleukin 6/signal transducer and activator of transcription 3 (IL-6/STAT3) signaling pathways (Han et al., 2014; Fabregat et al., 2016; Makino et al., 2018). Furthermore, previous studies have shown HSC activation to be regulated by the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Takashima et al., 2009; Bian et al., 2012). These characteristics of activated HSCs and the role of the TME lay the foundation for exploring the relationship between the hepatic TME, activated HSCs, and hepatocarcinogenesis. This review will focus on the mechanisms regulating HSC activation and how they contribute to fibrosis, cirrhosis and HCC development (Wright et al., 2014; Coll et al., 2015; Das et al., 2020) and promising clinical therapies associated with HSCs (Dong et al., 2018; Li et al., 2020).

# THE ROLE OF ACTIVATED HEPATIC STELLATE CELLS IN HEPATIC FIBROSIS, CIRRHOSIS, AND PROGRESSION TO HCC

Hepatic fibrosis is a major risk factor for HCC development. Furthermore, activation of HSCs is a driver of hepatic fibrosis, cirrhosis and HCC (Tsuchida and Friedman, 2017). Due to the lack of effective liver fibrosis treatments, there is a need to better understand the molecular pathways and mediators of HSC activation to develop advantageous, targeted therapies for liver disease. Hepatic fibrosis is a result of chronic inflammation (post liver injury) characterized by secretion of excess ECM components, resulting in a wound healing response that will produce a "scar" in the liver (Friedman, 2008b; Lee and Friedman, 2011). Chronic inflammation can be induced by HBV/HCV infection, alcohol abuse, FLD (including non-alcoholic fatty liver disease and non-alcoholic steatohepatitis) or other metabolic disorders of the normal liver (Higashi et al., 2017). During post chronic liver inflammation, HSC activation supports the development of fibrosis and later cirrhosis, characterized by liver function impairment, portal vein hypertension and jaundice (Gines et al., 1987; de Franchis, 2000; D'Amico et al., 2006). Therefore, preventing the activation and proliferation of HSCs in cases of hepatic fibrosis has the potential to delay progression to HCC.

## The Role of DNA Methylation in HSC Activation

Recently, studies have begun focusing on the epigenetic regulation of HSCs to uncover the complexity of HSC activation (Tsuchida and Friedman, 2017). The tumor suppressor gene PTEN is an important negative regulator of HSC activation, which is silenced through promoter hypermethylation in tumors (Cairns et al., 1997; Salvesen et al., 2001; Soria et al., 2002; Furuta et al., 2004; Roman-Gomez et al., 2004; Mirmohammadsadegh et al., 2006; Wiencke et al., 2007; Tao et al., 2011; van Eggermond et al., 2011; Bian et al., 2012). Furthermore, the inhibition of PTEN activity leads to a constitutive activation of HSCs, which can perpetuate hepatic fibrosis (Bian et al., 2012). In addition, activated HSCs have been shown to possess altered DNA methylation and hydroxymethylation marks. For example, Page et al. (2016) showed that activated HSCs lose their fibrogenic phenotype when DNA methyltransferase 3a (DNMT3a) is suppressed in a carbon tetra chloride ($CCl_4$) rat model of liver fibrosis *in vivo*. The continued discovery of novel mediators of HSC activation, including epigenetic regulators, will help better understand the role of activated HSCs in hepatic fibrosis.

## Pro-fibrogenic Cytokines in HSC Activation During Hepatic Fibrosis

Transforming growth factor beta is one of the key fibrogenic cytokines that drive hepatic fibrosis and regulate HSC activation (Fabregat et al., 2016; Tsuchida and Friedman, 2017; Dewidar et al., 2019). The bona-fide TGFB signaling pathway requires latent TGFB to be cleaved and activated by thrombospondin

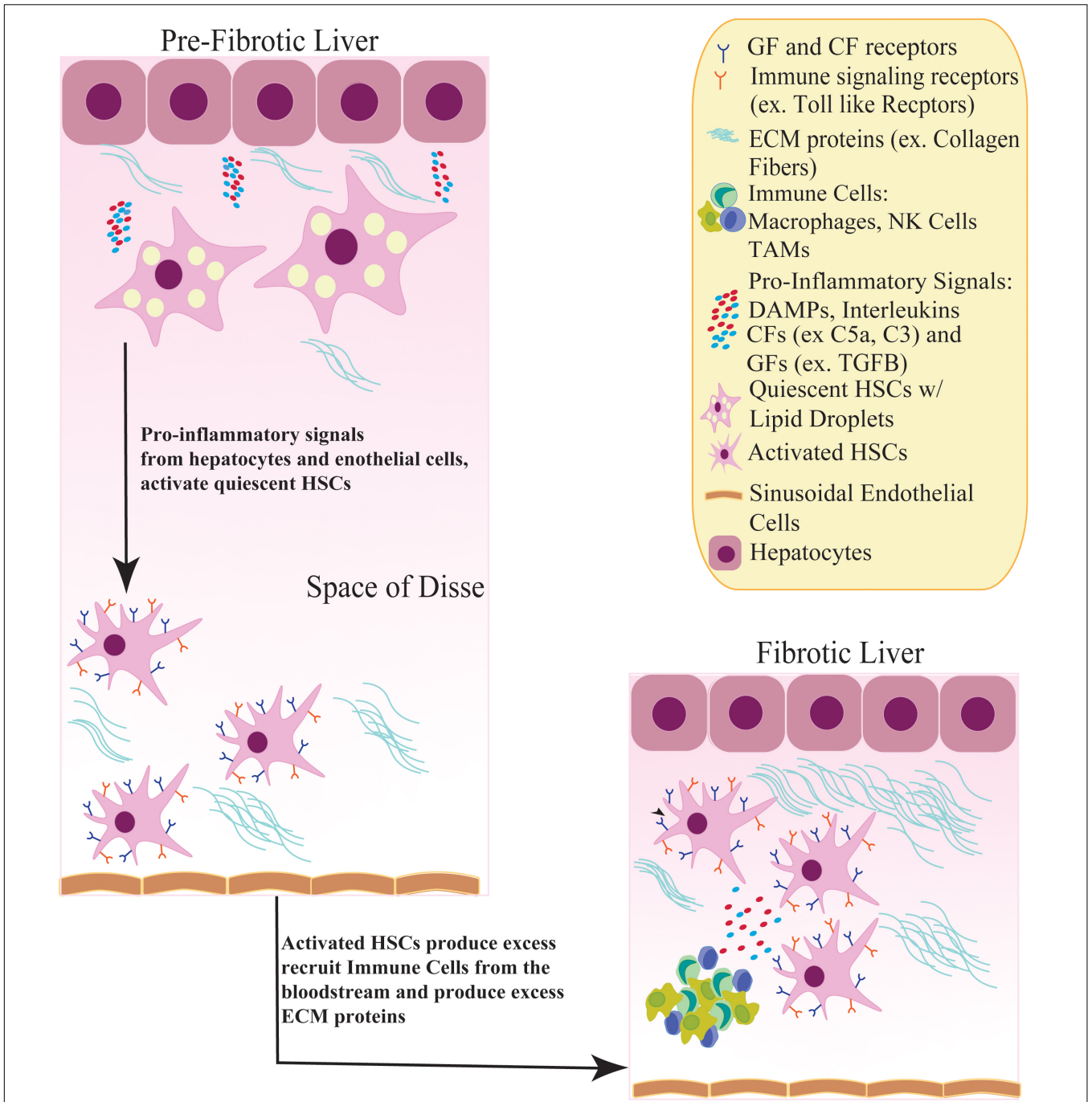


**FIGURE 1 |** Activation of quiescent HSCs contribute to a fibrotic liver. In a pre-fibrotic liver, quiescent HSCs in the Space of Disse receive inflammatory signals, such as Damaged Associated Molecular Patterns (DAMPs) and Complimentary factors (CFs), from nearby hepatocytes and sinusoidal endothelial cells. In response, HSCs become activated, adopting a myofibroblast phenotype and losing its lipid droplets containing Vitamin A. Activated HSCs induce growth factor receptor signaling (TGFB, PDGF) and recruit immune cells such as NK cells and tumor associated macrophages (TAMs) from the bloodstream which produce more inflammatory stimuli. These inflammatory signals stimulate HSCs to secrete excess ECM proteins (ex. Collagen fibers), creating a fibrotic environment.

1 (TSP1). Activated TGFB then binds to TGFB receptor 2 (TGFBR2), inducing phosphorylation of mothers against decapentaplegic homolog 2 and 3 (SMAD2, SMAD3) which translocate into the nucleus to regulate downstream gene expression of pro-fibrotic genes (Breitkopf et al., 2006; Friedman, 2008a; Liu et al., 2013; Meng et al., 2016; Tsuchida and Friedman, 2017; Murphy-Ullrich and Suto, 2018). TGFB can also activate the MAPK p38, ERK, and c-jun N-terminal kinase (JNK) pathways to regulate HSC activation (Engel et al., 1999; Hanafusa et al., 1999). Central to liver injury and HSC

activation is a pathological increase in expression and activation of TGFB in the ECM (Tsuchida and Friedman, 2017). During HSC activation, TGFB targets and binds to HSCs, inducing phosphorylation of SMAD3 to promote production of type I and II collagen (Breitkopf et al., 2006; Friedman, 2008a). Activated HSCs also promote increased production of TGFB1 and TSP1 further enhancing the profibrogenic activities of TGFB (Breitkopf et al., 2005). Moreover, attenuation of TSP1 activated TGFB can be achieved with a TSP1 antagonist peptide and has been shown to decrease liver fibrosis in a dimethyl-nitrosamine liver fibrosis model (Li et al., 2017; Murphy-Ullrich and Suto, 2018), demonstrating the importance of this relationship. Along with TGFB, the DNA demethylase tet methylcytosine dioxygenase 3 (TET3) is also upregulated in mouse and human fibrotic livers (Xu et al., 2020). Xu et al. (2020) established TGFB1 stimulation could increase TET3 levels along with increased profibrotic gene expression in LX-2 cells, a human HSC cell line. Furthermore, TET3 was shown to upregulate the TGFB pathway genes TSP1 and TGFBR2, suggesting a positive feedback loop between TET3 and TGFB1 to promote HSC activation and hepatic fibrosis (Xu et al., 2020). In addition to TGFB signaling influencing HSC activation, hepatocyte produced extracellular matrix protein 1 (ECM1) has been shown to suppress TGFB levels and prevent HSC activation in an *in vivo* ECM1 knockout mouse model (Fan et al., 2019). Although TGFB remains a strong mediator of HSC activation and fibrogenesis, additional profibrogenic cytokines contribute to this process as well.

Another essential cytokine involved in HSC activation is PDGF (Tsuchida and Friedman, 2017). PDGF levels are increased in human cirrhotic livers compared to normal, healthy livers (Pinzani et al., 1996; Ikura et al., 1997; Stock et al., 2007; Tsuchida and Friedman, 2017). Upon liver injury in both humans and rodents, PDGF receptor beta (PDGFRB) expression increases in HSCs to drive HSC activation, proliferation and migration (Wong et al., 1994; Borkham-Kamphorst et al., 2004). Pdgf-C, a member of Pdgf family, is highly expressed on membrane receptors of hepatocytes in a transgenic Pdgf-c mouse model that resulted in dynamic liver fibrosis (Wright et al., 2014), suggesting that HSC activation may include Pdgf-c signaling. Pdgf is further supported as an effective activator of HSCs through the fibrotic role of Agrin (Agrn), a secreted proteoglycan induced by Pdgf-induced HSC activation in HCC in Diethyl nitrosamine (DEN)-induced HCC Sprague Dawley rat model (Lv et al., 2017). In this study the authors showed that Pdgf acts as an activator of the HSCs, which was inhibited by blocking the binding of Pdgf to its receptor. The authors also demonstrated that Agrin from activated HSC supernatant increased proliferation, metastasis, and invasion of SMMC-7721 (a human HCC cell line) and promoted epithelial to mesenchymal transition (EMT) (Lv et al., 2017). Overall, this study supports the role of PDGF-induced HSC activation resulting in fibrosis and HCC.

The matricellular protein CCN2, known for mediating fibrosis in various organs including the liver (Hall-Glenn and Lyons, 2011; Jun and Lau, 2011; Kodama et al., 2011; Lipson et al., 2012), has also been shown to activate HSCs and promote tumor progression via HSC secretion of the IL-6 and STAT3 *in vitro* (Makino et al., 2018). A potential clinical player in the cellular crosstalk between HCSs and HCC cells is stroma-derived fibroblast growth factor 9 (FGF9). Interestingly, a 2020 study conducted by Seitz et al. found that only activated HSCs expressed FGF9 compared to HCC cells. In HCC tissues, activated HSC overexpression of FGF9 reduced sensitivity to therapeutic agents and was associated with poor prognosis (Seitz et al., 2020), suggesting FGF9 as a potential therapeutic target and prognostic tool for HCC. Altogether, these findings support the notion of the growth factors PDGF-C and CCN2 as activators of HSCs and FGF9 as a potential clinical target for HCC.

## Association of Resident Liver Lymphocytes With Activated HSCs During Hepatic Fibrosis and Cirrhosis

Pathogenesis of liver fibrosis also involves resident liver lymphocytes including Type I and Type II Natural Killer T (NKT), Natural Killer (NK) cells and innate lymphoid cells (ILCs) (Wang and Zhang, 2019). Specifically, the interaction between activated HSCs and these hepatic lymphocytes is important (Wang and Zhang, 2019). The innate role of NKT cells is to defend against pathogens by recruiting circulating lymphocytes (Racanelli and Rehermann, 2006). Once activated, NKT cells can induce HSC activation via production of pro-inflammatory cytokines and release osteopontin and Hedgehog (Hh) ligands (Syn et al., 2012; Wehr et al., 2013) to aid in fibrosis development (Wang and Yin, 2015; Bandyopadhyay et al., 2016). However, it has been established that NK cells, along with NKT cells, protect the liver by preventing infection, tumor formation (Racanelli and Rehermann, 2006) and fibrogenesis (Melhem et al., 2006; Radaeva et al., 2006) in liver fibrosis mouse models (Muhanna et al., 2011; Gur et al., 2012; Hou et al., 2012) and HCV patients in various clinical studies (Glassner et al., 2012; Gur et al., 2012; Kramer et al., 2012). Furthermore, NK cells have an anti-fibrotic effect on HSCs by inducing activated HSC apoptosis (Radaeva et al., 2006). However, this is a temporary effect, and could result in apoptosis- resistant activated HSCs (Radaeva et al., 2007). Additionally, type 3 innate lymphoid cells (ILC3s) function as pro-fibrotic effectors in the liver (Muhanna et al., 2007; Bjorklund et al., 2016). Through co-culturing experiments with LX-2 cells, ILC3s promoted fibrogenesis via Interleukin-17A (IL-17A) and Interleukin-22 (IL-22), resulting in IL-22 inhibition of interferon gamma (IFNG) to indirectly enhance fibrogenesis (Wang et al., 2018). These data suggest an important role for resident liver immune cells in liver fibrosis through interactions with activated HSCs (Schon and Weiskirchen, 2014). Furthermore, they suggest the involvement of the innate immune system in relation to HSC activation in fibrosis; thus, warranting further study of the roles of activated HSCs in enhancing and suppressing fibrosis.

Even though stimulation of innate immunity has been shown to have a pivotal role in anti-viral and anti-tumor defenses in addition to fibrosis suppression, the regulation of innate immunity during chronic liver injury still needs to be elucidated. In a $CCl_4$ mouse model, Infg induced NK cell activation was decreased in late liver fibrosis (advanced scarring) compared to early fibrosis (minimal scarring) (Jeong et al., 2011). The authors further demonstrated that the anti-fibrotic roles of NK cells

are suppressed during advanced livery injury through increased expression of suppressor of cytokine signaling 1 (Socs1) and Tgfb (Jeong et al., 2011). This study also showed *in vitro* evidence of early activated HSC (4 days co-culture of HSCs and liver NK cells) induced NK cell activation via natural killer group 2 member D (NKG2D), whereas this was abolished in intermediately activated HSCs (8 days co culture of HSCs and liver NK cells) due to increased levels of TGFB1 and downregulation of NKG2D (Jeong et al., 2011). These results establish that although NK cells interact with activated HSCs to mitigate liver fibrosis during chronic liver injury, this process can be suppressed through increased TGFB and SOCS1 produced by activated HSCs *in vitro*.

In a 2017 study, Shi et al. (2017) showed that in liver cirrhosis patients, activated HSCs interact with purified NK cells through HSC-derived TGFB regulation of emperipolesis (the presence of an intact cell within the cytoplasm of another cell). This process was mediated through TGFB and evidenced by significantly reduced NK cell emperipolesis when activated HSCs were treated with an anti-TGFB antibody. The NK cells inside activated HSCs were also apoptotic as observed through positive terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining which indicates DNA fragmentation, a trait of cellular apoptosis (Kyrylkova et al., 2012). This suggests that activated HSCs reduce the anti-fibrotic roles of NK cells *in vitro* through activated HSC-derived TGFB and programmed death of NK cells to promote fibrosis (Shi et al., 2017). This study demonstrates that activated HSC-derived TGFB and NK cells work together to diminish NK cell anti-fibrotic capabilities and promote fibrosis in liver cirrhosis patients.

## Cellular Senescence of Activated HSCs During Hepatic Fibrosis

Senescence of activated HSCs has been shown to suppress liver fibrosis (Hoare et al., 2010; Jin et al., 2016; Nishizawa et al., 2016). In cancer, cellular senescence is a known tumor suppressive mechanism (Sager, 1991) activated by oncogenic stress (Campisi and d'Adda di Fagagna, 2007) and may also be essential for regulating liver fibrosis and cirrhosis (Tsuchida and Friedman, 2017). Cancer cell senescence is maintained by the tumor suppressor proteins p53/p21, $p16^{Ink4a}$ and retinoblastoma (Rb) (Campisi and d'Adda di Fagagna, 2007; Collado et al., 2007). Furthermore, transgenic $p53^{-/-}$ mice, with p53 knocked out specifically in HSCs, that were treated with $CCl_4$ showed increased fibrosis compared to WT p53 mice. This phenotype indicates that activated HSCs have the ability to undergo senescence resulting in decreased liver fibrosis *in vivo* (Krizhanovsky et al., 2008). This study also revealed retained fibrotic lesions in $p53^{-/-}$; $Cdkn2a/Arf^{-/-}$ mice 20 days post $CCl_4$ treatment, along with increased smooth muscle actin (Acta2), Tgfb, and Ki67 (marker of proliferative cells) expression. These findings suggest that activated HSCs during liver fibrosis can also evade cellular senescence to proliferate and secrete ECM components to begin developing the hepatic TME (Krizhanovsky et al., 2008).

HSC activation is one of the first responses to LSEC (liver sinusoidal endothelial cell) injury in the liver. LSECs serve as a permeable barrier between hepatocytes and the bloodstream and are characterized by fenestrations and a disorganized basement membrane, making them one of the most permeable types of endothelial cells (DeLeve and Maretti-Mira, 2017; Poisson et al., 2017). This permeability allows for efficient transport of solutes and metabolites throughout the liver. In addition, LSECs are an active contributor to the production of excess ECM proteins during liver fibrosis (Natarajan et al., 2017). Recent data shows that removal of senescent LSECs promotes liver fibrosis (Grosse et al., 2020). Through genetic lineage tracing mouse models, Grosse, et al. demonstrated that the majority of senescent cells were vascular endothelial cells, mostly LSECs in liver sinusoids, in addition to macrophages and adipocytes to a reduced extent. The authors also showed that both continuous and acute elimination of senescent cells disrupted blood-tissue barriers resulting in liver and perivascular tissue fibrosis. Overall, this study establishes that senescent cells involved in preventing liver fibrosis are primarily LSECs, rather than hepatic stellate cells (Grosse et al., 2020). While senescent LSECs serve as a barrier against fibrosis, the activation, proliferation and transformation of HSCs caused by liver injury, in addition to their ability to evade senescence once activated, are important developmental processes that provide a suitable microenvironment required for fibrosis, cirrhosis and later HCC development (Lashen et al., 2020).

# ACTIVATED HEPATIC STELLATE CELLS WITHIN THE HEPATIC TUMOR MICROENVIRONMENT

The interplay between liver tumor cells and the hepatic TME is crucial to the initiation and progression of HCC (Hernandez-Gea et al., 2013; Zhou et al., 2019). The TME is defined as a peritumoral space (Alfarouk et al., 2011; Joyce and Fearon, 2015; Spill et al., 2016) contributing to the acquisition of various hallmark traits of cancer, including sustained proliferative signaling and activation of invasion, metastasis, and angiogenesis (Hanahan and Weinberg, 2011). Furthermore, the TME can be divided into two major components: (1) cellular and (2) non-cellular. Activated HSCs are a part of the cellular component and exhibit essential biological functions such as promotion of fibrogenesis and ECM remodeling to positively influence HCC tumorigenesis (Friedman, 2008a; Amann et al., 2009; Coulouarn and Clement, 2014).

In addition to HSCs, cellular components of the hepatic TME include stromal hepatocytes, immune cells such as myeloid-derived suppressor cells (MDSCs) (Fu et al., 2007; Hoechst et al., 2008), tumor associated macrophages (TAMs) (Budhu and Wang, 2006; Budhu et al., 2006; Jeong et al., 2011), and cancer associated fibroblasts (CAFs) (Yin et al., 2019). Non-cellular components include cytokines such as Interleukin-6 (IL-6) (Budhu and Wang, 2006; Budhu et al., 2006) and Interleukin-22 (IL-22) (Jiang et al., 2011), growth factors such as VEGF (Coulouarn et al., 2012), TGFB (Thompson et al., 2015), PDGF (Tsuchida and Friedman, 2017), and CCN2 (Makino et al., 2018). Additional non-cellular components include matrix metalloproteinases (MMPs), their inhibitors

(Novikova et al., 2017) and proteoglycans (Theocharis et al., 2010) (**Table 1**). The following studies will cover the different roles activated HSCs play in HCC progression through their interaction with the other cellular and non-cellular components of the hepatic TME.

## Cellular Crosstalk Between Activated HSCs and Cellular Components of the Hepatic TME

The hepatic TME consists of various immune cells to create an immunosuppressed environment in order to maintain HCC tumor growth (Lu et al., 2019). Activated HSCs contribute to this immunosuppressed environment by secreting cytokines which induce MDSC expansion (Maher, 2001; Hui et al., 2004; Yu et al., 2004; Pan et al., 2008; Gabrilovich and Nagaraj, 2009; Hsieh et al., 2013). In an orthotopic liver tumor mouse model, activated HSCs significantly increased regulatory T cell (Treg) and MDSC expression to benefit HCC growth in the spleen, bone marrow, and tumor tissues (Zhao et al., 2014). Furthermore, activated HSCs secrete angiogenic growth factors to form new vasculature within the TME (Coulouarn et al., 2012; Heindryckx and Gerwins, 2015). These functions of activated HSCs create a link to the circulatory system for supplying nutrients to the tumor. Immune cells may also regulate activation of HSCs *in vitro*, demonstrated by Interleukin 20 (IL-20) activation of HSCs, resulting in upregulation of TGFB1 and type I collagen, and increased proliferation and migration of activated HSCs (Chiu et al., 2014). The same study further indicated that these fibrogenic phenotypes could be attenuated with an anti-IL-20 receptor (IL-20R1) monoclonal antibody, proposing IL-20 as a significant activator of HSCs and fibrogenesis. Taken together, activated HSCs may have an important role in promoting an immunosuppressed and angiogenic hepatic TME to support aggressive HCC cell growth.

## Crosstalk Between Activated HSCs and Non-cellular Components of the Hepatic TME

In addition to interacting with other hepatic TME cellular components, HSCs also respond to the non-cellular components of the liver TME (Hall-Glenn and Lyons, 2011; Jun and Lau, 2011; Kodama et al., 2011; Lipson et al., 2012). An example of such is the response to CCN2 produced from hepatic tumor cells (Makino et al., 2018). Makino et al. (2018) demonstrated that elevated CCN2 expression positively correlated with activated HSCs, indicated by smooth muscle actin (ACTA2) expression, in both mouse and human liver tumors. Furthermore, the authors showed that anti-CCN2 reduced IL-6 production in LX-2 cells and inhibited STAT3 activation in HepG2 (human HCC cell line) cells (Makino et al., 2018). This study was the first to establish HCC-cell-derived CCN2 activates HSCs in the TME, thus, accelerating the progression of HCC through cytokine production. These results also support the need for further exploration of CCN2 and other ECM proteins involved in the activation of HSCs.

While HSCs respond to growth factors such as CCN2; once activated, HSCs can also modulate the ECM through secretion and upregulation of proteins such as MMPs (Lachowski et al., 2019), which are needed for HCC tumor migration (Scheau et al., 2019). Studies have shown MMP2 and MMP9 (the most commonly studied MMPs in HCC EMT) to be important for the invasive potential of HCC tumors through degradation and remodeling of collagen in the ECM (Wang et al., 2014; Sun et al., 2018). Moreover, signaling between FAK and MMP9 is considered to be one of the main pathways that promotes HCC cell invasion and metastasis (Chen et al., 2010; Jia et al., 2011). Thus, it is plausible to speculate whether this signaling pathway is promoted through HSC activation. This hypothesis was explored by Han et al. (2014), who investigated whether activated HSCs promote FAK-MMP9 signaling *in vitro*. First, elevated numbers activated HSCs were shown to associate with tumor invasion of the portal vein, advanced tumor node metastasis staging, and lesser tumor differentiation. Thereafter, the number of activated HSCs, quantified by cytoplasmic ACTA2 expression, were positively correlated with the expression levels of phosphorylated FAK (p-FAK) and MMP9 in HCC. Furthermore, the authors used a co-culture experiment to demonstrate the activation of FAK-MMP9 signaling in HCC cells in the presence of activated HSC conditioned medium and with co-culture of activated HSCs. Additionally, inhibition of FAK-MMP9 signaling via small interfering RNA (siRNA) for FAK (siFAK) abrogated the migratory and invasive effects of activated HSCs on HCC cells (Han et al., 2014). These data show that FAK-MMP9 signaling is promoted by activated HSCS and plays a role in modulating metastasis of HCC following activation of HSCs; thus, highlighting the crosstalk between tumor cells and activated HSCs in the hepatic TME.

## Micro-RNA Involvement in HSC Activation in the Hepatic TME

In addition to ECM components such as MMPs and growth factors, recent advances have emphasized the significant roles played by micro-RNAs (miRNAs) in the TME (Cheng et al., 2015). This is demonstrated through the ability of miRNAs in tumor cells to transform the microenvironment by sustaining cancer hallmark traits and non-cell-autonomous signaling pathways (Suzuki et al., 2015). miRNAs are small non-coding RNAs (20-25 nucleotides in length) that regulate gene expression by binding to target mRNA transcripts through a seed sequence at the 5′ end of the miRNA (Bartel, 2004). In cancer cells, miRNAs are aberrantly expressed compared to normal cells, with expression patterns varying between different types of cancer cells (Karube et al., 2005; Lu et al., 2005; Merritt et al., 2008; Garzon et al., 2009). Additionally, activation and inactivation of HSCs can be controlled by profibrogenic and antifibrogenic miRNAs (Tsuchida and Friedman, 2017). Interestingly, miRNAs have been shown to possess dual roles as oncogenes and tumor suppressors in cancer cells (Zhang et al., 2007). The following studies investigate these dual roles in relation to the activation of HSCs in the hepatic TME.

**TABLE 1 |** Components of the hepatic TME.

| Cellular | References | Non-cellular | References |
|---|---|---|---|
| Stromal hepatocytes: connective tissue cells that provide support to the epithelial cells of the liver | Tahmasebi Birgani and Carloni, 2017 | ECM Proteins (Matrix metalloproteinases, collagens, proteoglycans, lamins): key players in the invasive potential of HCC tumors by modulating the ECM | Novikova et al., 2017 |
| Hepatic stellate cells: mesenchymal cells found in the liver that contribute to the hepatic TME by proliferating and promoting fibrosis when activated | Friedman, 2008a; Coulouarn and Clement, 2014 | Growth factors (TGFB, PDGF, CCN2, VEGF, HGF): signaling proteins that stimulate the expression pathways of pro-fibrotic genes; stimulate HSC activation, proliferation and migration | Coulouarn et al., 2012; Thompson et al., 2015; Makino et al., 2018 |
| Immune cells (Ex. Tumor Associated Macrophages and MDSCs): components of the hepatic TME that interact with activated HSCs by creating an immunosuppressed environment promoting HCC tumor growth and maintenance | Budhu and Wang, 2006; Budhu et al., 2006; Fu et al., 2007; Hoechst et al., 2008; Jeong et al., 2011 | Cytokines (IL-6, IL-8, IL-22): small proteins involved in a range of cell signaling that help drive fibrosis, HSC activation, and contribute to angiogenesis | Chiu et al., 2014; Sevic et al., 2019 |
| Cancer associated fibroblasts: type of cancer stromal cell critical to tumorigenesis regulation by possessing the ability to remodel the ECM and secrete proteins such as cytokines and VEGF | Yin et al., 2019 | | |

*Both cellular and non-cellular components cooperate in the hepatic TME to aid in hepatocarcinogenesis.*

Oncogenic miRNAs (oncomiRs) derived from the extracellular vesicles (EV) of HCC cells mediate communication between HCC cells and activated HSCs (Daugaard et al., 2017). Interestingly, crosstalk between miRNAs and TME components is partly mediated by exosomes, a type of EV produced in the endosome of eukaryotic cells that can transfer DNA, RNA and proteins to other cells (Zhang et al., 2015; Kosaka, 2016). Results from Li J. et al. (2019) found that EVs released by HepG2 and Huh7 (human HCC cell line) cells contained elevated oncomiRs. As a result, activated HSCs released their own EVs which stimulated HCC invasion, epithelial to mesenchymal transition (EMT) and activation of the AKT/ERK signaling pathway (Li J. et al., 2019). This suggests a positive feedback loop between exosomal oncomiRs of activated HSCs and HCC cells to promote hepatocarcinogenesis. Moreover, the authors observed upregulation of three specific oncomiRs: miR-21, miR-221, and miR-151. These results are further examples of the crosstalk between HCC cells and activated HSCs creating a pro-metastatic phenotype (Li J. et al., 2019).

Likewise, miR-1426 is an oncomiR shown to promote tumorigenesis, metastasis, and migration in multiple cancer types (Xu et al., 2019). Recently, a miRNA expression microarray study revealed a robust increase in miR-1246 in HCC cell lines when co-cultured with activated HSCs (Huang et al., 2020). These results reflect *in vitro* evidence that activated HSCs induce miR-1246 expression in HCC cell lines to promote metastasis. Moreover, miR-1246 and its target RAR related orphan receptor alpha (RORA), promoted EMT *in vitro* and *in vivo* in nude mice indicated by enhanced HCC cell migration, decreased *E*-cadherin, and increased vimentin protein expression. As a result of miR-1246 overexpression in PLC cells (human liver hepatoma cell line), the binding of RORA and beta-catenin (CTNNB1) in the cytoplasm was increased. This process was reversed with RORA knockdown, suggesting that the binding of RORA to beta-catenin prevents beta-catenin nuclear translocation and activation of the Wnt/beta-catenin signaling pathway. Furthermore, both miR-1246 and RORA were effectively used as independent prognostic markers in HCC tissue (Huang et al., 2020). This data suggests that miR-1246:RORA is a key component in the tumorigenic influence of activated HSCs on HCC cells, and that targeting the miR-1246:RORA axis may slow HCC progression (Huang et al., 2020).

However, miRNAs can also function as tumor suppressors in cancer cells (Zhang et al., 2007). For example, miRNA-212-3p has been shown to suppress cancer cell growth in other forms of cancer such as renal cell carcinoma (Gu et al., 2017) non-small-cell lung cancer (Tang et al., 2017) and glioblastoma (Tang et al., 2017). However, the effects, if any, in HCC remained unclear. Thus, Chen et al. (2019) examined the relationship between miRNA-212-3p and CCN2 in the hepatic TME. The authors showed that microRNA-212-3p inhibited proliferation of HCC cell lines through suppression of CCN2. Additionally, miR-212-3p was downregulated in HCC cell lines and tissues, and negatively correlated with vascular invasion and the absence of a fibrous tumor capsule. This fibrous capsule is formed by host liver mesenchymal cells, instead of HCC cells, and prevents possible invasion of HCC to the host liver (Ishizaki et al., 2001). These findings demonstrate a tumor suppressor role of miRNA-212-3p through its interaction with CCN2, a significant ECM component of the hepatic TME. Moreover, results from this study raise the question of whether microRNA-212-3p also inhibits the CCN2 mediated cytokine production in activated HSCs, given that the experiments from this study were carried out in HCC cell lines and tissues only.

## Activated HSC Regulation of Angiogenesis Within the Hepatic TME

The effects of activated HSCs on angiogenesis in HCC have also been investigated over the past decade. Angiogenesis within the

TME is essential for tumor progression, metastasis and invasion (Mittal et al., 2014). Zhu et al. (2015) identified Interleukin-8 (IL-8) as a contributing factor to angiogenesis in HCC. Interestingly, IL-8 was highly expressed in HCC stroma and was mainly derived from activated HSCs rather than from HCC cells. Furthermore, an IL-8 neutralizing antibody was demonstrated to suppress tumor angiogenesis in Hep3B cells (a human HCC cell line) treated with conditioned media from activated HSCs. The authors also demonstrated similar results *in vivo* through a chick embryo chorioallantoic membrane (CAM) assay. Most recently, Lin et al. observed that activated HSCs are the primary source of secreted angiopoietin-1 (Ang-1) in human HCC cells *in vitro*. This not only describes the promotion of HCC angiogenesis through activated HSCs and Ang-1 expression, but also opens the potential of Ang-1 as an anti-angiogenic therapeutic target in HCC (Lin et al., 2020). These findings identify angiogenic factors produced by activated HSCs in the hepatic TME to promote hepatocarcinogenesis.

Intriguingly, a 2019 investigation found that activated HSCs induced angiogenesis in HCC via upregulation of glioma associated oncogene 1 (Gli-1), a member of the Hh signaling pathway (Yan et al., 2017). Furthermore, this study established that 3,5,4′-trihydroxy-*trans*-stilbene (trade name: Resveratrol), a polyphenol compound found in red-wine, grapes, berries and peanuts and believed to act as an antioxidant, also hindered HCC progression driven by HSCs through targeting Gli-1. Specifically, activated HSC induced angiogenesis in HCC via upregulation of Gli-1 expression, stimulated reactive oxygen species (ROS) production and increased HCC cell invasiveness. Resveratrol further abolished activated HSC-stimulated angiogenesis and suppressed ROS production and IL-6 and C-X-C chemokine receptor type 4 (CXCR4) expression in HepG2 cells by downregulating Gli-1 expression (Yan et al., 2017). In a separate study, Han et al. (2019) also described Resveratrol to possess tumor-suppressive effects through tumor microenvironment modulation across several types of cancers including HCC. This suggests the possibility of Gli-1 as a potential target for angiogenesis prevention in HCC.

## QUIESCENT HEPATIC STELLATE CELLS

While activated HSCs play a major role in the formation of fibrosis and the hepatic TME, recent studies have also delved into the role of quiescent HSCs (qHSCs), as they are needed to maintain a healthy liver (Coll et al., 2015; Das et al., 2020). Uncovering the mechanisms preserving this phenotype could increase the scope of HSC targeted therapies.

Coll et al. (2015) conducted a study which examined these mechanisms and included a miRNA microarray analysis of isolated human qHSCs. The microarray revealed that HSCs express 259 miRNAs. In contrast, when HSCs were activated *in vitro*, 212 of these miRNAs were upregulated and the other 47 miRNAs were downregulated (Coll et al., 2015) suggesting a role for miRNAs in maintenance of qHSCs. Furthermore, the interactions between the miRNA target genes in qHSCs were also associated with HSC activation. Specifically, miRNA-192 was chosen for further *in vivo* study due to having 28 target genes in qHSCs and demonstrating decreased expression in cirrhotic liver samples compared to healthy samples. To elucidate the role of miRNA-192 in qHSCs *in vivo*, HSCs were isolated from two liver fibrosis mouse models and showed decreased miRNA-192 expression compared to healthy mouse HSCs. Furthermore, miRNA-192 overexpression resulted in inhibited Tgfb1 signaling and Pdgf-induced HSC migration in primary mouse HSC cells. This data supports miRNA-192 as a regulator of qHSCs through suppressing target genes needed for HSC activation (Coll et al., 2015). Thus, these findings support additional study into the miRNA-192 targeted genes involved in HSC activation to increase the scope of HSC targeted therapies.

Further supporting the significance of qHSCs, Das et al. demonstrated *in vitro* qHSC induction of cancer cell apoptosis via a caspase-independent mechanism (Das et al., 2020). This mechanism was established in rat hepatoma cells treated with qHSC conditioned media. The study determined that qHSC induction of apoptosis required increased apoptosis-inducing factor (AIF) expression, nuclear localization and DNA fragmentation, and resulted in eventual cell death (Das et al., 2020). This data illustrates the ability of qHSCs to increase toxicity and sensitivity to established chemotherapeutic agents, such as doxorubicin (Das et al., 2020), and could lead to augmentation of therapeutic strategies already in existence to increase their success.

## CLINICAL IMPLICATIONS AND RELEVANCE

Incidence of HCC in the U.S. has increased substantially in the past two decades (Singal et al., 2019; Das et al., 2020; Wu et al., 2020), and the American Cancer Society estimates 32,107 new cases of HCC will be diagnosed in 2020 along with 22,620 deaths (Society, 2020). Additionally, HCC incidence is three times higher in men than in women and the highest incidence is observed amongst patients greater than 70 years old with a steep mortality observed in patients ages 55–69 and older (Beal et al., 2017; Society, 2020). Unfortunately, over 80% of HCC patients present in advanced stages are not amenable to potentially curative surgical therapy, which combined with a paucity of effective systemic therapies, leads to a high morbidity and mortality rate (Zhou et al., 2018; Li D. et al., 2019). Ongoing investigation has focused on the process of tumor progression and potential therapeutic targets to create clinically relevant treatments for patients with HCC. Implicated in this research is the critical role of activated HSCs, from which 80–90% of HCC cells develop (Shiraha et al., 2020).

### Metformin

As mentioned previously, TGFB and PDGF induce HSC activation to contribute to the cellular crosstalk between tumor and stromal cells (Zhang and Friedman, 2012; Tsuchida and Friedman, 2017). GDF15, a member of the TGFB superfamily, is a biomarker for stress responses as a result of cancer treatment damage such as hypoxia and chemotherapy

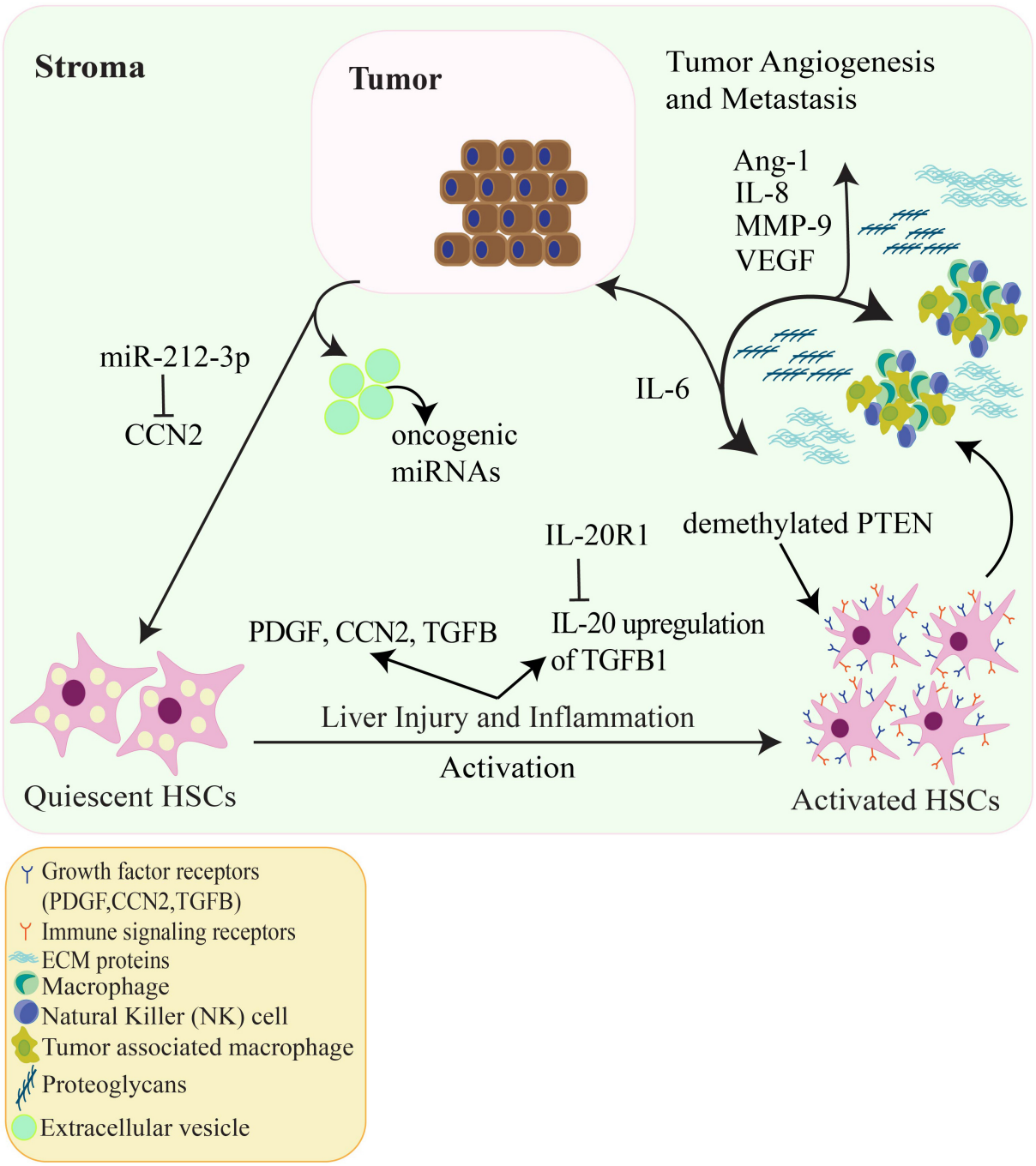


**FIGURE 2 |** The relationship amongst HCC tumor cells, tumor angiogenesis and HSCs in the hepatic TME. Quiescent HSCs are activated by numerous factors such as liver damage, inflammation, PDGF and TGFB signaling, hepatitis and viral infection. Additionally, HSC activation can be epigenetically regulated by demethylation of the tumor suppressor PTEN and IL-20 activation of HSCs via upregulation of TGFB1. Activated HSCs go on to produce ECM proteins and alter the ECM accompanied by proteoglycans and VEGF. Cytokines such as IL-6 are released which promote HCC tumor cell proliferation. CCN2 produced from HCC cells can activate HSCs, in contrast, CCN2 is inhibited through the oncogenic miRNA (oncomir) miR-212-3p which decreases HCC cell invasion *in vitro*. Other oncomirs secreted by HCC extracellular vesicles can regulate signaling between HSCs and HCC cells. In addition, IL-8, MMP-9, and Ang-1 contribute to tumor-associated angiogenesis. These factors allow for HCC tumor proliferation, invasion, and metastasis.

(Kelly et al., 2009; Corre et al., 2013) and may be a clinically relevant target for HCC. Common liver cancer therapy utilizes transarterial chemoembolization (TACE) that involves chemotherapy embolization-induced hypoxia to damage HCC cells; however, this therapy also induces stress in the surrounding HCC tissue (Dong et al., 2018). As a result of the intense

cellular stress, GDF15 is secreted by the treatment-damaged HCC cells, and may have the ability to promote fibrosis through activated HSCs (Dong et al., 2018). To elucidate this process, Dong et al. (2018) demonstrated that HCC cells under "TACE-like" conditions showed increased levels of GDF15 via activation of the p38 MAPK, ERK1/2 and JNK pathways. Metformin, a common FDA approved drug, is known to target the JNK/p38MAPK pathway (Wu et al., 2011). Dong et al. (2018) further illustrated that in activated HSC cells, GDF15 promoted HSC proliferation and collagen production indicated by increased type I collagen protein levels cellular 5-Ethynyl-2′-deoxyuridine (Edu) incorporation, a thymidine analog which incorporates into the DNA of dividing cells (Salic and Mitchison, 2008). Conversely, Metformin was able to target the JNK pathway and suppress GDF15 expression resulting in decreased collagen synthesis and proliferation of activated HSCs *in vitro* and *in vivo* (Dong et al., 2018). These results suggest an opportunity to slow the progression of HCC by targeting activated HSCs with Metformin, a widely available drug.

### Sorafenib

Another FDA approved drug, Sorafenib, is a multi-target anti-angiogenic tyrosine kinase inhibitor (Hasskarl, 2014). Sorafenib was the first systemic therapy approved for treatment of HCC after it was shown to increase mean survival time by 2–3 months (Llovet et al., 2008; Cheng et al., 2009). A recently published study suggested that a novel system of biodegradable dendritic polymeric nanoparticles loaded with Sorafenib enhanced HCC therapy (Li et al., 2020). Through the MTT assay, this system induced higher cytotoxicity of HCC cells than a PEG-conjugated nanoparticle system containing Sorafenib and free-Sorafenib *in vitro*. In addition, tumor growth was significantly subdued in mice with HepG2 xenografts, with minimal side effects (Li et al., 2020). Moreover, a previous nanoparticle related study published in 2018 demonstrated that combined delivery of Sorafenib with a mitogen activated protein kinase (MEK) inhibitor using C-X-C motif chemokine receptor 4 (CXCR4)-targeted nanoparticles reduced hepatic fibrosis and prevented tumor development (Sung et al., 2018). This study further assessed the effects of Sorafenib on activated HSCs and established that combined delivery of Sorafenib and a MEK inhibitor through CXCR4-targeted nanoparticles prevented ERK activation in activated HSCs and had anti-fibrotic effects in the $CCl_4$-mouse model (Sung et al., 2018).

### Bevacizumab

Continued interest in the critical role of activated HSCs in the regulation of angiogenesis will likely increase in light of recently released preliminary results of the IMbrave150 trial which challenges the longstanding paradigm of first line Sorafenib for advanced unresectable HCC. This study was a phase III randomized control trial of 336 patients who received either atezolizumab (PD-L1 inhibitor) plus Bevacizumab (VEGF-A inhibitor) or Sorafenib. Preliminary results from the trial demonstrated a significant overall survival benefit, with patients in the combination arm not reaching a median survival versus a median survival of 13.2 months with Sorafenib ($p < 0.0001$) (Cheng et al., 2019). Bevacizumab has been shown *in vivo* to decrease expression of profibrogenic genes TGFB and ACTA2, as well as decreasing overall HSC activation, altogether attenuating hepatic fibrosis in a $CCl_4$-rat model (Huang et al., 2013). Although final results of the study have not yet been published, it is widely expected to be practice changing and highlights the importance of both the immune and angiogenic crosstalk in the hepatic TME and HCC progression.

### Potential Alternative Therapies

Finally, there is an interest in therapeutically targeting the cytokine-HSC interaction. An IL-8-neutralizing antibody (Zhu et al., 2015) and an anti-CCN2 neutralizing antibody which lead to reduced IL-6 production (Makino et al., 2018) have both shown therapeutic potential in suppressing tumor progression of HCC *in vitro* and *in vivo* with a xenograft murine model. These neutralizing antibodies, which both target interleukin cytokines, are potential clinical therapies focusing on the relationship between activated HSCs and TME. Additionally, a 2015 study established the angiogenin inhibitor neomycin as a potential HCC therapy. This study demonstrated that neomycin decreased HSC activation with conditioned media or recombinant angiogenin *in vitro* (Barcena et al., 2015). Furthermore, neomycin administration reduced tumor growth of HepG2-LX2 cells co-injected into mice, suggesting that angiogenin secretion by HCC cells favors tumor development via induction of HSC activation and ECM remodeling. These findings not only suggest that targeting angiogenin signaling may be of potential relevance in HCC management, but also establishes neomycin as a potential clinical treatment for HCC.

## DISCUSSION

Hepatic stellate cells activation is the central event of hepatic fibrosis and the development of cirrhosis and HCC. A fundamental gap in knowledge is the crosstalk between activated HSCs, the hepatic ECM and HCC tumor cells. Recent studies have focused on targeted molecular therapeutic strategies for liver fibrosis and cirrhosis (van der Heide et al., 2019). Thus, utilizing multiple biomarkers may lead to optimized early detection of HCC (Ismail and Pinzani, 2011; Tuohetahuntila et al., 2017). Data published in 2017 suggested a new therapeutic option to target and increase NK activity in patients with chronic hepatitis infection preceding hepatic fibrosis (Shi et al., 2017). Other recent studies discussing potential targeted therapies such as PDGF-C and TGFB are under exploration. Ultimately, elucidating the mechanistic links between activated HSCs through all stages of fibrosis and cirrhosis will lead to a better understanding of HCC tumorigenesis.

Due to the intricate relationship between the hepatic TME, tumor development and HCC progression, recent studies have begun focusing on the role of activated HSCs, one of the prominent factors involved in the hepatic TME. The hepatic TME provides a niche which includes both cellular components, such as HSCs and non-cellular components, being the ECM and

ECM proteins. As activated HSCs become ECM producing cells during liver fibrosis, secreted chemokines, cytokines, and growth factors prime the overall TME for supporting HCC proliferation.

Owing to the complexity of the TME, it is difficult to therapeutically target one pathway as they all have functional redundancies; however, targeting a pivotal cellular component of the hepatic TME, such as activated HSCs, may be more feasible. Pertinent to this review, studies focusing on the role of activated HSCs in the TME could lead to activated HSC targeted therapies that may affect activated HSC related factors such as TGFB, PDGF, MMP-9, CCN2, and oncogenic miRNAs. Other targets that warrant further study and serve as promising areas for therapeutic exploration include IL-8, Ang-1, and Gli-1. A proposed schematic illustrating the relationship between activated HSCs in the hepatic TME and HCC is shown in **Figure 2**.

Collectively the literature covered in this review outline the significance of activated HSCs in the hepatic TME. In addition to cellular crosstalk within the TME, activated HSCs play a crucial role in HCC progression through the TME's non-cellular components. These recent studies provide examples of cytokines, growth factors, ECM components and microRNAs, which are all crucial non-cellular components of the hepatic TME involved in the relationship between activated HSCs and HCC development.

This review also concentrated on clinical implications that highlight potential therapies for HCC through targeting activated HSCs and their relationship with HCC cells. In addition to enhancing the efficacy of current therapeutic agents such as Metformin, Sorafenib, and Atezolizumab/Bevacizumab, potential alternative therapies include neomycin and neutralizing antibodies against IL-22 and CCN2.

A deeper understanding of how the hepatic TME, most notably activated HSCs, interacts with the primary tumor and non-tumor cells will propel advances in effective diagnostic and prognostic tools. Ongoing investigations are imperative in order to develop more effective treatments for HCC and augment current therapies to increase their success.

## AUTHOR CONTRIBUTIONS

AB and RB equally drafted the review, compiled the authors' contributions and references, wrote the manuscript, and produced the table and figures. RL, KP, KZ, DD, RB, AS, and KK contributed by writing and editing various sections within the manuscript. HD provided the overall support, intellectual input, critical evaluation, and all rounds of editing. All the authors read and approved the manuscript.

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# Tumor-Educated Neutrophils Activate Mesenchymal Stem Cells to Promote Gastric Cancer Growth and Metastasis

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In response to tumor signals, mesenchymal stem cells (MSCs) are recruited to tumor sites and activated to promote tumor progression. Emerging evidences suggest that in addition to tumor cells, non-tumor cells in tumor microenvironment could also interact with MSCs to regulate their phenotype and function. However, the mechanism for MSCs regulation in gastric cancer has not been fully understood. In this study, we reported that tumor-educated neutrophils (TENs) induced the transformation of MSCs into cancer-associated fibroblasts (CAFs) which in turn remarkably facilitated gastric cancer growth and metastasis. Mechanistic study showed that TENs exerted their effects by secreting inflammatory factors including IL-17, IL-23 and TNF-α, which triggered the activation of AKT and p38 pathways in MSCs. Pre-treatment with neutralizing antibodies to these inflammatory factors or pathway inhibitors reversed TENs-induced transformation of MSCs to CAFs. Taken together, these data suggest that TENs promote gastric cancer progression through the regulation of MSCs/CAFs transformation.

**Keywords: neutrophils, mesenchymal stem cells, cancer-associated fibroblasts, gastric cancer, progression**

## INTRODUCTION

Gastric cancer (GC) is one of the most common malignant tumors worldwide. Although the recent advances in curative resection and targeted therapy, the overall survival rate of patients with GC is still poor with a 5-year survival rate of 20–40% (Bray et al., 2018). The mechanisms for the pathogenesis of GC have not been fully understood. Intriguingly, recent studies demonstrate that the interactions between GC cells, immune cells, and stromal cells orchestrate a unique microenvironment that promotes tumor growth, metastasis, therapy resistance, and recurrence (Hanahan and Weinberg, 2011; Quail and Joyce, 2013).

Neutrophils are the first defense against infection and tissue damage. The studies over the past decade have revealed an important role of neutrophils in the pathogenesis of many cancers (Swierczak et al., 2015; Liang and Ferrara, 2016; Powell and Huttenlocher, 2016). Neutrophils infiltrating within tumor stroma were educated by signals from tumors to promote tumor growth and metastasis, enhance angiogenesis, and mediate immunosuppression through multiple mechanisms (Houghton et al., 2010; Antonio et al., 2015; Coffelt et al., 2015;

Wculek and Malanchi, 2015; Szczerba et al., 2019; Wisdom et al., 2019). Moreover, elevated neutrophil to lymphocyte ratio (NLR) in cancer patients has been linked to poor prognosis (Shen et al., 2014). Targeting neutrophils to inhibit their pro-tumor function has shown promising therapeutic effects in animal models (Nywening et al., 2018). Therefore, better understanding of the roles of neutrophils in cancer will help develop new strategies for cancer therapy.

Non-tumor cells within tumor microenvironment (TME) interact with each other and form an intricate network that is involved in tumor development and progression. The previous studies have shown that cancer-associated fibroblasts (CAFs) facilitate cancer growth and metastasis in various cancers including GC (Chen and Song, 2019; Kobayashi et al., 2019). Further studies suggest that mesenchymal stem cells (MSCs) are one of the major sources of CAFs (Timaner et al., 2020). MSCs are stem cells with self-renewal and multi-differentiation abilities and they have shown tumor tropism to participate in the formation of tumor stroma (Quante et al., 2011). MSCs-like cells have been isolated from tumor tissues of GC patients and these cells display CAFs phenotypes with strong pro-tumor activities (Cao et al., 2009; Wang et al., 2014). MSCs can be activated by various tumor signals to present CAFs phenotype and function (Ridge et al., 2017). However, the mechanisms for MSCs transformation to CAFs in GC still have not been well characterized.

In this study, we reported that tumor-educated neutrophils (TENs) could mediate the transformation of MSCs to CAFs by secreting inflammatory factors. MSCs activated by TENs promoted the proliferation, migration and invasion of GC cells *in vitro* and accelerated GC growth and metastasis *in vivo*. The induction of MSCs/CAFs transformation represents a new mechanism for the pro-tumor roles of neutrophils in cancer.

# MATERIALS AND METHODS

## Cell Culture

Mesenchymal stem cells were isolated from human umbilical cord and characterized as previously described (Cao et al., 2009; Wang et al., 2014). Cells at passage 3–5 were used for studies. The experimental protocol was approved by the Ethics Committee of Jiangsu University (2014280). Human GC cell lines SGC-7901 and BGC-823 were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco; Thermo Fisher Scientific, Waltham, MA, United States) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, United States).

## Isolation of Human Neutrophils

Peripheral blood samples were collected from healthy volunteers and the study was approved by the ethics committee of Jiangsu University (2014280). Neutrophils were isolated by using Polymorphprep (Axis-Shield Po CAS, Norway) as previously described (Zhu et al., 2014). RBCs were lysed using hypotonic lysing procedure. The purity of neutrophils was 98% after this procedure. Neutrophils ($1 \times 10^6$) were seeded in RPMI 1640 medium (Invitrogen, United States) containing 10% (v/v) FBS and 1% penicillin/streptomycin.

## Collection of Conditioned Medium (Cm)

Firstly, GC cells were cultured in DMEM with 10% FBS. When reached 80%, cells were changed to new serum-free medium. After 48 h of incubation, the conditioned medium of gastric cancer cells (GC-CM) was collected and centrifuged to remove cell debris. The CM was stored in −80°C refrigerator until use. Secondly, neutrophils were treated with GC-CM (1:1 ratio) for 24 h. Then, neutrophils were collected, washed with PBS (to remove factors present in GC-CM), and re-seeded in new serum-free medium for 48 h. The CM of tumor-educated neutrophils (TEN-CM) was collected, centrifuged to remove cell debris and stored in −80°C refrigerator until use. Finally, MSCs were co-cultured with TEN-CM (1:1 ratio) for 24 h. Then, MSCs were collected, washed with PBS (to remove factors present in TEN-CM), and re-seeded in new serum-free medium for 48 h. The CM of TEN-CM-primed MSCs was collected, centrifuged to remove cell debris and stored in −80°C refrigerator until use.

## Cell Colony Formation Assay

Human GC cells were plated in 6-well plates at 800 cells per well and allowed to attach overnight. The cells were then treated with CM from TENs-activated MSCs for 24 h. Then, the CM was changed and added new culture medium for the next 7 days. Finally, the cells were fixed in 4% paraformaldehyde, stained with crystal violet, and photographed under a microscope. The experiments were performed in triplicate for each group.

## Cell Proliferation Assay

The proliferative abilities of GC cells and MSCs were determined by CCK8 (Vazyme, Nanjing, China) assay according to the manufacture's instruction. Cells were seeded in 96-well plates at 3,000 cells per well and allowed to attach overnight at 37°C in 5% $CO_2$. Then, different CMs were added into the culture plate and cells were treated for different times. The culture medium was discarded and CCK8 was added into each well for the last 4 h. The absorbances of each well were read at 450 nm using an enzyme-linked immunosorbent plate assay reader (FLX800; BioTek Instruments, Winooski, VT, United States). The experiments were performed in triplicate for each group.

## Cell Migration and Invasion Assays

Cell migration and invasion assays were tested in transwell chemotaxis chambers (Corning, Union City, CA, United States). Briefly, an appropriate 50 microliters of Matrigel (1:4 dilution, BD Biosciences) was added into the upper chamber of the transwell plates for the invasion assay, while the plates without Matrigel in the upper chamber were used for the migration assay. Cells ($3–5 \times 10^4$) treated with different CMs for 24 h were seeded in the upper chamber in serum-free medium. Then, 600 μL of complete medium was added to the lower chamber and the cells were incubated for 24–48 h at 37°C in 5% $CO_2$. Cells which remained on the top side of the membrane were wiped off with

cotton swabs. The migrated or invaded cells on the bottom side of the membrane were fixed in 4% paraformaldehyde and stained with crystal violet. The number of migrated or invaded cells was counted under a microscope. At least six fields from each group were selected.

## Real-Time Quantitative PCR

Total RNA was extracted from cells using Trizol reagent (Thermo Fisher Scientific, United States) according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed into cDNA by Reverse Transcription System (Vazyme, Nanjing, China). To quantify the mRNA levels of inflammatory factors, real-time PCR was performed using SYBRGreen Kit (Cwbio, Beijing, China) on a Bio-Rad CFX96 Detection System. The relative gene expression was normalized to β-actin. Data were analyzed by using the comparative Ct method. The primers of target genes used in this study were listed in **Supplementary Table 1**.

## Western Blot

The total protein from GC cells and MSCs were obtained with RIPA lysis buffer containing protease inhibitor cocktail (Invitrogen, United States). Proteins from each group were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with 5% non-fat milk for 1 h, the membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies including FAP (SAB, United States), α-SMA (BioWorld, United States), β-actin (SAB, United States), AKT/p-AKT (CST, United States), p38/p-p38 (CST, United States), STAT3/p-STAT3 (CST, United States), p65/p-p65 (CST, United States), ERK/p-ERK (CST, United States), N-cadherin (BioWorld, United States), E-cadherin (BioWorld, United States), Cyclin D1 (Abcam, United States), and PCNA (BioWorld, United States). The membranes were washed with TBST and incubated with secondary antibodies. The secondary antibodies were HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies (SAB, United States). The bands were visualized by ECL reagent and the β-actin was served as the loading control.

## Animal Studies

All animal studies were performed by using 4–6 week female BALB/c nude mice (Model Animal Center of Nanjing University, Nanjing, China). GC cells (SGC-7901, $5 \times 10^6$ cells in 100 μL PBS per mouse) pre-treated with different CMs for 24 h were subcutaneously injected into the flank of mice ($n$ = 5/group). The volume (V) and weight of tumor were assessed every 5 days, and tumor volumes were calculated using the formula: $V = 0.5 \times a \times b^2$, where $V$ represents volume, $a$ represents longitudinal diameter and $b$ represents latitudinal diameter. To examine the metastatic ability of GC cells *in vivo,* the same number of GC cells was injected intraperitoneally into mice. All mice were killed at 4 weeks after injection, the metastases of tumor to different organs were observed and the number of visible metastatic nodules was recorded. The protocol was approved by the Animal Use and Care Committee of Jiangsu University (2014280).

## Immunohistochemistry

Tumor tissues were fixed with formaldehyde, embedded in paraffin and then cut into 5 μm sections. Briefly, the slides were dewaxed in xylene and rehydrated in graded ethanol solutions. Then, the sections were soaked in $H_2O_2$ to block endogenous peroxidase activity and autoclaved in a citrate buffer of pH6.0. The sections were incubated with primary monoclonal antibody against Ki-67 (CST, United States) followed by incubation with the secondary antibody for 30 min at room temperature and stained with H&E, respectively. The histological changes of tumor tissue were examined under an optical microscope (DP73; Olympus, Tokyo, Japan).

## Immunofluorescence

Immunofluorescence was performed to determine the expression of proteins related to CAFs markers. Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, then blocked with 5% bovine serum albumin, and incubated with goat anti-rabbit FAP (1:100, SAB, United States) and goat anti-mouse α-SMA antibody (1:150, BioWorld, United States) at 4°C overnight. The MSCs were then washed and incubated with Alexa Fluor 555 conjugated donkey anti-rabbit IgG or FITC conjugated goat anti-Rabbit IgG (Invitrogen, United States) for 1 h. The nuclei were stained with DAPI (1:200, Sigma-Aldrich) and images were acquired using a fluorescent microscope (Nikon, Japan). The experiments were repeated in triplicate for each group.

## Statistical Analysis

Statistical analyses were carried out by GraphPad Prism Software (version 4). All values were presented as mean values ± SD. Two-way ANOVA for multiple groups and unpaired Student's $t$ test for two groups were applied for statistical analysis. $P < 0.05$ was considered statistically significant.

# RESULTS

## Neutrophils Treated With the Supernatant of Gastric Cancer Cells Stimulate the Transformation of MSCs to CAFs

We first wanted to know whether TENs could activate MSCs. Human peripheral blood derived neutrophils were treated with the supernatant from GC cells and the CM of primed neutrophils was collected. After then, MSCs isolated from human umbilical cord were treated with TEN-CM for 24 h and the alterations in their phenotype and function were examined. The results of western blot showed that treatment with CM from TENs induced the expression of CAFs markers, including fibroblast

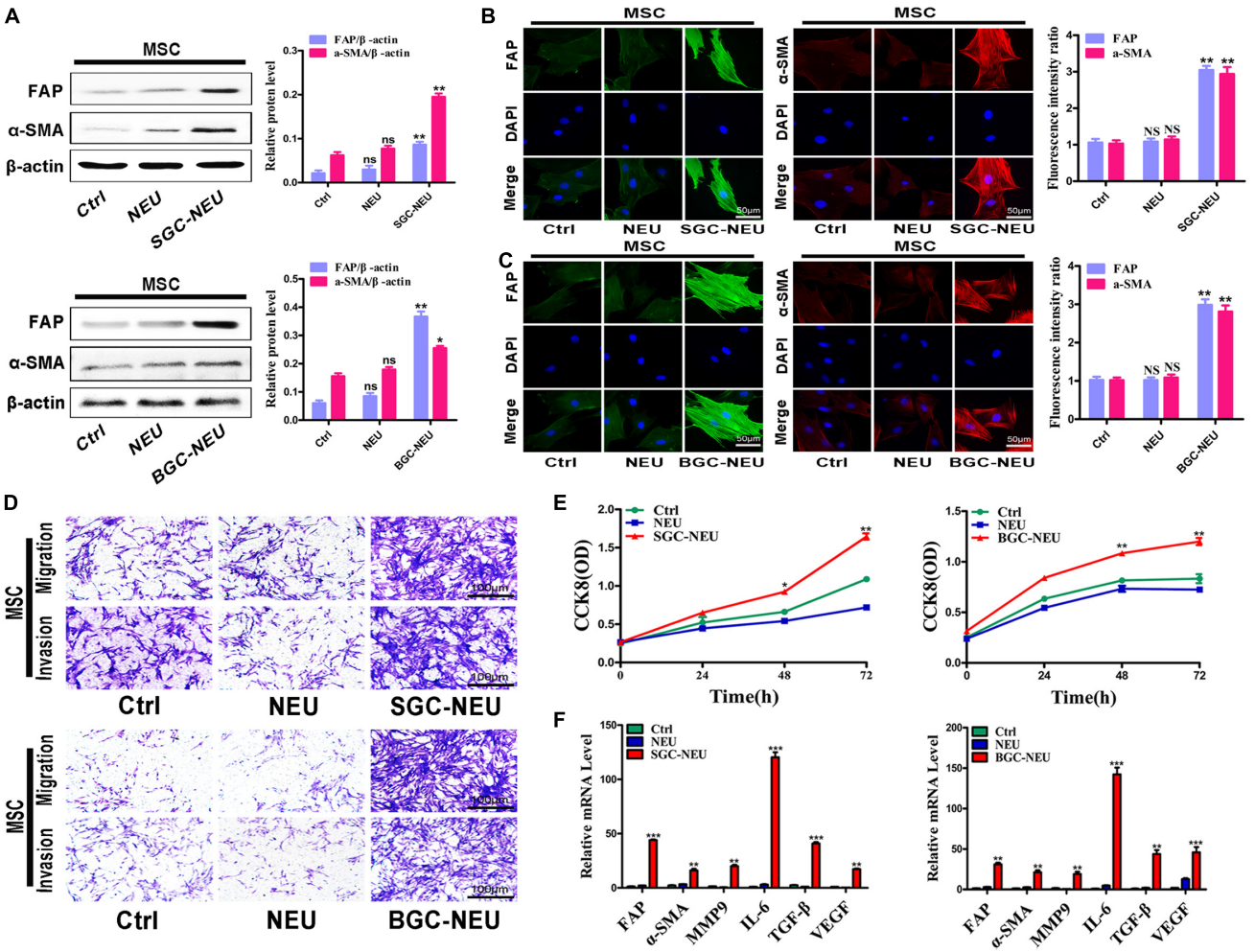


**FIGURE 1 |** Tumor-educated neutrophils stimulate the transformation of MSCs to CAFs. **(A)** Western blot assays for the expression of FAP and α-SMA in MSCs treated with supernatant from neutrophils. **(B,C)** Immunofluorescent staining of FAP and α-SMA in MSCs after pretreated with different supernatant from SGC-NEU **(B)** and BGC-NEU **(C)**. **(D)** Cell migration/invasion assays for MSCs treated with supernatant from neutrophils. **(E)** CCK8 assay to detect the effect of supernatant from neutrophils on the proliferation of MSCs. **(F)** qRT-PCR analyses of the expression of FAP, α-SMA, MMP9, IL-6, TGF-β, and VEGF genes in MSCs treated with supernatant from neutrophils. Ctrl, MSCs; NEU, MSCs treated with CM of neutrophils; SGC/BGC-NEU, MSCs treated with CM from TENs. Magnification: 40×. ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to control group).

activating protein (FAP) and α-smooth muscle actin (α-SMA), in MSCs (**Figure 1A**). Quantitative analysis of western blot also revealed an increase in FAP and α-SMA expression, in TEN-CM-treated MSCs (**Figure 1A**). Immunofluorescent staining and the statistical analysis of immunofluorescence intensity further demonstrated that TEN-CM stimulated the transformation of MSCs to CAFs (**Figures 1B,C**). Moreover, the migration and invasion capacities of MSCs were remarkably enhanced after incubation with TEN-CM (**Figure 1D**). We then performed CCK8 assay to determine the effect of TEN-CM on MSCs growth. As shown in **Figure 1E**, TEN-CM significantly promoted the proliferation of MSCs at 48 and 72 h after incubation. In addition, TEN-CM-treated MSCs showed increased expression of FAP, α-SMA, MMP9, IL-6, TGF-β, and VEGF genes (**Figure 1F**). Taken together, these results indicate that TEN-CM could induce MSCs to differentiate into CAFs and promote its proliferation and migration *in vitro*.

## Tumor-Educated Neutrophils Induce MSCs/CAFs Transformation Through AKT and p38 Signaling Pathways

To clarify the mechanism for MSCs activation by TENs, we treated MSCs with different TEN-CMs for 24 h and measured the responses of cell signaling pathway. TEN-CM treatment increased the expression of phosphorylated AKT (p-AKT) and p38 (p-p38) in MSCs and the statistical analysis of western blot (**Figures 2A,B**), indicating activation of AKT/p38 pathways. Moreover, pre-treatment with LY294002 and SB203580 restricted the increases in the proliferation abilities of MSCs after TEN-CM treatment (**Figure 2C**). In consistent with these observations,

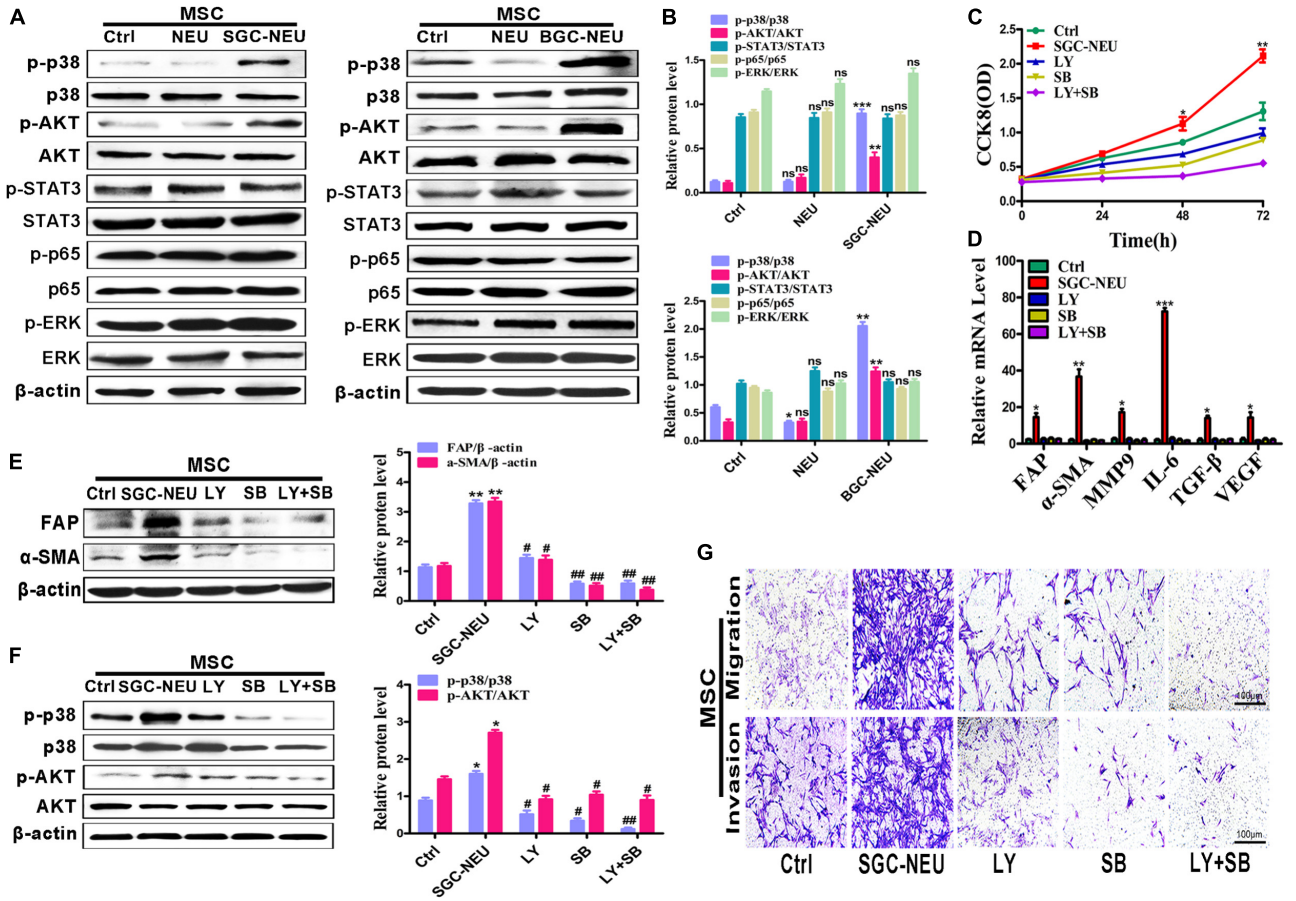


**FIGURE 2 |** Tumor-educated neutrophils induce MSCs/CAFs transformation through AKT and p38 signaling pathways. **(A,B)** The expression of cell signaling pathway in MSCs treated with supernatant from neutrophils was determined by western blot **(A)** and the quantification of protein expression level **(B)**. **(C)** CCK8 assay to assess the proliferation of MSCs treated with supernatant from TENs in the presence or absence of AKT and p38 pathway inhibitors. **(D)** qRT-PCR analyses of the expression of FAP, α-SMA, MMP9, IL-6, TGF-β, and VEGF genes in MSCs treated with supernatant from TENs in the presence or absence of AKT and p38 pathway inhibitors. **(E)** MSCs were pre-treated with AKT and p38 pathway inhibitors followed by incubation with supernatant from TENs. The expression of FAP and α-SMA in MSCs was detected by western blot. **(F)** MSCs were pre-treated with AKT and p38 pathway inhibitors followed by incubation with supernatant from TENs. The expression of p-AKT and p-p38 in MSCs was detected by western blot. **(G)** MSCs were incubated with supernatant from TENs in the presence or absence of AKT and p38 pathway inhibitors and then used for cell migration/invasion assays. Ctrl, MSCs; NEU, MSCs treated with CM of neutrophils; SGC/BGC-NEU, MSCs treated with CM from TENs; LY, MSCs treated with CM from TENs in the presence of AKT pathway inhibitor (LY294002); SB, MSCs treated with supernatant from TENs in the presence of p38 pathway inhibitor (SB203580); LY + SB, MSCs pre-treated with AKT and p38 pathway inhibitors followed by incubation with CM from TENs. Magnification: 40×. ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to control group; $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared to SGC-NEU group).

LY294002 and SB203580 almost completely blocked TEN-CM-induced expression of FAP, α-SMA, MMP9, IL-6, TGF-β, and VEGF genes in MSCs (**Figure 2D**). The induced expression of CAFs markers (FAP and α-SMA) in MSCs by TEN-CM was also reversed by specific inhibitors of AKT and p38 pathways (**Figure 2E**). Notably, pre-treatment with inhibitors of AKT (LY294002) and p38 (SB203580) pathways blocked the activation of AKT and p38 (**Figure 2F**) by TEN-CM. In addition, we also detected the effects of AKT (LY294002) and p38 (SB203580) pathway inhibitors on MSCs (control cells) alone. The pre-treatment with LY294002 and SB203580 shown no remarkable difference on the proliferation and the expression of inflammatory factor in MSCs (**Supplementary Figures S1A,B**). Consistent with that observation, LY294002 and SB203580 have no obvious impact on the expression of CAFs markers (FAP and α-SMA) and the activation of AKT and p38 in MSCs (**Supplementary Figures S1C,D**). Besides, the migration abilities of MSCs were inhibited after pretreated with LY294002 and SB203580 (**Figure 2G**). Taken together, these results indicate that TENs induce MSCs transformation to CAFs via activating AKT/p38 pathways.

## MSCs/CAFs Transformation Induced by Tumor-Educated Neutrophils Promotes the Proliferation, Migration, and Invasion of Gastric Cancer Cells *in vitro*

We then wanted to know the functional roles of MSCs/CAFs transformation induced by TENs. MSCs were treated with TEN-CM and the culture supernatant from MSCs was collected

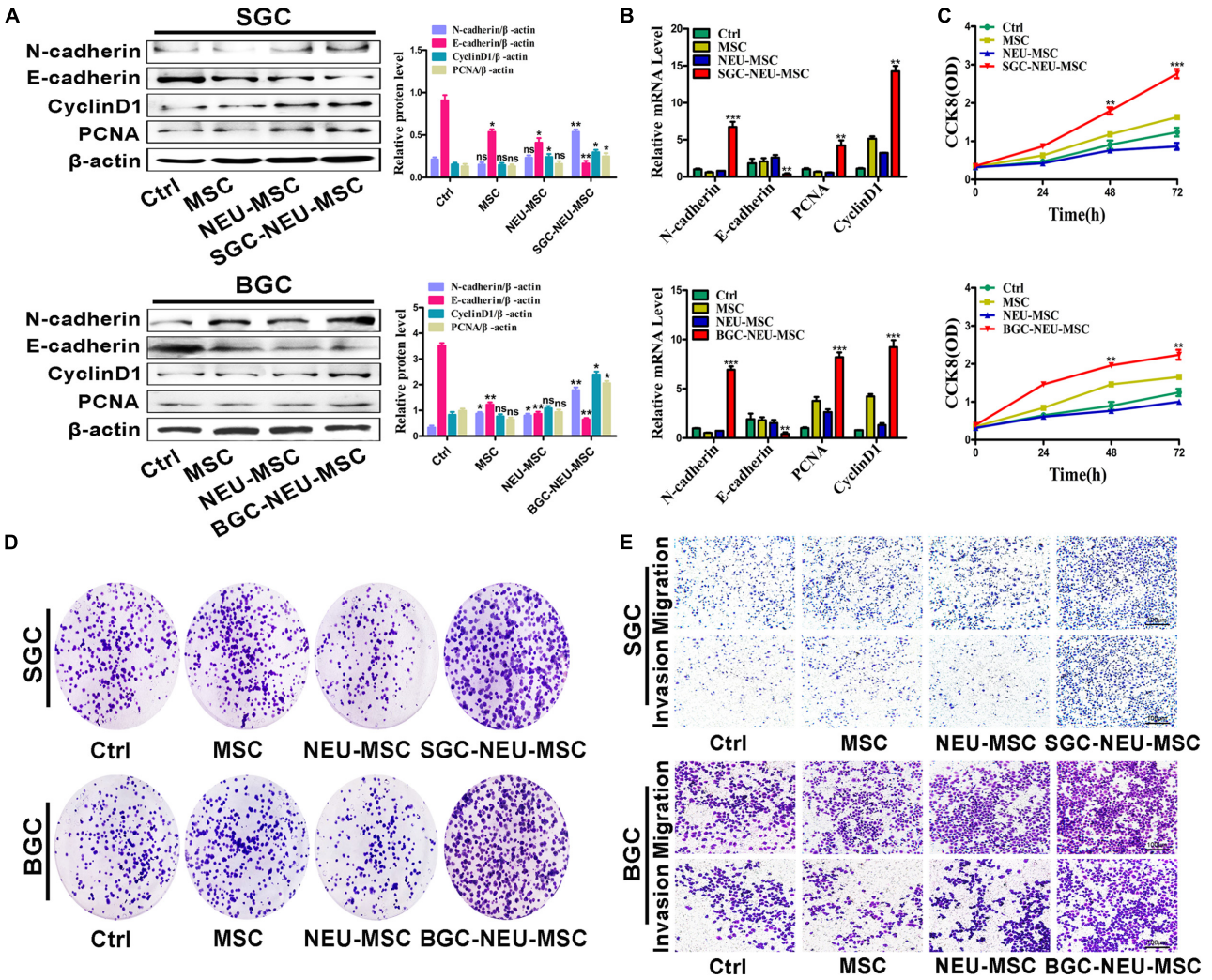


**FIGURE 3 |** MSCs/CAFs transformation induced by gastric cancer-activated neutrophils promotes the proliferation, migration and invasion of gastric cancer cells *in vitro*. **(A)** Western blot assays for E-cadherin, N-cadherin, Cyclin D1, and PCNA expression in gastric cancer cells treated with different supernatant from neutrophils-activated MSCs. **(B)** The expression of E-cadherin, N-cadherin, Cyclin D1, and PCNA genes in gastric cancer cells treated with different supernatant from neutrophils-activated MSCs was determined by qRT-PCR. **(C)** The proliferation of gastric cancer cells treated with different supernatant from neutrophils-activated MSCs was detected by CCK8 assay. **(D)** Cell colony formation assays for gastric cancer cells that had been incubated with distinct supernatant from neutrophils-activated MSCs. **(E)** Transwell migration and matrigel invasion assays for gastric cancer cells treated with distinct supernatant from neutrophils-activated MSCs. Ctrl, SGC-7901/BGC-823; MSC, Gastric cancer cells treated with CM of MSCs; NEU-MSC, Gastric cancer cells treated with CM from neutrophils-primed MSCs; SGC/BGC-NEU-MSC, Gastric cancer cells pre-treated with the CM of MSCs activated by TENs. Magnification: 40×. ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to control group).

to act on GC cells for 24 h. We first tested the expression of proliferation and migration related proteins and genes in GC cells. We found that the expression of N-cadherin, Cyclin D1 and PCNA was increased, and the expression of E-cadherin was decreased in GC cells after treatment (**Figures 3A,B**). Then, the proliferation of GC cells was detected by CCK8 assay and cell colony formation assay. The proliferation abilities of GC cells treated with culture supernatant from TEN-CM-primed MSCs for 24 h were obviously higher than that of control cells (**Figures 3C,D**). The results of cell migration/invasion assays showed that the culture supernatant from TEN-CM-primed MSCs significantly increased the migration and invasion abilities of GC cells (**Figure 3E**). Collectively, these data indicate that TENs-induced MSCs/CAFs transformation promotes the proliferation, migration, and invasion of GC cells.

## Tumor-Educated Neutrophils Induce MSCs/CAFs Transformation Through the Release of Inflammatory Factors

To explore which molecules released by TENs induce MSCs/CAFs transformation, we measured the expression of inflammatory factors in neutrophils after treatment with the

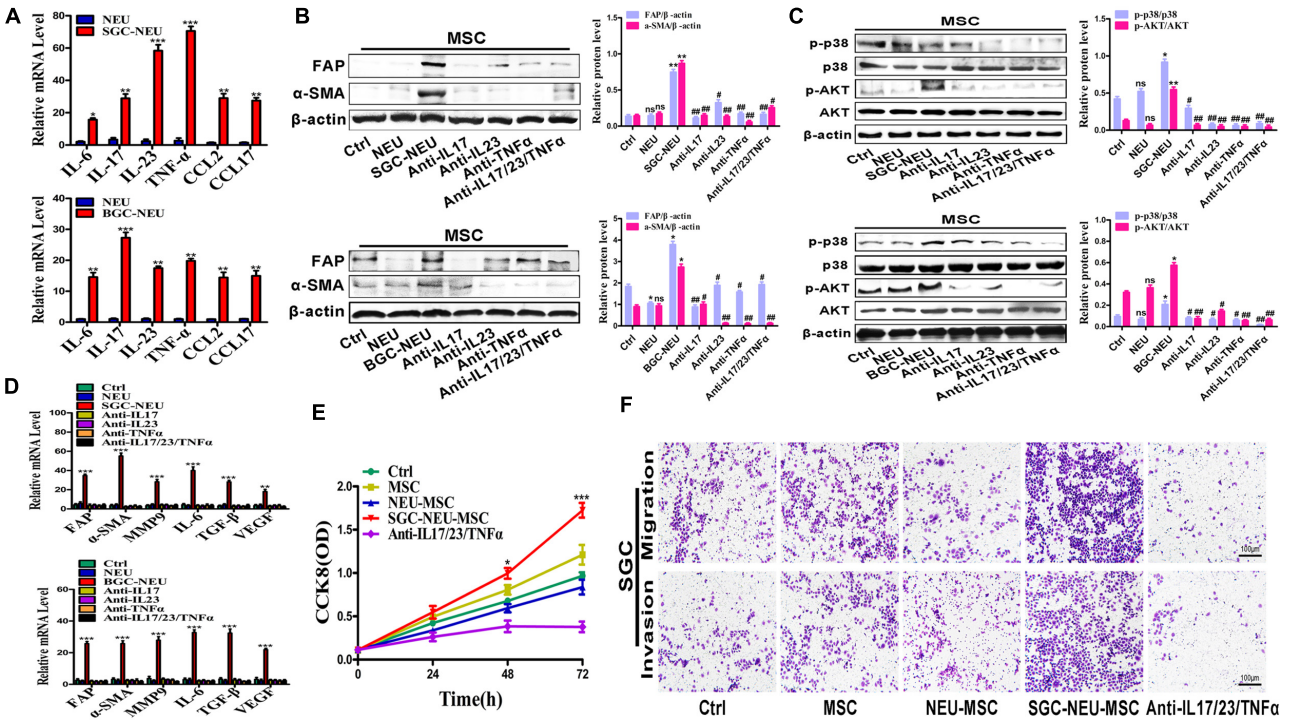


**FIGURE 4 |** Tumor-educated neutrophils induce MSCs/CAFs transformation through the release of inflammatory factors. **(A)** The expression of inflammatory factor genes (IL-6, IL-17, IL-23, TNF-α, CCL2, and CCL17) in neutrophils was determined by qRT-PCR. **(B)** The expression of FAP and α-SMA in MSCs treated with supernatant from TENs in the presence or absence of neutralizing antibodies was determined by western blot. **(C)** The expression of p-AKT and p-p38 in MSCs treated with supernatant from TENs in the presence or absence of neutralizing antibodies was detected by western blot. **(D)** qRT-PCR analyses of the expression of FAP, α-SMA, MMP9, IL-6, TGF-β, and VEGF genes in MSCs treated with supernatant from TENs in the presence or absence of neutralizing antibodies. Ctrl, MSCs; NEU, MSCs treated with CM of neutrophils; SGC/BGC-NEU, MSCs treated with CM from TENs; Anti-IL17/23/TNFα, MSCs treated with supernatant from TENs in the presence of neutralizing antibodies. **(E)** CCK8 assay for the proliferation of gastric cancer cells treated with supernatant from TEN-CM-primed MSCs in the presence or absence of neutralizing antibodies. **(F)** Transwell migration and matrigel invasion assays for gastric cancer cells treated with supernatant from TEN-CM-primed MSCs in the presence or absence of neutralizing antibodies. Ctrl, SGC-7901 cells; MSC, SGC-7901 cells treated with CM of MSCs; NEU-MSC, SGC-7901 cells treated with CM from neutrophils-primed MSCs; SGC-NEU-MSC, SGC-7901 cells treated with CM of MSCs activated by TENs; Anti-IL17/23/TNFα, SGC-7901 cells treated with supernatant from TEN-CM-primed MSCs in the presence of neutralizing antibodies. Magnification: 40×. ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to control group; $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared to SGC/BGC-NEU group).

supernatant from GC cells. Several genes of interest such as IL-17, IL-23, and TNF-α were identified to be highly expressed in the treated neutrophils (**Figure 4A**). We chose these three factors for the following studies due to their known relationships with pro-tumor roles of neutrophils and MSCs activation. Then, MSCs were treated with TEN-CM in the presence or absence of neutralizing antibodies against IL-17 (0.1 μg/mL), IL-23 (0.7 μg/mL) and TNF-α (0.05 μg/mL). Compared to SGC/BGC-NEU group, the expression of FAP and α-SMA proteins in neutralizing antibody group were significantly reduced (**Figure 4B**). The activation of AKT and p38 signaling pathway in MSCs by different TEN-CMs was significantly inhibited when neutralizing antibodies were added and the statistical analysis of western blot showed the same conclusion (**Figure 4C**). In addition, the expression of FAP, α-SMA, MMP9, IL-6, TGF-β, and VEGF genes in MSCs in neutralizing antibody group was also lower than that in SGC/BGC-NEU group (**Figure 4D**). Then, we detected the effects of anti-IL17, anti-IL23 and anti-TNFα on MSCs in control group. We found that there was almost no significant difference in the expression of FAP, α-SMA, MMP9, IL-6, TGF-β, and VEGF genes in MSCs between the groups (**Supplementary Figure S2A**). Compared to MSC group, the expression of CAFs markers and the activation of AKT and p38 signaling pathway in neutralizing antibody group had no remarkable difference (**Supplementary Figures S2B,C**). Moreover, the promotion of GC cell proliferation, migration and invasion abilities by culture supernatant from TEN-CM-primed MSCs was inhibited by the addition of neutralizing antibodies (**Figures 4E,F**). In summary, these data suggest that TENs induce MSCs/CAFs transformation through the release of IL-17/IL-23/TNF-α.

## Tumor-Educated Neutrophils Induce MSCs/CAFs Transformation to Promote Gastric Cancer Growth and Metastasis *in vivo*

To further confirm the significance of MSCs/CAFs transformation induced by TENs in tumor progression, we treated GC cells for 24 h with culture supernatant from MSCs

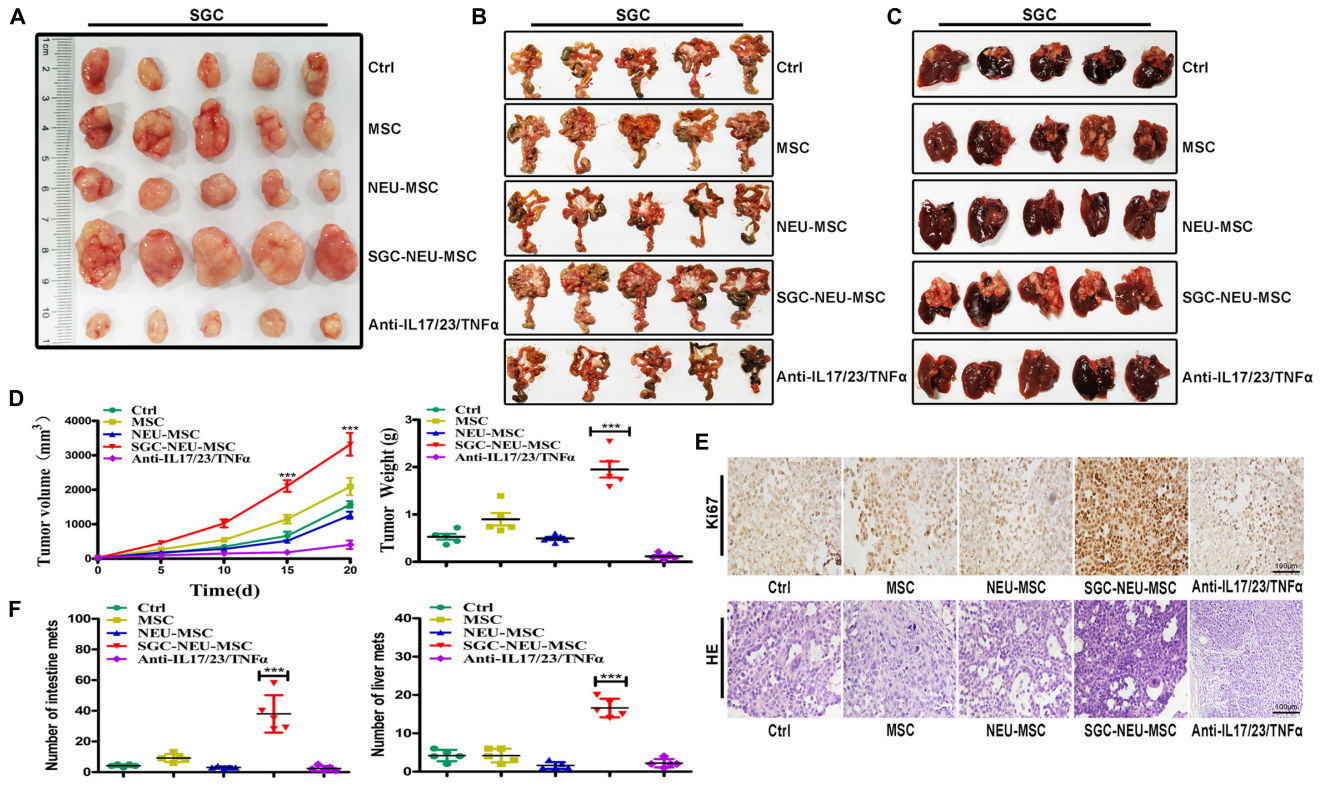


**FIGURE 5 |** MSCs/CAFs transformation induced by tumor-educated neutrophils promotes gastric cancer growth and metastasis *in vivo*. **(A)** Representative images of tumor tissues in each group. **(B)** The metastatic tumor nodules in intestinal tissues of nude mice in each group. **(C)** The metastatic tumor nodules in livers of nude mice in each group. **(D)** Tumor volumes and weights of nude mice in each group ($n$ = 5 per group). **(E)** Immunohistochemical staining of Ki-67 and H&E staining for mouse tumor tissues in different groups. **(F)** The number of metastatic tumor nodules in intestines and livers of nude mice in different groups. Ctrl, SGC-7901 cells; MSC, SGC-7901 cells treated with CM of MSCs; NEU-MSC, SGC-7901 cells treated with CM from neutrophils-primed MSCs; SGC-NEU-MSC, SGC-7901 cells pre-treated with the CM of MSCs activated by TENs; Anti-IL17/23/TNFα, SGC-7901 cells treated with supernatant from TEN-CM-primed MSCs in the presence of neutralizing antibodies. Magnification: 40×. ($n$ = 5; $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control group).

that had been primed with or without TEN-CM in the presence or absence of neutralizing antibodies. Then, GC cells were subcutaneously injected into BALB/c nude mice to establish xenograft tumor models. As shown in **Figure 5A**, significantly larger tumors were formed in mice injected with GC cells that had been treated with culture supernatant from TEN-CM-activated MSCs. However, the volume and weight of tumors formed by GC cells in neutralizing antibody group were remarkably smaller than that in TEN-CM-primed MSCs group (**Figure 5D**). Furthermore, the results of immunohistochemical analyses showed that tumor cells in TEN-CM-primed MSCs group had stronger Ki-67 staining than those in control group (**Figure 5E**). On the contrary, the positivity and intensity of Ki-67 staining in tumors was decreased in neutralizing antibodies group compared to TEN-CM-primed MSCs group (**Figure 5E**). To further investigate the metastatic potential of GC cells *in vivo*, we established mouse peritoneal metastasis model. We found that treatment with culture supernatant from TEN-CM-primed MSCs promoted the colonization of GC cells in the intestines (**Figure 5B**) and enhanced the capacity of GC cells to metastasize to the livers (**Figure 5C**). The number of visible metastatic nodules was recorded and analyzed statistically. Consistent with that observation, the number of tumor nodules in the intestines and livers of mice in TEN-CM-primed MSCs group were more than that in control and unprimed MSCs groups (**Figure 5F**). However, the number of tumor nodules in the intestines and livers of mice in neutralizing antibody group was less than that in TEN-CM-primed MSCs group (**Figure 5F**). In conclusion, these findings suggest that TENs induce MSCs/CAFs transformation to promote GC growth and metastasis *in vivo*.

## DISCUSSION

In this study, we reported that TENs induced MSCs/CAFs transformation to promote GC progression. TENs derived factors trigged the differentiation of MSCs to CAFs by activating AKT/p38 pathways. In turn, TENs-activated MSCs accelerated GC growth and metastasis both *in vitro* and *in vivo*. When the neutralizing antibodies of IL-17, IL-23, and TNF-α were added before priming, the transformation of MSCs to CAFs by TENs was blocked. Then, the promoting roles of MSCs in tumor growth and metastasis were also greatly reduced (**Figure 6**). The findings of our study provide new evidence for the pro-tumor roles of

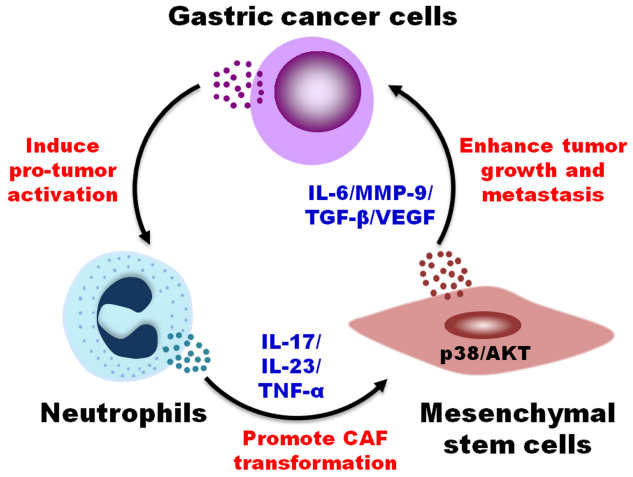


**FIGURE 6 |** A proposed model for the regulation of MSCs/CAFs transformation by tumor-educated neutrophils in gastric cancer progression. Neutrophils are educated by gastric cancer cells in tumor microenvironment to acquire a pro-tumor phenotype and function. Tumor-educated neutrophils induce the transformation of MSCs to CAFs through the production of inflammatory factors (i.e., IL-17, IL-23, and TNF-α) and the activation of AKT and p38 pathways, which in turn promotes the proliferation, migration, and invasion of gastric cancer cells, finally accelerating gastric cancer growth and metastasis.

neutrophils in cancer and reveal a novel mechanism for MSCs regulation in GC.

Cancer-associated fibroblasts are one of the key components of TME and are critically involved in tumor progression (Chen and Song, 2019; Kobayashi et al., 2019). Accumulating studies indicate that cancer development depends not only on malignant cancer cells, but also on stromal activation (Quante et al., 2011). The interplay between CAFs and cancer cells is critical for cancer cell proliferation, invasion, metastasis, and other malignant biological behaviors. We have previously shown that neutrophils educated by GC cells displayed pro-tumor phenotype and function (Zhang et al., 2018). TENs could promote the migration and invasion of GC cells through direct induction of EMT (Zhang et al., 2017). However, whether TENs could regulate CAFs are not clear. In this study, we aimed to investigate whether TENs could induce MSCs activation to CAFs. The roles of CAFs and TENs in cancer have been firmly established (Coffelt et al., 2016; Chen and Song, 2019). In this study we reported that TENs could induce the transformation of MSCs into CAFs, while activated MSCs in turn promote the proliferation, migration and invasion of GC cells. These data suggest that there may be a positive feedback loop among cancer cells, neutrophils, and MSCs that synergistically drives GC progression, which provides new evidence for the important role of inflammation in cancer.

Neutrophils have been reported to recruit regulatory T cells and macrophages to promote the progression of hepatocellular carcinoma and resistance to sorafenib (Zhou et al., 2016). Wang et al. (2017) suggest that neutrophils could inhibit T cell function through PD-L1/PD-1 interaction to promote GC growth, suggesting that neutrophils can regulate other non-tumor cells in TME to promote tumor progression. In the current study, we showed that TENs could induce the MSCs/CAFs switch, which further enhanced the malignant behaviors of GC cells, suggesting that TENs could reprogram MSCs to create a favorable microenvironment for tumor progression.

In human cancers, neutrophils exert tumor-promoting functions by producing a variety of factors, including HGF, CCL17, BMP2, among others (Coffelt et al., 2016; Shaul and Fridlender, 2019; Zhou et al., 2019). Our data showed that TENs induced MSCs/CAFs transformation by producing IL-17, IL-23, and TNF-α. Li et al. (2017) demonstrate that IL-17 is primarily expressed by neutrophils in GC tissues. They also found that neutrophils were enriched predominantly in the invasive margin, and neutrophil levels were a powerful predictor of poor survival in patients with GC (Li et al., 2017). Clinical studies have suggested that elevated numbers of IL-17-producing cells infiltrating in tumors are an independent marker of adverse prognosis in patients with cancer (Chung et al., 2013). IL-17 stimulates GC cells to express CXC chemokines, which recruits neutrophils into GC tissue to promote angiogenesis. Another study suggests that neutrophils derived IL-17 induce EMT in GC cells to promote their migration and invasion (Li et al., 2019). In addition, neutrophils infiltrated in the colon tissues of patients with IBD have been reported to be the main source of IL-23 and increased IL-23 expression is associated with the pathogenesis of IBD (Kvedaraite et al., 2016). Intriguingly, IL-23, when combined with IL-6, could induce IL-17 expression in neutrophils in GC (Li et al., 2017). Increased TNF-α expression in neutrophils has also been reported in late-stage tumor mouse model and neutrophils induce apoptosis of $CD8^+$ T cells through TNF-α (Mishalian et al., 2013; Michaeli et al., 2017). We found that when IL-17/IL-23/TNF-α were neutralized, TENs failed to induce MSCs/CAFs transformation and the effects of TENs-activated MSCs on promoting GC growth and metastasis were significantly attenuated, indicating that these factors are important for TENs-induced MSCs/CAFs transformation. The secreted factors that lead to tumor cells activation of the neutrophils have been widely reported, including CXCL5, HGF, OSM, HA, among others (Zhou et al., 2016, 2019; Liang et al., 2018). Recently, we reported that GC cells derived exosomal HMGB1 could activate neutrophils (Zhang et al., 2018). We have previously shown that MSCs resident in GC tissues could induce pro-tumor activation of neutrophils via IL-6 (Zhu et al., 2014). Yu et al. (2017) have shown that TNFα-activated mesenchymal stromal cells promote breast cancer metastasis by recruiting $CXCR2^+$ neutrophils. These findings, when combined together, suggest that a bidirectional crosstalk may exist between neutrophils and MSCs in TME and that neutrophils cooperate with MSCs to promote cancer progression.

Recent studies demonstrate that inflammatory factors play an active role in tumor growth and progression (Viola et al., 2012). Tumor-infiltrating neutrophils in melanoma are associated with poor outcome (Jensen et al., 2012). In addition, high densities of neutrophils in tumor are identified as an independent risk factor for poor prognosis (Shen et al., 2014). Several inflammatory factors have been previously shown to be involved in AKT/p38

signaling pathway activation and MSCs/CAFs transformation (Ridge et al., 2017; Timaner et al., 2020). Our observations showed that TENs induced MSCs/CAFs transformation by activating AKT/p38 pathways. When the activation of AKT/p38 pathways was inhibited by specific inhibitors, TENs-induced CAFs transformation and pro-tumor functions in MSCs were reversed, suggesting a key role of these pathways in MSCs/CAFs transformation in this setting. CAFs can secrete a variety of factors, including TGF-β, MMPs and interleukins, to promote EMT of cancer cells and stimulate the invasive and metastatic potential of cancer cells (Chen and Song, 2019; Kobayashi et al., 2019). Herein, we found that TENs-activated MSCs expressed higher levels of IL-6, MMP9, TGF-β, and VEGF, which have been previously shown to be associated with the pro-tumor functions of MSCs (McAndrews et al., 2015; Scherzad et al., 2015; Ridge et al., 2017; Timaner et al., 2020). Shrestha et al. (2016) demonstrate that neutrophils activate MSCs in an ERK-dependent manner and neutrophils-activated MSCs enhance the growth of germinal center B-cell lymphoma cells more efficiently. In this study, we measured the expression of cell signaling pathway in MSCs treated with supernatant from neutrophils. We found that TENs induce MSCs transformation to CAFs via activating AKT/p38 pathways. These findings suggest that neutrophils may regulate MSCs activation and function through distinct mechanisms in different cancers.

## CONCLUSION

In summary, our study suggests that TENs are able to induce MSCs transition to CAFs via increased expression of inflammatory factors IL-17/IL-23/TNF-α and the subsequent activation of AKT and p38 signaling pathways, which finally leads to enhanced GC growth and metastasis. These findings provide mechanistic insights into the regulation of MSCs by neutrophils in GC as well as offer novel strategies for the design of new therapeutic strategies.

## 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 University Committee on Use and Care of Animals of Jiangsu University (2012258).

## AUTHOR CONTRIBUTIONS

JZ, CJ, and HS completed all the experiments and wrote the manuscript. WX, HQ, and XZ designed the outline of the experiments. WL, ZM, YS, and RJ reviewed and corrected the draft. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00788/full#supplementary-material

**FIGURE S1 |** Effects of AKT and p38 pathway inhibitors on MSCs. **(A)** CCK8 assay for the proliferation of MSCs treated with LY294002 and SB203580. **(B)** qRT-PCR analyses of the expression of FAP, α-SMA, MMP9, IL-6, TGF-β, and VEGF genes in MSCs treated with LY294002 and SB203580. **(C)** The expression of FAP and α-SMA in MSCs treated with LY294002 and SB203580 was detected by western blot. **(D)** Western blot assay for the expression of p-AKT and p-p38 in MSCs pre-treated with LY294002 and SB203580.

**FIGURE S2 |** Detecting the effects of anti-IL17, anti-IL23 and anti-TNFα on MSCs. **(A)** The expression of FAP, α-SMA, MMP9, IL-6, TGF-β, and VEGF genes in MSCs treated with neutralizing antibodies was detected by qRT-PCR. **(B)** Western blot assays for the expression of FAP and α-SMA in MSCs treated with neutralizing antibodies. **(C)** The expression of p-AKT and p-p38 in MSCs treated with neutralizing antibodies was detected by western blot.

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REVIEW
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# Cellular Interactions and Inflammation in the Pathogenesis of Cutaneous T-Cell Lymphoma

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Cutaneous T-cell lymphoma (CTCL) comprises a group of lymphoproliferative diseases characterized by the accumulation of malignant T cells in chronically inflamed skin lesions. In early stages, the disease presents as skin patches or plaques covering a limited area of the skin and normally follows an indolent course. However, in a subset of patients the cutaneous lesions develop into tumors and the malignant T cells may spread to the lymphatic system, blood and internal organs with fatal consequences. Despite intensive research, the mechanisms driving disease progression remain incompletely understood. While most studies have focused on cancer cell-intrinsic oncogenesis, such as genetic and epigenetic events driving malignant transformation and disease progression, an increasing body of evidence shows that the interplay between malignant T cells and non-malignant cells plays a crucial role. Here, we outline some of the emerging mechanisms by which tumor, stromal and epidermal interactions may contribute to the progression of CTCL with particular emphasis on the crosstalk between fibroblasts, keratinocytes and malignant T cells.

**Keywords: cutaneous T-cell lymphoma, malignant T cells, fibroblasts, keratinocytes, tumor microenvironment**

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## INTRODUCTION

Cutaneous T-cell lymphoma (CTCL) represents a heterogeneous group of extranodal non-Hodgkin's lymphomas that are characterized by the accumulation of malignant T cells in chronically inflamed skin lesions. The classical clinical variants of CTCL are mycosis fungoides (MF) and Sézary syndrome (SS). Early stages of MF present as erythematous skin patches or plaques covering a limited area of the skin and closely resemble benign inflammatory skin conditions such as psoriasis and chronic eczema (Kim et al., 2005; Willemze et al., 2019). The majority of patients diagnosed with early MF experience an indolent disease course with a favorable prognosis. However, about a third of the patients progress to advanced stages which can result in a fatal outcome (Scarisbrick et al., 2014; Hristov et al., 2019). As the disease progresses, the malignant T cells accumulate, the skin lesions expand and distinctive fungus-like tumors may develop. Eventually, the malignant T cells can spread to the blood, lymph nodes, bone marrow and internal organs. With advancing clinical stages, the disease becomes increasingly aggressive, the prognosis worsens and the median life expectancy of patients with late stage CTCL drops to less than 5 years (Kim et al., 2005; Scarisbrick et al., 2014; Hristov et al., 2019; Willemze et al., 2019). SS is a leukemic form of CTCL involving generalized erythroderma, lymphadenopathy and the presence of atypical

T cells with cerebriform nuclei (Sézary cells) in the peripheral blood (Kim et al., 2005; Willemze et al., 2019). It may develop *de novo* or occasionally in patients with long-term chronic MF, and is considered a late stage of CTCL due to its high aggressiveness and poor prognosis (Kim et al., 2005; Scarisbrick et al., 2014; Hristov et al., 2019; Willemze et al., 2019).

The malignant T cells in MF and SS typically exhibit the phenotype of skin-homing CD4 T cells expressing receptors such as cutaneous lymphocyte antigen (CLA) and CC chemokine receptor 4 (CCR4) (Ferenczi et al., 2002; Campbell et al., 2010; Sugaya et al., 2015). Yet, as highlighted by recent single-cell RNA sequencing studies the malignant T cells display substantial inter- and intra-patient phenotypic heterogeneity (Buus et al., 2018; Gaydosik et al., 2019). Extensive inter-patient heterogeneity is also observed at the genetic level and based on current data the disease is generally not caused by a few specific recurrent genetic aberrations (Choi et al., 2015; da Silva Almeida et al., 2015; Kiel et al., 2015; McGirt et al., 2015; Ungewickell et al., 2015; Wang et al., 2015; Woollard et al., 2016; Iyer et al., 2020; Phyo et al., 2020). Moreover, a nationwide study of Danish twins did not detect any familial aggregation of CTCL, arguing against heredity as a dominant etiologic factor (Odum et al., 2017). Somatic genetic alterations are, however, frequently observed in genes involved in certain cellular processes and signaling pathways. In particular, genes involved in epigenetic regulation, DNA damage response, cell cycle control and programmed cell death as well as in the T cell receptor (TCR), nuclear factor-kappa B (NF-κB) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathways (Choi et al., 2015; da Silva Almeida et al., 2015; Kiel et al., 2015; McGirt et al., 2015; Ungewickell et al., 2015; Wang et al., 2015; Woollard et al., 2016; Iyer et al., 2020; Phyo et al., 2020). Importantly, extensive experimental data from cell lines, primary cells and clinical samples corroborate that dysregulation of these cellular processes and signaling pathways plays a central functional role in the pathogenesis of CTCL.

For long, it has been the general view that CTCL is a monoclonal disease with MF originating from skin-resident memory T cells and SS from mature central memory T cells (Kim et al., 2005; Campbell et al., 2010). Challenging this view, Iyer et al. (2019) recently reported the existence of multiple malignant T cell clones in both the skin and blood of MF patients with substantial variation in the clonotypes between patients and different lesions within the same patient. They further found evidence of extensive genetic intratumoral heterogeneity showing a branched phylogenetic relationship pattern (Iyer et al., 2020). Stage progression was associated with increased intratumoral heterogeneity and divergent subclonal evolution (Iyer et al., 2020). The authors proposed that MF skin lesions are formed by seeding of circulating malignant T cell clones which expand and undergo additional mutational evolution in the skin leading to the appearance of new genetically different subclones, some of which may reenter the circulation and seed other skin lesions (Iyer et al., 2020). If correct, this theory could bear significant implications for the understanding of the disease and the development of new therapeutic strategies.

The only known treatment with the potential to cure CTCL is allogenic bone marrow transplantation which is only suitable for a fraction of patients with advanced disease (Hosing et al., 2015; Johnson et al., 2019; Novelli et al., 2019). Therefore, the current therapeutic aim is primarily to control the disease, reduce symptoms and improve cosmetics while minimizing toxic effects. Early disease stages are often treated with skin-directed therapies such as topical corticosteroids and UV light therapy, whereas advanced disease usually is treated with systemic therapies (Belloni et al., 2012; Trautinger et al., 2017; Hristov et al., 2019; Trager and Geskin, 2019). However, even with proper treatment a considerable subset of CTCL patients develop or suffer from progressive disease (Belloni et al., 2012; Scarisbrick et al., 2014; Hristov et al., 2019). In view of the increased aggressiveness and poor survival rate in advanced clinical stages, it is critical to gain a better understanding of the mechanisms that drive the transition from early indolent to progressive and advanced disease.

Despite the heterogeneous nature of CTCL, consistent changes are typically observed in the lesional skin when comparing early and advanced stages. These changes appear to be facilitated by complex cellular interactions between the malignant T cells and their microenvironment and to play a central role in the progression of the disease (Miyagaki and Sugaya, 2014; Gonzalez et al., 2016; Krejsgaard et al., 2017). While most reviews have focused on the malignant T cells and their interactions with benign immune cells, we here outline some of the mechanisms by which malignant, stromal and epidermal interactions may contribute to the progression of CTCL with particular focus on emerging data highlighting the significance of the crosstalk between the malignant T cells, fibroblasts and keratinocytes.

## TH2-BIAS DURING DISEASE PROGRESSION

In early stages of MF, the majority of immune cells in the lesional skin are benign and the malignant T cells only constitute a minor fraction. A substantial proportion of the benign immune cells are reactive T helper 1 (Th1) cells and cytotoxic CD8 T cells expressing interferon gamma (IFNγ) and cytotoxic molecules (Wood et al., 1994; Asadullah et al., 1997; Bagot et al., 1998; Echchakir et al., 2000; Vermeer et al., 2001; Kim et al., 2005; Hsi et al., 2015). These cells have the capacity to kill autologous malignant T cells *ex vivo* and high numbers of lesional CD8 T cells are associated with a favorable prognosis, indicating that the cellular immune reaction in early disease represents an anti-tumor response that keeps the malignant population in check (Hoppe et al., 1995; Berger et al., 1996; Bagot et al., 1998; Vermeer et al., 2001; Abeni et al., 2005). However, with advancing clinical stages there is a decline in Th1-associated markers as well as in the numbers of activated Th1 and CD8 T cells, whereas the levels of Th2-associated markers including GATA-3, IL-4, IL-5 and IL-13 increase (Vowels et al., 1994; Hoppe et al., 1995; Vermeer et al., 2001; Papadavid et al., 2003; Hahtola et al., 2006; Johnson et al., 2014; Litvinov et al., 2014; Geskin et al., 2015; Hsi et al., 2015). The shift from a Th1- to a Th2-biased tumor microenvironment (**Figure 1**) is

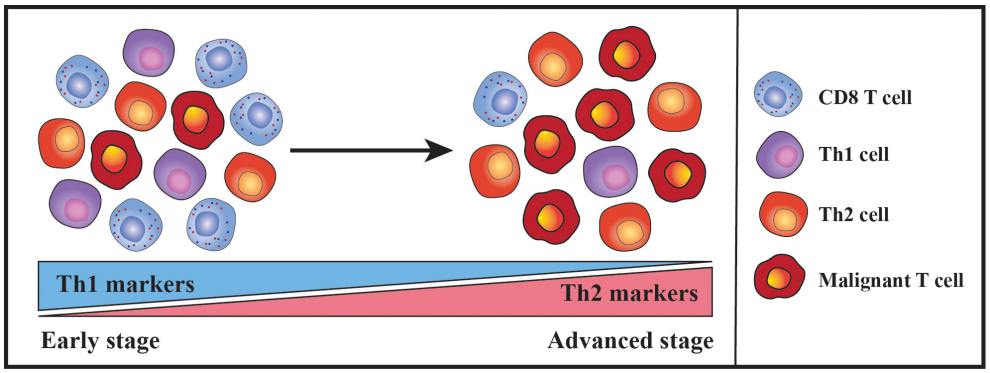


**FIGURE 1 |** Schematic illustration portraying the shift in the nature of the inflammatory milieu during the progression of CTCL. In early disease stages, CTCL skin lesions typically contain few malignant T cells within a dense infiltrate of benign immune cells. A substantial proportion of the benign immune cells are reactive T helper 1 (Th1) cells and cytotoxic CD8 T cells and accordingly Th1-associated markers are highly expressed. However, during disease progression there is a decline in the expression of Th1-associated markers along with the numbers of infiltrating Th1 and CD8 T cells. In contrast, the malignant T cells accumulate and the levels of Th2-associated markers increase, eventually leading to a Th2-dominated inflammatory milieu in advanced disease stages.

thought to play a critical role in the transition from indolent to progressive disease by impairing cellular anti-tumor responses whilst fostering the proliferation of malignant T cells. Indeed, administration of Toll-like receptor (TLR) agonists boosting cellular immunity has shown clinical efficacy, and treatment with IL-12 and IFNγ can induce regression of CTCL lesions which is associated with increased numbers of CD8 T cells in the resolving skin (Rook et al., 1999, 2001, 2015; Suchin et al., 2002; Dummer et al., 2004; Duvic et al., 2006; Wysocka et al., 2007; Kim et al., 2010; Accart et al., 2013). Of notice, recent case reports have surprisingly described that long-term treatment with dupilumab, a neutralizing antibody targeting IL-4 receptor alpha, may exacerbate CTCL and possibly even trigger the disease in certain patients with severe atopic dermatitis (AD) (Chiba et al., 2019; Espinosa et al., 2020; Miyashiro et al., 2020; Tran et al., 2020; Umemoto et al., 2020). While substantial data currently support that the transition from a Th1- to a Th2-biased tumor microenvironment contributes to the progression of CTCL, these new findings suggest that the role of the cytokine milieu might be more complex than appreciated thus far. Further studies are, however, required to gain a better understanding of the clinical and biological effects of dupilumab in patients with CTCL.

## CELLULAR INTERACTIONS ENHANCE MALIGNANT STAT ACTIVATION, PROLIFERATION AND TH2 CYTOKINE EXPRESSION

The progression of CTCL is associated with a gradual dysregulation of the JAK/STAT pathway, and evidence pinpoints this dysregulation as a driving force in mediating the shift toward a Th2-biased tumor microenvironment. Normally, STAT3, STAT5 and STAT6 become persistently activated in the malignant T cells which have been shown to fuel their expression of Th2 cytokines (Zhang et al., 1996, 2000; Nielsen et al., 2002; Sommer et al., 2004; Krejsgaard et al., 2006; Choi et al., 2015; Geskin et al., 2015; Kiel et al., 2015; Woollard et al., 2016; Gaydosik et al., 2020). Furthermore, activation of STAT6 upregulates the malignant expression of the Th2-associated transcription factor GATA-3 (Gaydosik et al., 2020). Whereas the expression of GATA-3 increases during disease progression, STAT4, which promotes Th1 differentiation and IFNγ expression, is often lost (Nebozhyn et al., 2006; Johnson et al., 2014; Litvinov et al., 2014; Hsi et al., 2015). Accordingly, malignant T cells isolated from patients with advanced leukemic disease typically express Th2 cytokines such as IL-4 and IL-13 but are negative for IFNγ (Guenova et al., 2013).

While it remains unclear what initially triggers an increase in the lesional levels of Th2 cytokines, evidence suggests that this may ignite a positive feedback loop between the malignant, stromal and epidermal cells that further enhances the malignant activation of STAT proteins and expression of Th2 cytokines. For example, it has been shown that IL-4 and IL-13 stimulate dermal fibroblasts from CTCL patients to secrete increased levels of the extracellular matrix protein periostin (Takahashi et al., 2016). Periostin is known to induce expression of thymic stromal lymphopoietin (TSLP) from keratinocytes and both periostin and TSLP are elevated in CTCL skin lesions and serum when compared with skin and serum from healthy control subjects (Miyagaki et al., 2009; Tuzova et al., 2015; Takahashi et al., 2016). The lesional levels of IL-4, periostin and TSLP correlate, indicating a scenario where Th2 cytokines stimulate dermal fibroblasts to secrete periostin which induces expression of TSLP from the epidermal keratinocytes. Completing the circle, TSLP has in turn been shown to activate STAT5 in malignant CTCL cells thereby promoting both their proliferation and production of IL-4 and IL-13 (Takahashi et al., 2016). Notably, STAT5 can also downregulate the malignant expression of STAT4 and the chromatin organizer and transcription factor SATB1 through induction of microRNA-155 (miR-155) (Litvinov et al., 2014; Fredholm et al., 2018; Herrera et al., 2020). As SATB1 represses

the expression of IL-5 and IL-9 in the malignant T cells, activation of STAT5 may in this manner indirectly promote the expression of these cytokines while concurrently suppressing IFNγ (Fredholm et al., 2018; Herrera et al., 2020).

Periostin, IL-4 and IL-13 have in addition been reported to stimulate primary human keratinocytes to express increased levels of the cytokine IL-25, which promotes Th2 immunity and cytokine production (Xu and Dong, 2017; Nakajima et al., 2018). Accordingly, the expression of IL-25 is increased in the epidermal keratinocytes in advanced CTCL skin lesions and enhances the expression of IL-13 in IL-25 receptor-positive malignant T cells via activation of STAT6 (Nakajima et al., 2018). Extending these data, Geskin et al. (2015) demonstrated that malignant T cells in the skin and blood of CTCL patients express receptors for IL-13. The authors further provided evidence that IL-13 promotes the proliferation of malignant T cells isolated from the blood of leukemic CTCL patients, suggesting that IL-25, TSLP and the general skewing toward a Th2-polarized inflammatory environment may fuel the malignant expansion indirectly by augmenting the levels of IL-13 (Geskin et al., 2015). Taken together, these findings illustrate how an initial increase in Th2 cytokines may elicit a complex loop of continuous signaling between fibroblasts, keratinocytes and malignant T cells that sustains and enhances the activation of STAT proteins, the proliferation and the Th2 cytokine expression of the malignant T cells (**Figure 2**).

## CHANGES IN THE MALIGNANT CYTOKINE EXPRESSION FOSTER A TH2-BIASED INFLAMMATORY ENVIRONMENT

Accumulating evidence indicates that the skewing of the malignant cytokine expression toward a Th2 profile fosters the development of a Th2-biased inflammatory environment – both via direct effects on the benign immune cells and indirectly by modulating the chemokine expression pattern in the tumor microenvironment (Miyagaki and Sugaya, 2014; Gonzalez et al., 2016; Krejsgaard et al., 2017). The capacity of the malignant T cells to directly modulate the inflammatory response of benign immune cells was elegantly demonstrated by Guenova et al. (2013). The authors found that malignant and benign T cells from leukemic CTCL patients expressed lower levels of IFNγ and higher levels of IL-4 and IL-13 than T cells from healthy controls. However, when the benign and malignant T cells were cultured separately, the benign T cells expressed enhanced levels of IFNγ and lower levels of IL-4, whereas the expression of these cytokines remained constant in the malignant T cells (Guenova et al., 2013). Peripheral blood mononuclear cells (PBMCs) from leukemic CTCL patients were further shown to suppress the expression of IFNγ from healthy donor PBMCs. The suppressive effect was completely abrogated by neutralizing antibodies against IL-4 and IL-13, suggesting that the malignant T cells directly repress benign Th1 responses via their expression of Th2 cytokines (Guenova et al., 2013). Supporting that the malignant T cells play a key role in suppressing Th1 responses in CTCL patients, different treatment modalities which reduced the numbers of malignant T cells through distinct mechanisms of action were invariably associated with enhanced Th1 and reduced Th2 responses (Guenova et al., 2013). Of notice, the malignant T cells may also inhibit anti-tumor immunity by inducing apoptosis in the benign T cells. It was, for example, recently reported that the malignant T cells secrete galectin-9 and that increased expression of galectin-9 in CTCL skin lesions is associated with a reduced infiltration of CD8 T cells while high serum levels are correlated with disease severity markers (Nakajima et al., 2019). Likewise, the malignant T cells frequently express Fas ligand (FasL) and have been shown to trigger FasL-dependent T cell apoptosis *in vitro* (Ni et al., 2001, 2005). CD8 T cells were reported to be inversely distributed with FasL-expressing malignant T cells in CTCL skin lesions, implying that the malignant T cells might use FasL to eliminate tumor-reactive CD8 T cells (Ni et al., 2001).

The malignant T cells may not only foster a Th2-dominated tumor microenvironment through direct effects on the benign immune cells but also indirectly by modulating the chemokine expression pattern of fibroblasts, macrophages, dendritic cells (DCs) and keratinocytes. In early disease the keratinocytes and dermal fibroblasts express high levels of chemokines such as CXCL9 and CXCL10 which preferentially attract CD8 T cells and Th1 cells. The expression of these Th1-associated chemokines is, however, strongly reduced in advanced disease stages, whereas the expression of chemokines such as CCL17, CCL18, CCL22 and CCL26 that preferentially attract Th2 cells is increased (Sarris et al., 1995; Tensen et al., 1998; Kakinuma et al., 2003; Bromley et al., 2008; Miyagaki et al., 2010, 2012b, 2013; Tuzova et al., 2015). Providing a putative explanation for the decreased expression of CXCL9 and CXCL10 in advanced disease, Miyagaki et al. (2012b) demonstrated that IFNγ induces secretion of these chemokines from dermal fibroblasts. IFNγ-induced synthesis of CXCL9 and CXCL10 was strongly potentiated by the TNF superfamily member LIGHT. Although LIGHT was expressed both in early and advanced disease stages, the expression of its receptor, HVEM, was decreased on fibroblasts in advanced disease, potentially contributing to their reduced expression of CXCL9 and CXCL10. In contrast to IFNγ, IL-4 has been shown to stimulate dermal fibroblasts from CTCL patients to express high levels of the Th2-recruiting chemokine CCL26 *in vitro* and, accordingly, the expression levels of IL-4 and CCL26 were found to correlate in the lesional skin (Miyagaki et al., 2010). IL-4 and IL-13 are correspondingly potent inducers of CCL26 expression in human keratinocytes, whereas IFNγ stimulates keratinocytes to secrete CXCL9 and CXCL10 (Giustizieri et al., 2001; Ohta et al., 2008; Gaspar et al., 2013). The increased expression of Th2 cytokines combined with the decreased secretion of IFNγ and HVEM may therefore, at least partially, explain the observed changes in the expression pattern of CXCL9, CXCL10 and CCL26 during disease progression. In addition, Th2 cytokines are known to stimulate the secretion of CCL18 and CCL22 from antigen-presenting cells (Vulcano et al., 2001; van Lieshout et al., 2006; Tsicopoulos et al., 2013; Furudate et al., 2016). These chemokines are indeed expressed by macrophages and

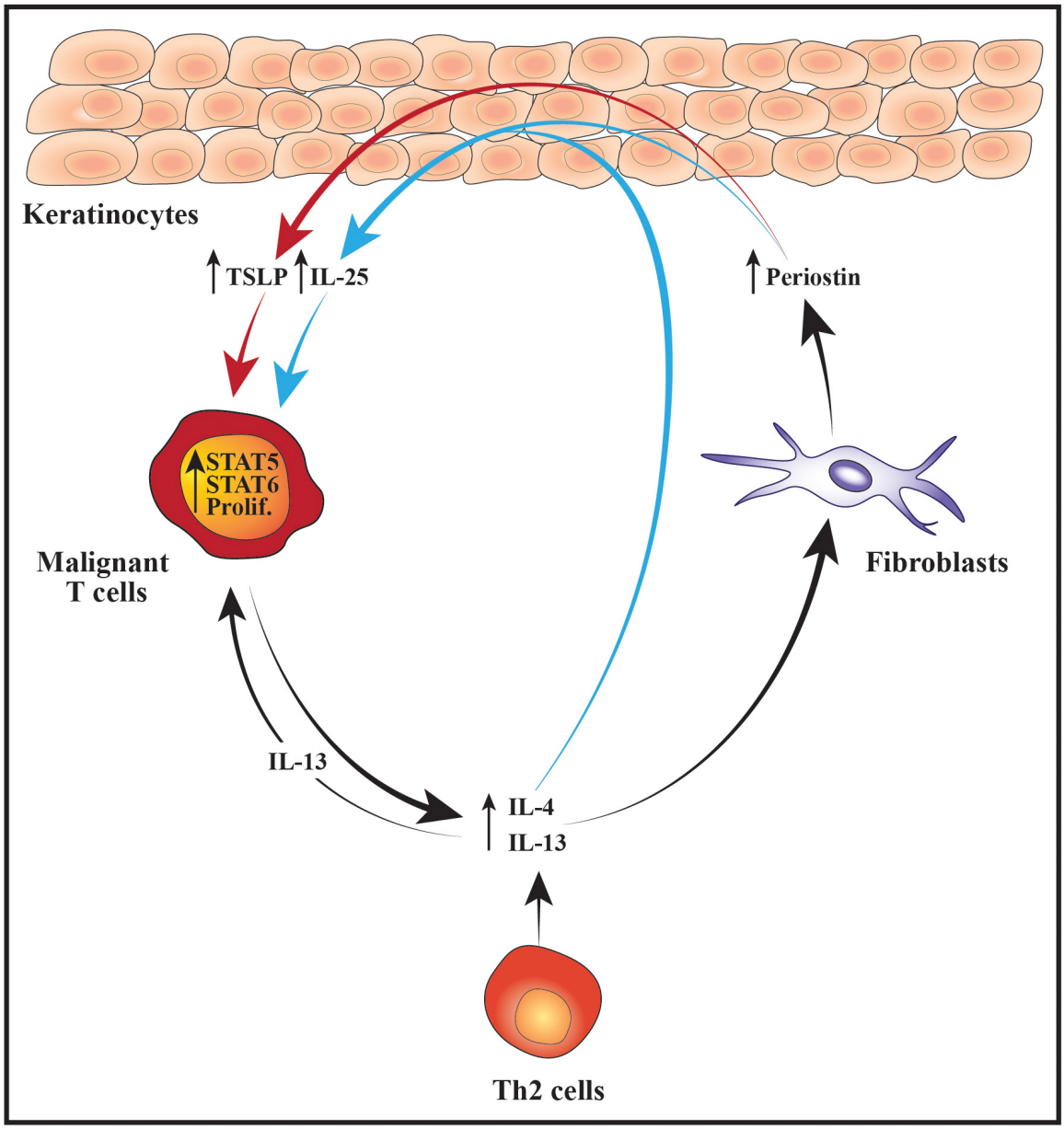


**FIGURE 2 |** Schematic illustration of cellular interactions that may fuel the malignant STAT activity, proliferation and Th2 cytokine expression. Increased expression of IL-4 and IL-13 from malignant T cells and/or benign Th2 cells stimulates dermal fibroblasts to produce higher levels of periostin which subsequently promotes the secretion of TSLP from the epidermal keratinocytes. In parallel, IL-4, IL-13 and periostin cooperatively stimulate the keratinocytes to express IL-25. The elevated levels of TSLP and IL-25 boost the activity of STAT5 and STAT6 in the malignant T cells which fuel their proliferation (prolif.) and production of Th2 cytokines such as IL-4 and IL-13. In turn, IL-13 further augments the malignant proliferation in an autocrine and paracrine manner. Moreover, the increased expression of Th2 cytokines leads to enhanced secretion of periostin, TSLP and IL-25 thus giving rise to a positive feedback loop nurturing the malignant T cells.

DCs in CTCL skin lesions and the levels of CCL18 correlate with those of IL-4 (Gunther et al., 2011; Miyagaki et al., 2013; Tanita et al., 2019; Vieyra-Garcia et al., 2019). A recent study provided evidence suggesting that lesional c-Kit$^{+}$ DCs recruit benign Th2 cells via secretion of CCL18 which leads to formation of an inflammatory synapse between the DCs, benign Th2 cells and the malignant T cells (Vieyra-Garcia et al., 2019). In this synapse, the benign Th2 cells are activated by OX40/OX40L cell interactions with the DCs and by CD40/CD40L interactions with both DCs and malignant T cells resulting in increased skin inflammation (Vieyra-Garcia et al., 2019). Collectively, these data illustrate how changes in the malignant expression of Th1 and Th2 cytokines may modulate the chemokine expression of benign immune cells, fibroblasts and keratinocytes to favor skin trafficking and activation of Th2 cells (**Figure 3**). By adding to the increasing levels of Th2 cytokines, this fuels a vicious cycle that

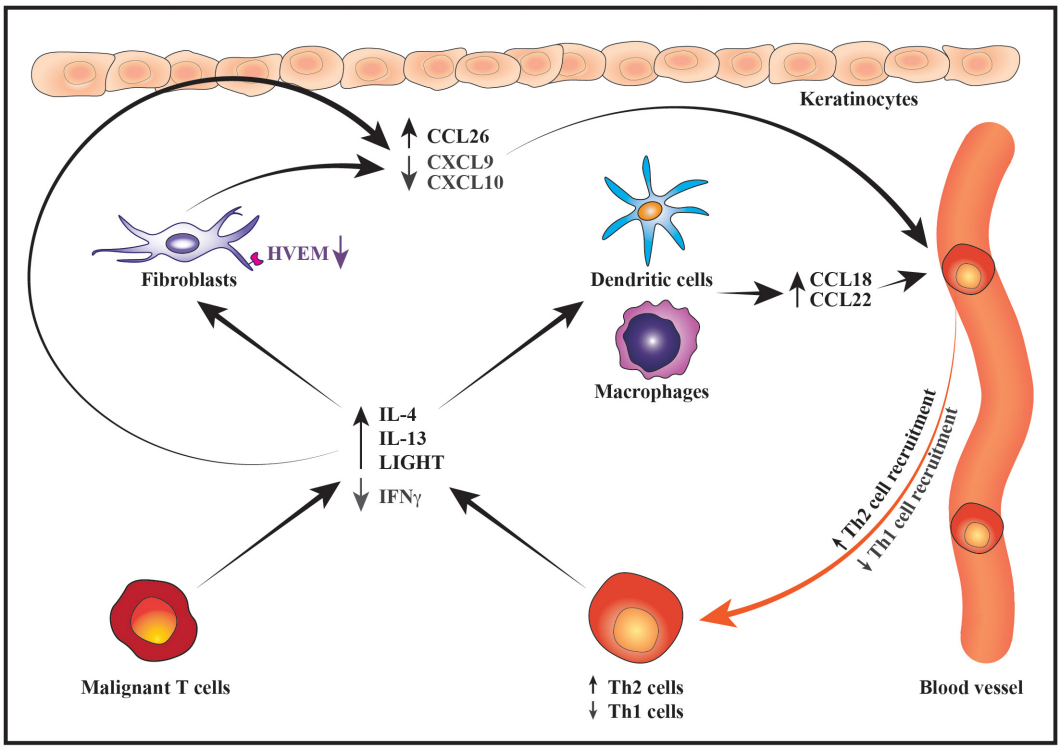


**FIGURE 3 |** Schematic illustration of how changes in the malignant cytokine secretion alter the chemokine expression pattern to promote a Th2-biased inflammatory microenvironment. During disease progression, the malignant T cells express increased levels of IL-4 and IL-13 whereas their expression of IFNγ decreases. Lower production of IFNγ by malignant T cells together with downregulation of HVEM receptor on fibroblasts leads to suppressed expression of the Th1-recruting chemokines CXCL9 and CXCL10 by fibroblasts and keratinocytes resulting in reduced recruitment of Th1 cells into the tumor microenvironment. In contrast, increased secretion of IL-4 and IL-13 stimulates the production of Th2-recruting chemokines such as CCL26 by keratinocytes and fibroblasts as well as CCL18 and CCL22 by macrophages and DCs. In turn, the recruitment of more Th2 cells to the lesional skin contributes to the increasing levels of Th2 cytokines thereby sustaining the development of a Th2-dominated tumor microenvironment.

reinforces the development of a Th2-biased inflammatory tumor microenvironment (**Figure 3**).

## THE MALIGNANT T CELLS INDUCE ANGIOGENESIS AND LYMPHANGIOGENESIS

Angiogenesis and lymphangiogenesis are critical processes in tumor growth and metastasis (Stacker et al., 2014; De Palma et al., 2017). Accordingly, biopsies from the lesional skin of CTCL patients exhibit a stage-dependent increase in microvessel density and the lymphatic marker podoplanin (Vacca et al., 1997; Mazur et al., 2004; Kawaguchi et al., 2014; Jankowska-Konsur et al., 2016, 2017). High expression of podoplanin is associated with shorter overall survival in CTCL patients, and the expression levels of vascular and proliferative markers correlate *in situ* (Jankowska-Konsur et al., 2016, 2017). Moreover, high expression of endothelial and lymphatic markers is associated with nodal involvement, altogether indicating that angiogenesis and lymphangiogenesis may contribute to the expansion and dissemination of the malignant T cells (Jankowska-Konsur et al., 2016, 2017).

The increase in podoplanin-positive lymphatic vessels during the clinical progression of CTCL strongly correlates with the expression levels of vascular endothelial growth factor C (VEGF-C) which is a key stimulator of lymphangiogenesis (Stacker et al., 2014; Jankowska-Konsur et al., 2017). While neoplastic T cells stain positive for VEGF-C in CTCL skin lesions, malignant CTCL cells were reported not to produce VEGF-C *in vitro* (Pedersen et al., 2013). However, when the malignant T cells were inoculated into the skin of immunodeficient mice a proportion of them displayed clear expression of VEGF-C during tumor formation *in vivo* but this expression was not retained *ex vivo* (Pedersen et al., 2013). VEGF-C-positive malignant T cells were predominantly observed in close proximity to stromal cells within the tumor microenvironment. Interestingly, *in vitro* co-culture of malignant CTCL cell lines with skin fibroblasts enhanced the secretion of VEGF-C by the latter, jointly suggesting that the tumor and stromal cells may engage in a cross-talk that promotes synthesis of VEGF-C which, in turn, stimulates lymphangiogenesis (Pedersen et al., 2013).

In line with the growing microvessel density, a variety of angiogenic factors are increased in the lesional skin of CTCL patients when compared with normal skin or skin from patients with benign inflammatory skin conditions

(Krejsgaard et al., 2006; Miyagaki et al., 2012a, 2017; Kawaguchi et al., 2014; Lauenborg et al., 2015; Sakamoto et al., 2018; Suzuki et al., 2020). Although some of these are expressed by stromal cells, the majority mainly appear to be produced by the malignant T cells. For instance, atypical T cells in CTCL lesions stain positive for the highly angiogenic protein VEGF-A, and malignant CTCL cell lines produce VEGF-A via a JAK- and c-Jun N-terminal kinase (JNK)-dependent mechanism *in vitro* (Krejsgaard et al., 2006; Miyagaki et al., 2017; Sakamoto et al., 2018). Besides being a potent stimulator of angiogenesis, VEGF-A also has the capacity to enhance the expression of TSLP in keratinocytes and, accordingly, the serum concentrations of VEGF-A correlate with the lesional expression of TSLP in erythrodermic CTCL patients (Sakamoto et al., 2018). VEGF-A may thus both contribute to the pathogenesis of CTCL by promoting angiogenesis, and by stimulating TSLP production by epidermal keratinocytes. Alongside VEGF-A, the malignant T cells also express lymphotoxin α (LTα) and its cognate receptor, tumor necrosis factor receptor (TNFR)2 (Lauenborg et al., 2015). It was demonstrated that LTα can function in an autocrine manner to induce malignant secretion of IL-6 which together with LTα and VEGF-A stimulate endothelial sprouting and tube formation (Lauenborg et al., 2015). The malignant T cells have additionally been reported to express a number of other pro-angiogenic factors such as IL-17F, angiopoietin-2 (Ang-2), placental growth factor (PlGF) and YKL-40 (Krejsgaard et al., 2013; Kawaguchi et al., 2014; Lauenborg et al., 2017; Miyagaki et al., 2017; Suzuki et al., 2020). The secretion of IL-17F by malignant T cells has been shown to stimulate angiogenesis *in vitro*, and the numbers of Ang-2-positive cells correlate with the numbers of blood vessels in erythrodermic CTCL lesions (Kawaguchi et al., 2014; Lauenborg et al., 2017). Furthermore, PlGF and YKL-40 were demonstrated to promote blood vessel formation and lymphoma growth in murine xenograft models (Miyagaki et al., 2017; Suzuki et al., 2020). In conjunction with angiogenic factors, the malignant T cells express matrix metalloproteinases (MMP) such as MMP2 and MMP9 which may facilitate the angiogenic process and spread of the malignant T cells (Vacca et al., 1997; Rasheed et al., 2010). MMPs were also found to be strongly expressed by stromal cells in the vicinity of the malignant T cells, suggesting that malignant T cells secrete factors that stimulate the expression of MMPs by the surrounding stroma (Vacca et al., 1997; Rasheed et al., 2010). Collectively, these data highlight how the malignant T cells may facilitate their own growth and dissemination through the production of factors that directly or indirectly stimulate blood and lymph vessel formation in the tumor stroma.

## THE MALIGNANT T CELLS MAY INDUCE CHANGES IN THE EPIDERMAL ARCHITECTURE

In addition to the increased microvessel density, the lesional skin of CTCL patients often exhibits changes in the epidermal architecture and an impaired barrier function (Suga et al., 2014; Fatima et al., 2020). Accordingly, Suga et al. (2014) found that keratinocytes in CTCL skin lesions express lower levels of skin barrier proteins than keratinocytes in healthy skin. This was more prominent in advanced disease and the levels of skin barrier proteins correlated inversely with the expression of IL-4, CCL17 and CCL18. Using an organotypic skin model of CTCL, Thode et al. (2015) provided evidence that the malignant T cells secrete factors which affect the behavior of keratinocytes leading to increased proliferation, disorganized stratification and decreased resistance to mechanical stress. The malignant T cells have also been shown to produce IL-31, which together with IL-4, IL-5 and IL-13, is believed to stimulate the sensation of pruritus in CTCL patients (Ohmatsu et al., 2012; Singer et al., 2013; Cedeno-Laurent et al., 2015; Nattkemper et al., 2016; Lewis et al., 2018). At such, the malignant T cells may facilitate mechanical disruption of the skin integrity indirectly by producing factors that elicit scratching. Yet, as many of the cutaneous features of CTCL resemble those of chronic inflammatory skin diseases it is likely that interactions between the benign immune cells and keratinocytes also contribute significantly to the changes in the epidermal architecture (Ralfkiaer et al., 2011; Willemze et al., 2019). Indeed, Miyagaki et al. (2011) reported that benign T cells in CTCL produce IL-22 which may promote STAT3 activation, CCL20 expression and epidermal hyperplasia. In general, this area of investigation remains poorly explored and further studies are needed to gain a better understanding of how the interplay between malignant T cells, benign immune cells and keratinocytes affects the epidermis.

## INTERACTIONS BETWEEN BACTERIAL TOXINS AND MALIGNANT T CELLS

Considering the compromised cellular immunity and weakened skin barrier, it is not surprising that CTCL patients exhibit increased susceptibility to cutaneous bacterial infections. The lesional skin is, in particular, frequently colonized by enterotoxin-producing *Staphylococcus aureus* (*S. aureus)* which constitutes a major cause of morbidity and mortality (Axelrod et al., 1992; Tokura et al., 1995; Jackow et al., 1997; Nguyen et al., 2008; Talpur et al., 2008; Mirvish et al., 2011; Willerslev-Olsen et al., 2013; Lebas et al., 2017; Blaizot et al., 2018; Scarisbrick, 2018). A series of *in vitro* studies have uncovered that staphylococcal enterotoxins can trigger a complex crosstalk between the malignant and non-malignant T cells. This cross-talk leads to increased proliferation, cytokine production, IL-2 receptor alpha chain (IL2Rα) expression and STAT3 activation in the malignant T cells, suggesting that staphylococcal enterotoxins may modulate the inflammatory environment and fuel disease progression (Woetmann et al., 2007; Krejsgaard et al., 2014; Willerslev-Olsen et al., 2016, 2020; Lindahl et al., 2019). *S. aureus* also produces other factors that may play a pathogenic role in CTCL. It was for example shown that *S. aureus* isolates from CTCL skin lesions express the pore forming toxin, alpha-toxin, and that the malignant T cells in many patients are considerably more resistant to alpha-toxin-induced cell death than non-malignant CD4 and CD8 T cells (Blümel et al., 2019, 2020; Lindahl et al., 2019). It is thus possible that alpha-toxin

impedes CD8 T cell-mediated anti-tumor responses and tilts the balance between the malignant and non-malignant CD4 T cells. Supporting that toxinogenic *S. aureus* infections exacerbate the disease activity, antibiotic treatment leading to successful eradication of *S. aureus* is associated with significant clinical improvement in most patients with advanced CTCL (Tokura et al., 1995; Jackow et al., 1997; Talpur et al., 2008; Willerslev-Olsen et al., 2013; Lindahl et al., 2019). A recent study further demonstrated that patients exhibiting clinical improvement after transient antibiotic therapy, display diminished STAT3 signaling, IL-2Rα expression and cell proliferation in the lesional skin (Lindahl et al., 2019). Antibiotic treatment not only reduced the disease activity at the clinical and histological level but also resulted in a significant decrease in the fraction of malignant T cells in the lesional skin in the majority of patients (Lindahl et al., 2019). These findings indicate that there may be a mutual sustenance between the malignant T cells and toxin-producing *S. aureus* where the former compromise the local immunity and the bacteria, in turn, mitigate anti-tumor responses while concurrently fueling the expansion of malignant T cells.

## CONCLUSION

Accumulating evidence suggests that malignant, stromal and epidermal interactions play a central role in the pathogenesis of CTCL. In this review, we have outlined some of the emerging mechanisms by which these interactions may contribute to the progression of the disease. As highlighted, complex signaling networks between fibroblasts, keratinocytes and the tumor cells may fuel diverse pathological processes including the malignant activation of STAT proteins, the development of a Th2 dominated inflammatory microenvironment, neovascularization of the tumor tissue and changes in the skin architecture. These processes can through different pathways facilitate the malignant proliferation and dissemination while impeding anti-tumor responses. Despite recent progress, the interactions between tumor, stromal and epidermal cells in CTCL remain poorly characterized and additional studies are warranted to substantiate and further illuminate their role in the pathogenesis of the disease. We believe that research into this field of investigation may pave the way for novel therapeutic strategies that can be of clinical benefit for patients with progressive or advanced CTCL.

## AUTHOR CONTRIBUTIONS

VS, MN, SH, MG, TB, AW-O, NØ, and TK wrote the manuscript. VS and TK made the figures. All authors contributed to the article and approved the submitted version.

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# Stromal Expression of the Core Clock Gene *Period 2* Is Essential for Tumor Initiation and Metastatic Colonization

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The circadian clock regulates diverse physiological processes by maintaining a 24-h gene expression pattern. Genetic and environmental cues that disrupt normal clock rhythms can lead to cancer, yet the extent to which this effect is controlled by the cancer cells versus non-malignant cells in the tumor microenvironment (TME) is not clear. Here we set out to address this question, by selective manipulation of circadian clock genes in the TME. In two different mouse models of cancer we find that expression of the core clock gene *Per2* in the TME is crucial for tumor initiation and metastatic colonization, whereas another core gene, *Per1,* is dispensable. We further show that loss of *Per2* in the TME leads to significant transcriptional changes in response to cancer cell introduction. These changes may contribute to a tumor-suppressive microenvironment. Thus, our work unravels an unexpected protumorigenic role for the core clock gene *Per2* in the TME, with potential implications for therapeutic dosing strategies and treatment regimens.

**Keywords: circadian clock, *Per2*, tumor microenvironment, colon cancer, chronobiology, metastasis, liver, MC38 colon cancer cells**

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## INTRODUCTION

The circadian clock is an endogenous, evolutionally conserved and ubiquitously expressed pacemaker, consisting of cell autonomous clocks and a central pacemaker located in the hypothalamus suprachiasmatic nucleus (SCN). Together these clocks synchronize numerous biological processes between the organism and its environment. Amongst these processes are DNA damage repair, metabolism, and cell cycle (Blakeman et al., 2016). The circadian clocks act as oscillators to drive 24-h rhythms in gene expression and protein function, and these rhythms help the organism maintain a homeostatic relationship with the environment (Padmanabhan and Billaud, 2017). Disruption of circadian rhythms has been associated with various forms of cancer in humans, and it has been shown that a disordered circadian clock, whether genetically or due to environmental signals (e.g., changes of dark/light exposure) accelerates tumor progression (Savvidis and Koutsilieris, 2012; Hadadi et al., 2019). Additionally, epidemiological studies show that women who work in irregular shift work may be at higher risk of developing breast cancer (Schernhammer et al., 2003; Hansen and Stevens, 2012; Blakeman et al., 2016). Moreover, the toxicity and efficacy of various cancer therapies is dictated by time-of-day (Kobayashi et al., 2002;

Lauriola et al., 2014; Borniger et al., 2017). Cancer cells with a disrupted clock show increased growth in culture, and mice with a disrupted clock tend to develop more radiation-induced tumors than wild type (WT) mice (Shostak, 2017). Mutations in genes that regulate the molecular clock have been found in human cancer samples, indicating a gene-specific causal relation between the circadian clock and cancer (Kettner et al., 2014). At the cellular level, most cancer cell-lines show loss of rhythmicity, specifically of genes that are related to proliferation and apoptosis, thus enabling their uncontrolled proliferation (Relógio et al., 2014).

The currently held molecular model for circadian rhythmicity is based on a transcription-translation feedback loop. This mechanism includes the transcription factors BMAL1 and CLOCK that drive transcription of the Period (*Per*) and Cryptochrome (*Cry*) genes. Then, PER and CRY proteins dimerize, translocate to the nucleus and repress *Clock-Bmal1* transcription. This orchestrated machinery results in periodic expression of approximately half of the mammalian genes (Zhang et al., 2014). Previous studies found that *Per1* and *Per2* are essential for the proper function of the circadian clock, while the contribution of *Per3* is not as important (Bae et al., 2001). *Per2* was shown to be essential for proper function of the circadian clock under conditions of constant dark or light, as mice lacking *Per2* exhibit a faulty clock that is reflected by a shorter period and rhythmic instability (Zheng et al., 1999; Tamiya et al., 2016). *In vitro*, loss of *Per1* or *Per2* was suggested to have a much milder effect on cell rhythmicity (Ramanathan et al., 2014), compared to the effect observed *in vivo*.

It is well established that the non-malignant components of the tumor, also termed the tumor microenvironment (TME) are an integral and essential part of the tumor. The TME is composed of many cell types, including immune cells, cancer associated fibroblasts (CAF), endothelial cells, and extracellular matrix (ECM). The TME is essential for tumor formation, homeostasis, and progression (Quail and Joyce, 2013; Yuan et al., 2016; Jin et al., 2017). It is dynamically reshaped, thus certain cell populations in the TME can be detrimental for early stages of tumor initiation, while protumorigenic in later stages of tumor progression (Lu et al., 2012; Bercovici et al., 2019; Lin et al., 2019; Wei et al., 2020). Previous studies have shown that cancer cells within a tumor lose their rhythmicity, yet there is sparse data about rhythmicity of the TME. This is partially due to the fact that the vast majority of published studies on cancer and circadian clocks used mouse models in which both the TME and the cancer cells are affected by a disrupted clock, either by genetic mutations or by environmental cues. Recently it was shown that mice with environmentally disrupted clocks had a more protumorigenic immune microenvironment in breast cancer (Hadadi et al., 2019) and thoracic cancer (Yang et al., 2019). Pan-cancer analysis supported these findings, and suggested that circadian genes are involved in the regulation of cancer immunity (Zhou et al., 2020). Not only the immune microenvironment, but also CAFs were proposed to play a role in clock regulation in cancer, when shown that co-culturing colon cancer cells with CAFs improved the rhythmicity of cancer cells (Fuhr et al., 2019). Gaining a better understanding of how circadian clocks in the TME affect the development and progression of the tumor could lead to novel therapeutic advances and approaches. It is possible that synchronization, or lack of synchronization, between the TME and the cancer cells, plays a role in cancer progression. For example, tumors may rely on the TME maintaining intact circadian rhythms to compensate for imbalances resulting from loss of rhythmicity in the cancer cells. Alternatively, loss of rhythmicity may be essential for cells of the TME to shift from antitumorigenic to protumorigenic. In this study we set out to explore the role of the circadian clock in the TME. Using orthotopic injection of cancer cells into clock-deficient mice, we find that stromal *Per2*, but not *Per1*, is required for tumor growth and metastatic colonization. Loss of *Per2* in the TME leads to transcriptional rewiring at early stages of metastases formation, and suppresses subsequent metastatic tumor progression, highlighting *Per2* as an unexpected protumorigenic mediator in the TME.

# RESULTS

## The Circadian Rhythm in Cancer Cells Is Disrupted, While Normal Fibroblasts Maintain a Robust Rhythm, *in vitro*

A disrupted circadian clock is associated with tumorigenesis, and cancer cells are thought to express a less robust clock compared to non-malignant cells (Welsh et al., 2004; Kiessling et al., 2017). To test this, we set out to evaluate the rhythmicity of NIH3T3, a non-malignant immortalized fibroblast cell line, and of three cancer cell lines – MC38 (colon), LLC (lung), and E0771 (breast) – using continuous bioluminescence monitoring of cells. Cells were transduced with a *Per2* promotor-driven luciferase reporter (Yoo et al., 2004; Liu et al., 2008), synchronized by the synthetic glucocorticoid Dexamethasone, and longitudinally assessed using a LumiCycle luminometer (Yamazaki and Takahashi, 2005). To better understand the rhythmic behavior of cells, we developed a regression model that calculates the period length ($\pi$) of the curve (see "Materials and Methods"). The coefficient of determination $R^2$ can be used as a readout for how closely our empiric data follows a sinusoidal pattern. As evident by the period and the coefficient of determination, NIH3T3 fibroblasts exhibited robust rhythmicity (**Figure 1A**), while all three cancer cell lines were less rhythmic than NIH3T3 (**Figures 1B–D**). E0771 cells exhibited the highest coefficient of determination of all three cancer cell lines, indicating that their rhythmicity is the most intact, while still less robust than that of non-malignant cells. The period length of all three cancer cell lines was longer than that of the fibroblasts, with LLC having the longest period (28.3 h), followed by E0771 (27.2 h), and MC38 (26.3 h). Next, we asked whether the rhythmicity of the NIH3T3 fibroblasts could be disrupted by loss of *Per1*/*Per2*. We knocked down *Per1* and *Per2* using siRNA and assessed their rhythmicity using bioluminescence. Indeed, we found that deletion of *Per1* and *Per2* impairs cell rhythmicity (**Figures 1E,F**). These results suggest that the rhythmicity of cancer cells is disrupted, *in vitro*, while normal cells maintain

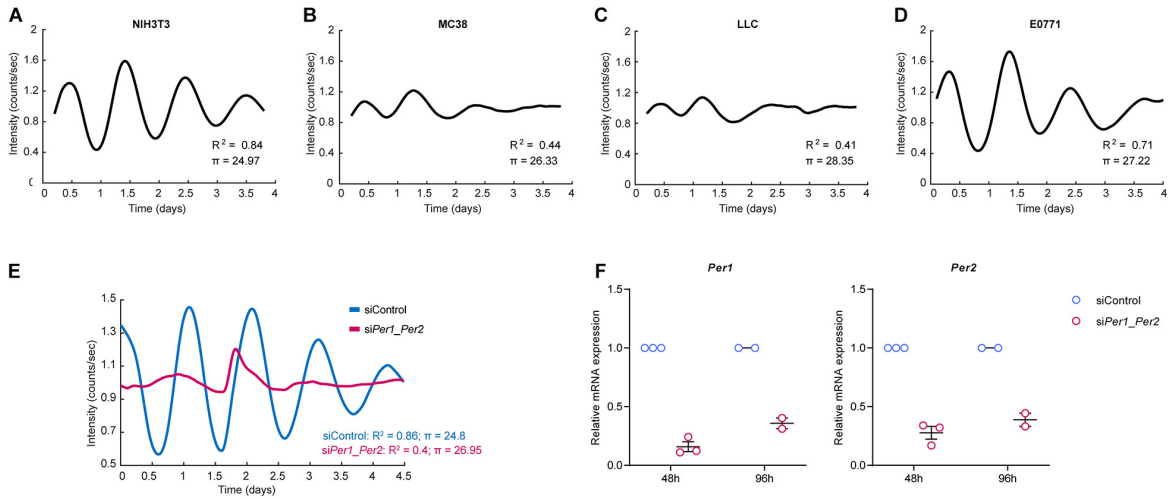


**FIGURE 1 |** Cancer cells show disrupted rhythmicity compared to normal fibroblasts. **(A–D)** Per2::luc expressing NIH3T3 fibroblasts or cancer cells, as indicated, were synchronized by dexamethasone and their luminescence signal was measured continuously (every 12 min) in a LumiCycle luminometer. The period and non-linear regression model coefficient of determination are presented (see "Materials and Methods"). The curves are an average of 3 technical repeats, and are representative of at least two independent experiments. **(E,F)** *Per1* and *Per2* were knocked down in Per2::luc-expressing NIH3T3 cells using siRNA. The cells were synchronized by dexamethasone and their luminescence was measured continuously (every 12 min) in a LumiCycle luminometer. **(E)** Period and the non-linear regression model coefficient of determination are presented. The curves are an average of 3 technical repeats, and are representative of two experiments. **(F)** Validation of *Per* gene knockdown efficiency at two time points by RT-PCR is presented as mean ± SEM.

a functional clock, the activity of which is dependent on the expression of the core clock genes *Per1* and *Per2*.

## Loss of *Per1* and *Per2* Inhibits Metastatic Colonization

Several reports have shown that depletion of the *Per* family genes enhances cancer cell growth and tumor progression (Fu et al., 2002; Gery et al., 2006; Yang et al., 2009; Papagiannakopoulos et al., 2016). To understand how loss of rhythmicity in the TME affects tumor growth, we used a mouse model of liver metastasis, employing a syngeneic colorectal cancer cell line (MC38) in C57Bl/6 mice. Since the liver functions in a circadian manner (Reinke and Asher, 2016, 2019), and is affected directly by the feeding patterns of the organism, it serves as a good model to investigate the circadian clock *in vivo* (Tahara and Shibata, 2016). In our model, 20,000 cancer cells were injected to the hepatic portal vein of mice, producing liver metastases 21 days post injection. We applied this model to WT and $Per1^{-/-}Per2^{-/-}$ mice to assess the effect of a clock-impaired TME on metastatic colonization. Surprisingly, $Per1^{-/-}Per2^{-/-}$ mice had a lower metastatic burden than WT mice, as measured by the reduced number of metastases and lower liver/body weight ratio (**Figures 2A–C** and **Supplementary Figure 1A**). WT mice developed dozens of liver metastases, which exhibited substantial ECM rearrangements, as evident by Sirius red staining for collagen, and were heavily infiltrated by α-SMA-positive CAFs (**Figure 2D**). In stark contrast, the livers of $Per1^{-/-}Per2^{-/-}$ mice had significantly fewer metastases, and stained mostly negative for α-SMA and Sirius Red (**Figure 2D**). These results suggest that loss of *Per* genes in the stroma inhibits metastatic colonization and ECM-remodeling of the metastatic niche.

T-cells are a major component of the TME, and have a key role in the cytotoxic activity against cancer cells. To assess whether T cells mediate the tumor growth inhibition observed in $Per1^{-/-}Per2^{-/-}$ mice, as well as to exclude the possibility that $Per1^{-/-}Per2^{-/-}$ mice reject the MC38 cancer cells due to their expression of *Per1* and *Per2* genes, we injected cancer cells into WT or $Per1^{-/-}Per2^{-/-}$ mice in which T cells were depleted by CD4 and CD8 antibodies (**Supplementary Figure 1B**). CD4 and CD8 antibodies were injected 2 days prior to the injection of cancer cells, and every 5 days until the endpoint, and the efficiency of depletion was verified by FACS analysis (**Supplementary Figure 1B**). $Per1^{-/-}Per2^{-/-}$ mice were significantly more resistant to metastatic colonization than WT mice, even upon T cell depletion (**Figures 2E–G**). The effect of $Per1^{-/-}Per2^{-/-}$ loss in the TME was similar in the presence and absence of T cells (**Figure 2H**). Together, these results suggest that T cells do not mediate stromal $Per1^{-/-}Per2^{-/-}$-associated tumor inhibition.

## Stromal *Per2* Regulates Tumor Progression

It is well established that both *Per1* and *Per2* are essential for maintaining proper circadian clock activity (Bae et al., 2001). To test whether both genes are also essential for the stromal regulation of tumor progression, we injected MC38 cancer cells to the portal vein of mice in which each of the *Per* genes (*Per1, Per2*) was knocked-out separately, and assessed metastatic colonization. WT mice were used as control. $Per2^{-/-}$ mice exhibited significantly lower metastatic burden than WT mice (**Figures 3A–C**). Loss of *Per1*, on the other hand, did not affect metastasis, and $Per1^{-/-}$ mice exhibited similar metastatic

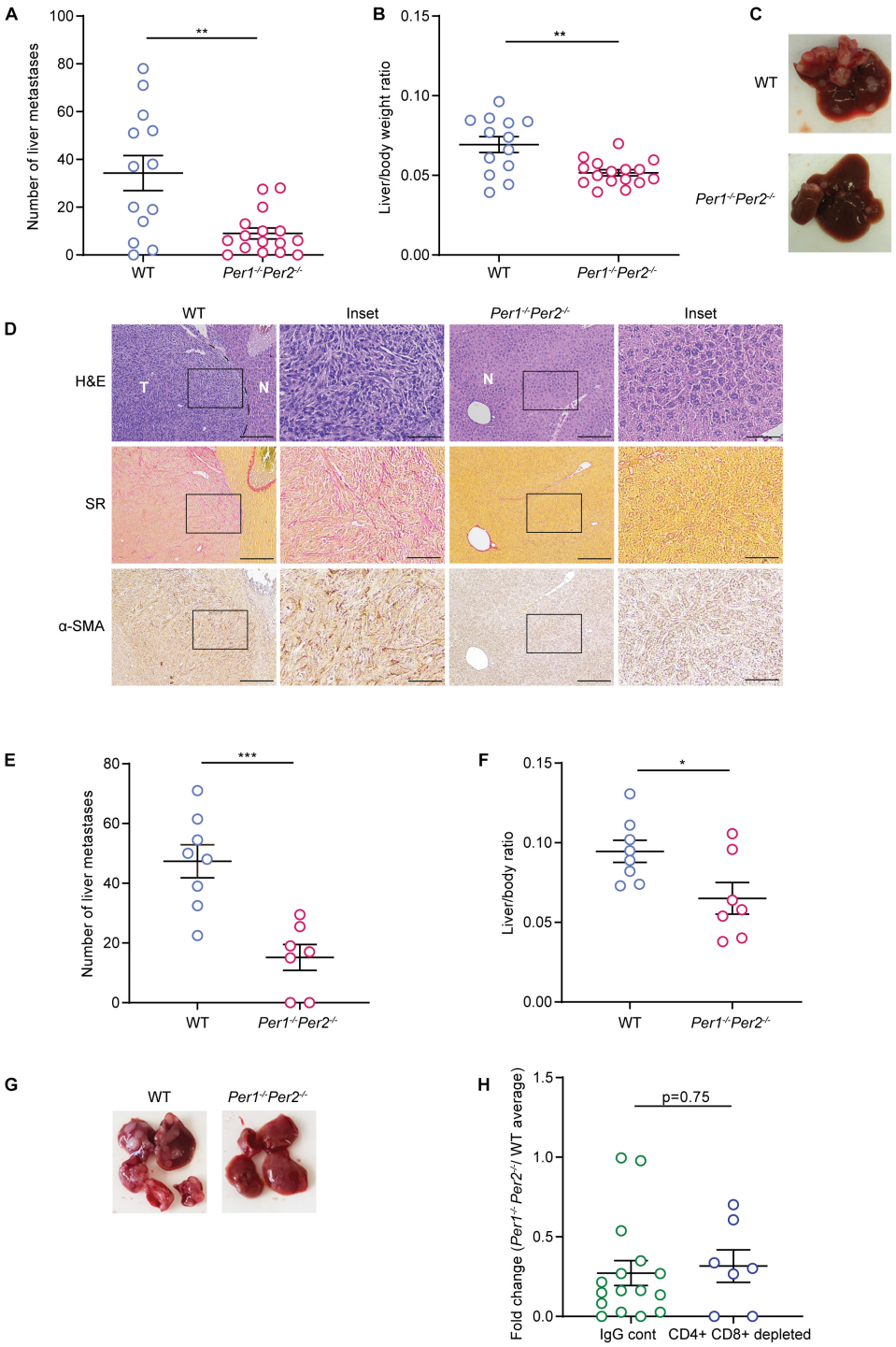


**FIGURE 2 |** Clock deficient mice exhibit lower metastatic burden. **(A–D)** WT and *Per1*$^{-/-}$*Per2*$^{-/-}$ mice were injected with MC38 cancer cells into the hepatic portal vein. At 21 days post injection macrometastases were quantified by visual inspection **(A)** and the ratio of liver weight to body weight was calculated **(B)**. $n$ = 13–16 mice per genotype combined from three independent experiments. $P$-value was calculated using unpaired Student's $t$-test with Welch's correction. Error bars represent SEM. **(C)** Representative images of livers from WT and *Per1*$^{-/-}$*Per2*$^{-/-}$mice. **(D)** Representative Hematoxylin and Eosin (H&E), Sirius red (SR), and α-SMA staining of livers from WT and *Per1*$^{-/-}$*Per2*$^{-/-}$ mice. Scale bar – 200 μm; inset – 67 μm; T-tumor, N-normal tissue. **(E–H)** WT and *Per1*$^{-/-}$*Per2*$^{-/-}$ mice were injected with antibodies of CD4+ and CD8+ 2 days prior to MC38 injection and every 5 days until the end point. At 21 days post injection macrometastases were quantified by visual inspection **(E)** and the ratio of liver weight to body weight was calculated **(F)**. $n$ = 7–8 per genotype combined from two independent experiments. $P$-value was calculated using unpaired Student's $t$-test. **(G)** Representative images of livers from WT and *Per1*$^{-/-}$*Per2*$^{-/-}$ mice with T cell depletion are shown. **(H)** The number of metastases in normal and T cell depleted *Per1*$^{-/-}$*Per2*$^{-/-}$ mice was normalized to the average number of metastases in WT mice. $P$-value was calculated using unpaired Student's $t$-test. In all figures error bars represent SEM, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

burden to that of WT (**Figures 3D–F**). *Per2*$^{-/-}$ livers also showed less collagen deposition and CAF infiltration than WT (**Figure 3G**), while the amount and the structure of collagen and infiltrating CAFs was similar between *Per1*$^{-/-}$ and WT livers (**Figure 3H**). These results suggest that stromal *Per2* is essential for metastatic colonization, while *Per1* is dispensable, thus implying that the effect of stromal *Per2* knock-out on metastatic colonization may be clock-independent.

## Transcriptional Analysis of WT and *Per2*$^{-/-}$ Livers Suggests a Role for *Per2* in Eliciting a Pre-metastatic Niche

We hypothesized that stromal *Per2* plays a role in preparing the hepatic niche for the initiation of metastasis. To test this hypothesis, we injected MC38 cancer cells (or PBS, as control) to the portal vein of *Per2*$^{-/-}$ and WT mice, harvested the livers 1 week following injection, and performed RNA-sequencing on total liver extracts (**Supplementary Table 1**). At this early stage only micrometastases ($\sim$0.0005 mm$^3$), and not macrometastases ($\sim$5 mm$^3$ at 3 weeks) were visible in WT livers (**Figure 4A**). The average area covered by metastases in the liver was 0.7% in WT mice and 0.05% in *Per2*$^{-/-}$ mice at this early time point (compared to 34.3% in WT mice and 8.7% in *Per2*$^{-/-}$ mice at 3 weeks post injection; **Figure 4B**). Therefore, we assume that the sequencing data represents mostly the normal liver and the pre-metastatic niche, and not cancer cells. Principal component analysis (PCA) showed that the basal transcriptional landscape of WT and *Per2*$^{-/-}$ livers is different, and further changes following injection of cancer cells (**Supplementary Figure 2B**). Hierarchical clustering highlighted 1222 differentially expressed genes between WT and *Per2*$^{-/-}$ livers injected with PBS or with MC38 cancer cells (**Figure 4C** and **Supplementary Table 1**). In the control mice (PBS), loss of *Per2*$^{-/-}$ led to upregulation of the response to interferon-beta (*Ifit1, Stat1, Tgtp1, Eif2ak2*), catabolism (*Adam9, Pik3c2a, Tnfaip3, Rock1*), wound healing (*Cd36, Cldn1, Pdpn, Pdgfra, Pten*), recycling of bile acids and salts (*Slc10a2, Abcb11, Slco1b2*), and neutrophil degranulation (*Adam10, Ctsc, Degs1*; **Figure 4C** and cluster 4 and **Supplementary Table 2**). Apoptosis (*Akt1, Bad, Bcl2l1, Bnip3*), carboxylic acid biosynthesis (*Aldoa, Apoa1, Apoa4, Ccnd3*), and the cellular response to reactive oxygen species (*Sod3, Pdk2, Park7, Ccs*) were downregulated in the *Per2*$^{-/-}$ livers (**Figure 4C** and cluster 3 and **Supplementary Table 2**). The same genes and pathways were also downregulated (though to a lesser extent) following injection of MC38 cells to either WT or *Per2*$^{-/-}$ mice, suggesting that these are genes involved in normal liver functions that fail to function properly upon loss of *Per2*$^{-/-}$ or in early cancer stages (**Figure 4C**, cluster 3). We could not identify pathways upregulated in WT livers 1 week after cancer cell injection. In *Per2*$^{-/-}$ livers, however, we observed upregulation of genes involved in autophagy (*Ulk1, Plk3, Sesn2*), circadian regulation (*Ngfr, Klf10, Atf5*), and inhibition of cell proliferation (*Ngfr, Hes1, Tob1*) following cancer cell injection (**Figure 4C** and cluster 1 and **Supplementary Table 2**). This analysis suggests that in early stages of metastases formation, house-keeping genes are downregulated in WT livers, while only minor transcriptional upregulation is observed. Loss of *Per2,* however, rearranges the premetastatic liver niche, in a manner that activates tumor suppressing pathways in response to the presence of cancer cells, resulting in attenuated metastatic spread.

We further interrogated our RNA-sequencing data using CIBERSORT. This tool provides an estimation of the abundance of various cell types in a mixed cell population, based on expression of cell-type specific hallmark genes (Newman et al., 2019). We compiled several published single-cell datasets to create a reference dataset with which we examined the composition of our samples [**Supplementary Table 3** and references (Heng and Painter, 2008; Dobie et al., 2019; Xiong et al., 2019)]. We found that the cellular composition is similar across samples (**Supplementary Table 4**), with one exception: cholangiocytes were significantly elevated in *Per2*$^{-/-}$ livers compared to WT livers (**Supplementary Figure 2C** and **Supplementary Table 4**). While this analysis did not highlight changes in cholangiocyte numbers between MC38 treated and non-treated livers, these findings suggest that *Per2* plays a role in cholangiocyte homeostasis. These findings are in line with previous reports showing that deletion of *Per2* exacerbates cholestatic liver injury and fibrosis (Chen et al., 2013). The majority of cells detected in all of our samples were hepatocytes (>95%, **Supplementary Table 4**), suggesting that transcriptional rewiring of these cells, rather than changes in cell composition, leads to the observed changes in gene expression.

## Loss of *Per2* in the Stroma Impairs Primary Tumor Growth

Next, we asked whether stromal *Per2* is essential not only for metastatic colonization, but also for primary tumor formation. We used two orthotropic models: The E0771 triple negative breast cancer cell line injected to the mammary fat pad, and the MC38 colon cancer cell line (used for the liver metastasis model) injected into the colon submucosa by an endoscopy-guided procedure [as described in Zigmond et al. (2011)]. In both models, cancer cells were injected to *Per2*$^{-/-}$ and WT mice, and tumor volume was assessed at the end point. *Per2*$^{-/-}$ mice exhibited significantly smaller E0771 tumors compared to WT mice (**Figure 5A**), and a similar trend was seen for the MC38 colon model (**Figure 5B**). Taken together with our results from the liver metastasis model, these results suggest that stromal *Per*2 is required for tumor formation both in the primary site as well as in the metastatic site. These findings also imply that PER2 is required throughout the course of tumor progression and metastasis.

# DISCUSSION

Disrupted circadian rhythms are generally thought to contribute to tumorigenesis and poor prognosis (Savvidis and Koutsilieris, 2012; Hadadi et al., 2019). However, much of the work performed on cancer and rhythmicity, to date, was done in a context in which both cancer cells and their microenvironment have disrupted clocks. Here we found that expression of the core clock gene *Per2* in the TME supports different stages of cancer

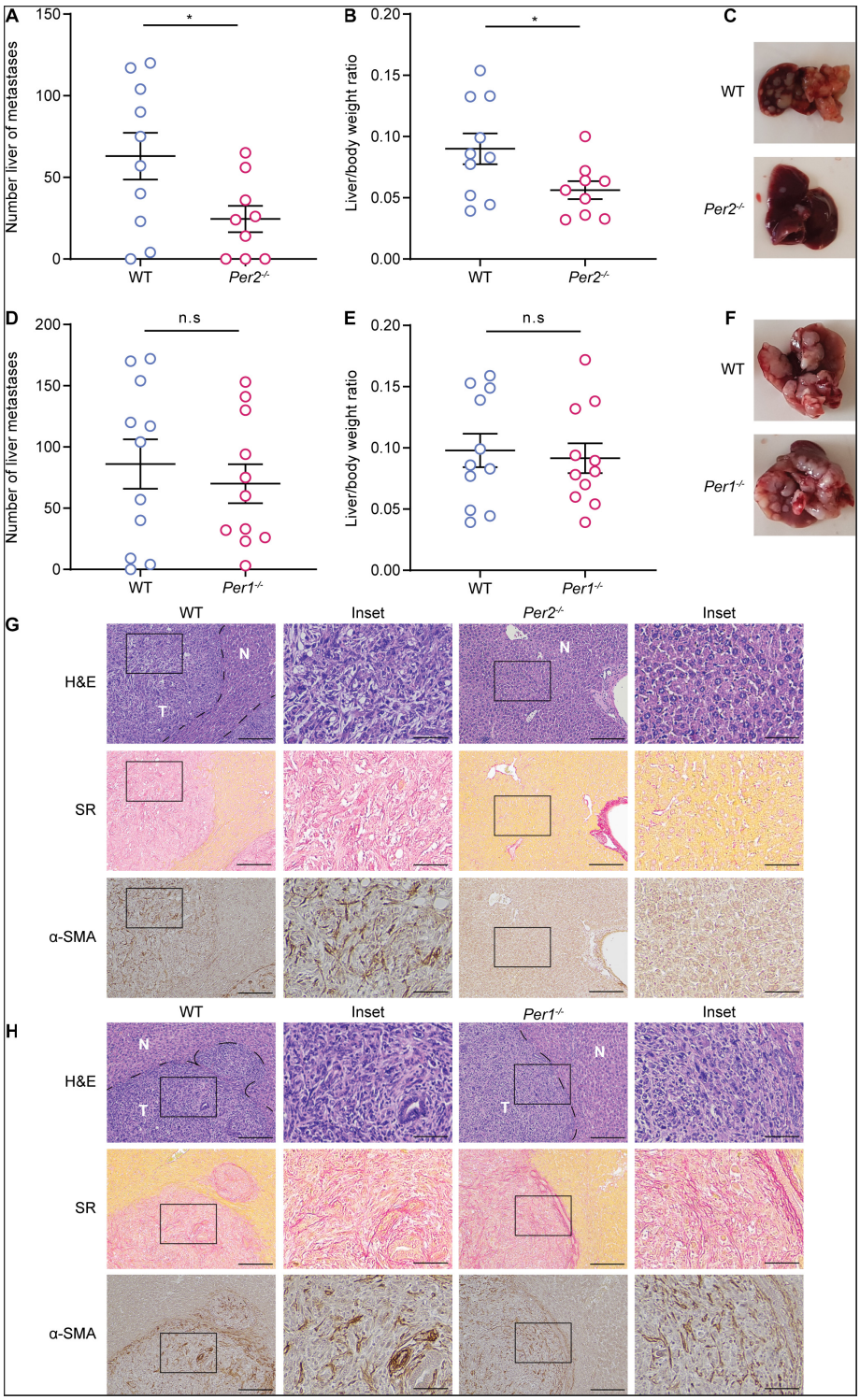


**FIGURE 3 |** Stromal *Per2*, and not *Per1* promotes metastatic colonization. **(A–C)** WT and *Per2*$^{-/-}$ mice were injected with MC38 cancer cells into the hepatic portal vein. At 21 days post injection **(A)** macrometastases were quantified by visual inspection and **(B)** the ratio of liver weight to body weight was calculated. $n$ = 9–10 mice per genotype combined from three independent experiments. **(C)** Representative images of livers from WT and *Per2*$^{-/-}$ mice are shown. **(D–F)** WT and *Per1*$^{-/-}$ mice were injected with MC38 cancer cells into the hepatic portal vein. At 21 days post injection macrometastases were quantified by visual inspection **(D)** and the ratio of liver weight to body weight was calculated **(E)**. $n$ = 11 mice per genotype combined from three independent experiments. **(F)** Representative images of livers from WT and *Per1*$^{-/-}$ mice are shown. **(G,H)** Representative H&E, Sirius red (SR), and α-SMA staining of livers from WT, *Per2*$^{-/-}$ and *Per1*$^{-/-}$ mice harvested at day 21 post injection. Scale bar – 200 μm, inset – 67 μm; T-tumor N-normal tissue. $P$-value was calculated using unpaired Student's $t$-test. Error bars represent SEM, $^{*}p < 0.05$.

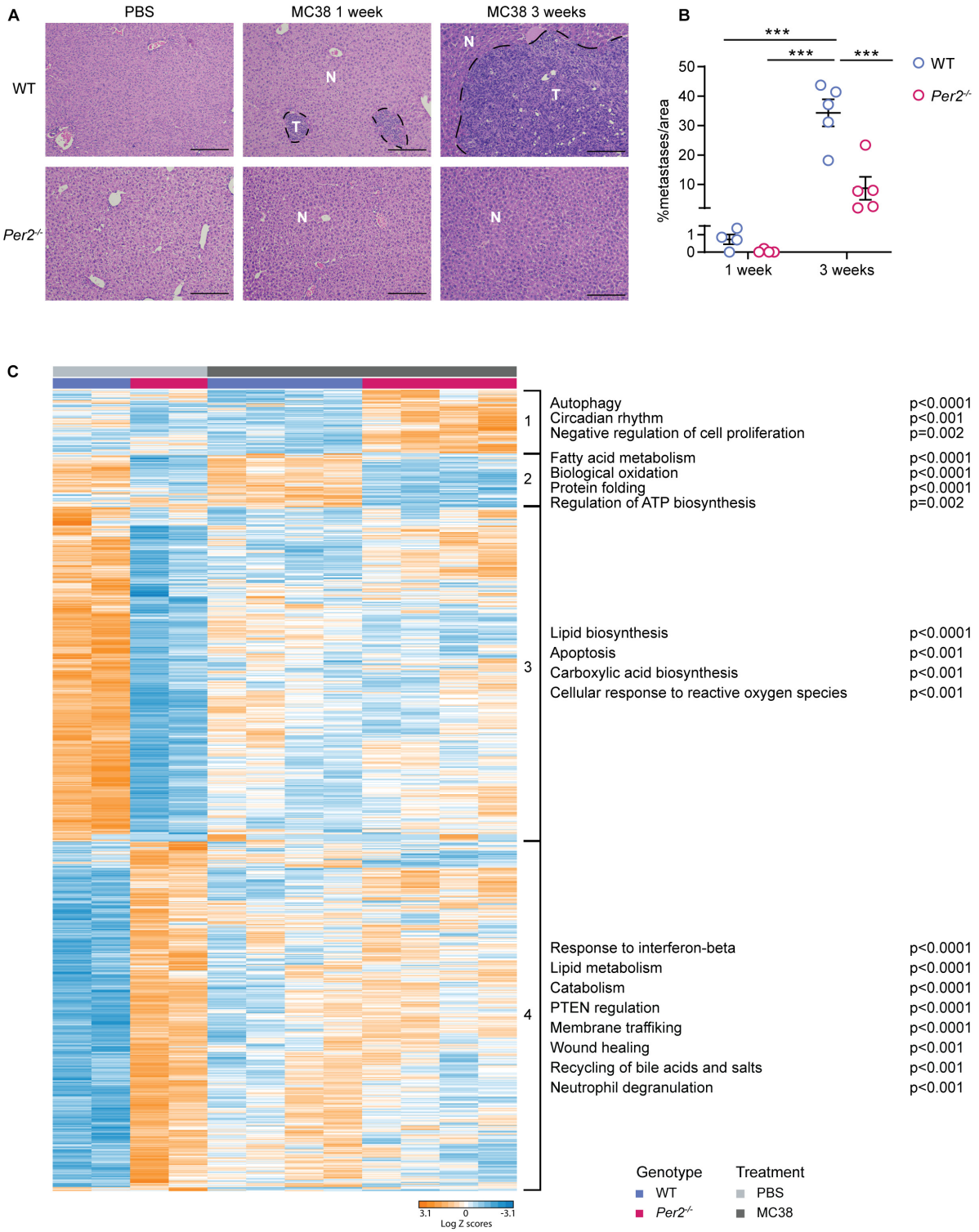


**FIGURE 4 |** Stromal *Per2*$^{-/-}$ is essential for the formation of the pre-metastatic niche. WT and *Per2*$^{-/-}$ mice were injected with MC38 cancer cells into the hepatic portal vein and sacrificed 1 or 3 weeks post injection, as indicated. PBS was injected to control mice which were sacrificed 1 week post injection. $n$ = 4–5 mice per genotype. **(A)** Representative H&E staining of livers from WT and *Per2*$^{-/-}$ mice post injection of PBS or MC38 cancer cells. Scale bar – 200 μm, inset – 67 μm; T-tumor, N-normal tissue. **(B)** The area covered by metastases in MC38-injected livers was calculated by QuPath based on H&E images. *P*-value was calculated using two way ANOVA. Error bars represent SEM, ***$p < 0.0005$. **(C)** RNA-sequencing was performed on livers of WT and *Per2*$^{-/-}$ mice injected with MC38 ($n$ = 4 per genotype) and PBS ($n$ = 2 per genotype). Heatmap showing hierarchical clustering of 1222 differentially expressed genes between all four conditions. Pathway analysis was performed using Metascape. Significant pathways are shown; $p < 0.05$, FDR < 0.05 (for details see **Supplementary Table 2**).

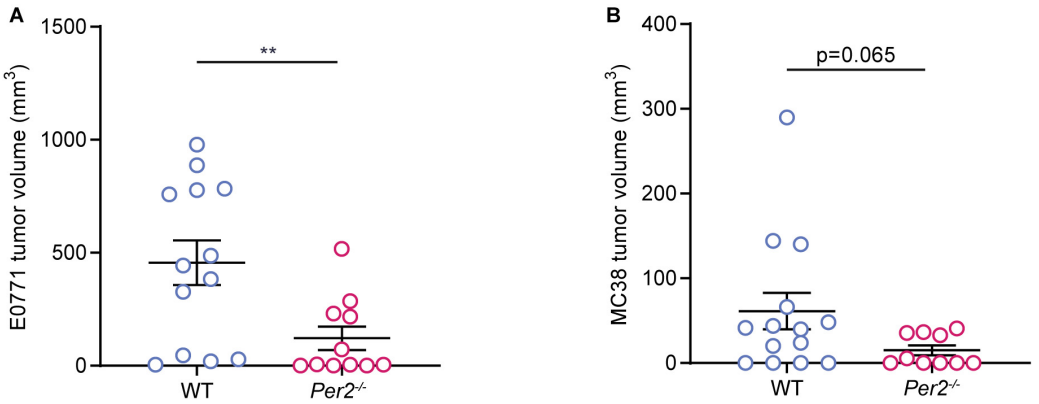


**FIGURE 5 |** Stromal *Per2* controls primary tumor growth. **(A)** WT and $Per2^{-/-}$ mice were injected with E0771 cancer cells into the mammary fat pad. The mice were sacrificed at day 32, and tumor volume was calculated. $n$ = 11–13 per genotype, combined from two independent experiments. **(B)** WT and $Per2^{-/-}$ mice were injected with MC38 cancer cells into the colon wall. Mice were sacrificed at day 14 and tumor volume was calculated. $n$ = 10–14 per genotype combined from two independent experiments. $P$-value was calculated using unpaired student's $t$-test with Welch's correction. Error bars represent SEM, $^{**}p < 0.005$.

progression. Employing different orthotopic tumor models, we showed that loss of stromal *Per2*, but not *Per1*, inhibits tumorigenesis and metastasis.

The circadian clock is a major regulator of metabolism and cell cycle and its disruption is well known to promote cancer. In cancer cells, both PER1 and PER2 were shown to act as tumor suppressors (Fu et al., 2002, 2016; Yang et al., 2009; Su et al., 2017; Zhu et al., 2019; Hou et al., 2020; Liu et al., 2020) and mutations in these genes were associated with human cancer (Chen et al., 2005; Kettner et al., 2014; Wu et al., 2019). However, whether these effects are clock-dependent is not clear. Moreover, despite the expansive knowledge of *Per1* and *Per2* roles in cancer cells, their roles in the TME were largely overlooked. Here we show that selective loss of the core clock genes *Per1* and *Per2* in the TME not only does not promote cancer but actually inhibits tumor growth and metastatic colonization. These findings suggest that the tumor-suppressive effects of PER1 and PER2 are most likely restricted to the cancer cells themselves, whereas in the TME PER1 and PER2 play different roles, that may be clock-independent.

The TME is comprised of different cell types. Which of these cell types contributes to the observed inhibitory effect of *Per2* loss on tumor growth and metastasis? Recent studies suggested an immune-regulatory role for *Per* genes in the TME, as mice with disrupted clocks have more protumorigenic immune microenvironments. Yet we find that T cell depletion does not compensate for the $Per1^{-/-}Per2^{-/-}$-driven inhibition of tumor growth, nor do we detect immune cell transcriptional signatures in early stages of metastases formation, suggesting that the adaptive immune microenvironment does not mediate this effect. Various other cell types play protumorigenic roles in the TME. We observed increased numbers of cholangiocytes in $Per2^{-/-}$ mice, which may contribute to the anti-tumorigenic TME. Additionally, we detect massive transcriptional changes in hepatocytes of the pre-metastatic niche. Indeed, hepatocytes were recently reported to drive the formation of a pro-metastatic niche in the liver (Lee et al., 2019). CAFs were also proposed to play a role in clock regulation of cancer (Fuhr et al., 2019), and are generally known to have key roles in cancer progression. We observed massive infiltration of α-SMA-positive CAFs and collagen secretion in WT tumors which were not seen in $Per2^{-/-}$ or $Per1^{-/-}Per2^{-/-}$ mice. Whether this CAF infiltration is driven by *Per2* in fibroblasts, or perhaps by *Per2* in other cells of the TME, remains to be determined in future studies.

The liver is a circadian organ, which allows for the circadian regulation of different metabolic functions, such as the synthesis and metabolism of glucose, lipid, cholesterol, and bile acid (Bass and Takahashi, 2010; Ferrell and Chiang, 2015). These pathways are massively deregulated in $Per2^{-/-}$ mice. Injection of cancer cells leads to further deregulation of these pathways, and induces other pathways (autophagy and inhibition of proliferation) which may contribute to the tumor-suppressive environment that prevents subsequent metastatic tumor growth. These processes may explain why, while PER2 serves as a tumor suppressor in cancer cells, it serves as a protumorigenic factor in the TME. In circadian biology, PER1, and PER2 function together to regulate clock functions. Our results show that *Per2*, but not *Per1*, plays an important role in the stromal regulation of tumor progression. These findings may imply a non-circadian role for *Per2*, differentiating it from *Per1*, and offering a new attractive therapeutic target in the TME.

## MATERIALS AND METHODS

### Ethics Statement

All animal studies were conducted in accordance with the regulations formulated by the Institutional Animal Care and Use Committee (IACUC; protocols #02100220-2 and #02550418-3).

### Mice

$Per1^{-/-}Per2^{-/-}$ (Zheng et al., 2001) were back crossed to C57BL/6 (Adamovich et al., 2019) and single $Per1^{-/-}$, $Per2^{-/-}$, were generated (kindly provided by Gad Asher, WIS). These

mice and WT C57Bl/6 controls (ENVIGO RMS, Israel) were maintained under specific-pathogen-free conditions at the Weizmann Institute's animal facility.

## Cell Culture

MC38 mouse colon cancer cells (kindly provided by Lea Eisenbach, WIS), LLC mouse lung carcinoma cells (kindly provided by Zvika Granot, HUJI), E0771 mouse breast cancer cells (kindly provided by Ronen Alon, WIS), and NIH3T3 mouse fibroblasts cell line (ECACC, #93061524) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, 01-052-1A) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% pen-strep and 1% L-glutamine (Biological Industries). All cell lines were maintained at 37°C in 5% CO2 and regularly tested for mycoplasma by PCR assay (Biological Industries). For orthotropic and portal vein injections, the cells were suspended at 80–90% confluence by treatment with 0.25% trypsin, 0.02% EDTA and washed with 1x phosphate buffered saline (PBS).

## Generation of Per2::Luc Cells by Lentiviral Infection

pLenti6-B4B2 construct expressing *Per2-dLuc* (Liu et al., 2008) (kindly provided by Gad Asher, WIS) was inserted into MC38, LLC, NIH-3T3, and E0771 cells using lentiviral infection. After infection, the cells were selected by blasticidin-containing complete DMEM for 10 days.

## Real-Time Luminescence Monitoring

Cells were seeded into black, clear bottom 24-well plates (Provair) and synchronized with 100 nM Dexamethasone treatment for 20 min. Bioluminescence was measured by a LumiCycle luminometer (Actimetrics) every 12 min for at least 3 days in the presence of luciferin (Promega) in the cell culture medium. For siRNA experiments, 24 h after seeding, NIH3T3 Per2::luc cells were transfected with siRNA for the specified genes (SMART Pool siRNA, Dharmacon). After 24 h cells were synchronized by Dexamethasone and bioluminescence was measured as described above.

## Mathematical Analysis of Bioluminescence Data

To evaluate the rhythmicity of Per2::luc cells we created a CircadLib package for the MATLAB R2017b environment using a non-linear regression model. The model includes a constant level and one sinusoid to estimate the curve parameters – amplitude, phase and period. To remove effects of cell proliferation and signal decline the algorithm uses a de-trending function. To remove technical noise, the moving average method was used. This analysis yields two parameters: $R^2$ – indicating how similar the curve is to a sinusoid, and π – indicating the period length.

## Quantitative RT–PCR Analysis

Total RNA was extracted from cells using Bio-Tri reagent (Bio-Lab). mRNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

**TABLE 1 |** Primers used in this study.

| | Forward | Reverse |
|---|---|---|
| HPRT | CATAACCTGGTTCATCATCGC | TCCTCCTCAGACCGCTTTT |
| TBP | CCCTATCACTCCTGCCACACCAGC | GTGCAATGGTCTTTAGGTCA AGTTTACAGCC |
| b2m | ACCGGCCTGTATGCTATCCAGAAA | GGTGAATTCAGTGTGA GCCAGGAT |
| Per1 | ACCAGCCATTCCGCCTAAC | CGGGGAGCTTCATAACCAGA |
| Per2 | GAAAGCTGTCACCACCATAGAA | AACTCGCACTTCCTTTTCAGG |

Quantitative RT–PCR analysis was performed using Fast SYBR Green Master mix (Applied Biosystems) with the primers listed in **Table 1**.

## Liver Metastatic Colonization via Portal Vein Injection

Sixteen weeks old WT C57Bl/6, $Per1^{-/-}$, $Per2^{-/-}$, and $Per1^{-/-}Per2^{-/-}$ mice, $n$ = 9–16 per genotype, were injected with an analgesic agent (Buprenorphine, 0.1 mg/kg SC), and anesthetized using isoflurane 2.5%. To form liver experimental metastases, 20,000 MC38 cells were injected into the hepatic portal vein. To prevent bleeding, light pressure was applied to the injection site with a cotton applicator for 4 min. Then, the muscle and skin were sutured using 6/0 polypropylene monofilament sutures. At day 21 post-injection, mice were sacrificed, and body weights were measured. Livers were harvested and weighed, and macrometastases were quantified by visual inspection. To normalize liver weights, liver/body weight ratios were calculated. Then, livers were formalin-fixed and paraffin-embedded for histological analysis.

## Quantification of Liver Metastasis Analysis

Hematoxylin and Eosin (H&E) slides of liver tissues were scanned by a Pannoramic SCAN II scanner, 20×/0.8 objective (3DHISTECH, Budapest, Hungary). Quantification of liver metastases area was done by QuPath (version 0.2.0-m8) (Bankhead et al., 2017) with pixel classification using the simple threshold method, with prefilter Gaussian, smoothing sigma 4 and a threshold of 180.

## Total RNA Isolation From Livers of WT and $Per2^{-/-}$ Mice

$Per2^{-/-}$ or WT C57Bl/6 male mice were injected at the age of 16 weeks, under anesthesia, with 20,000 MC38 cells ($n$ = 4 mice per genotype) and PBS ($n$ = 2 control mice per genotype) into the hepatic portal vein as described above. At day 7 post-injection, mice were sacrificed, and body weights were measured. Livers were weighed and dissociated by Bead Ruptor Elite (OMNI International) with metal beads in Microtube (Sarstedt #72.694.006). RNA isolation was performed by RNeasy Fibrous Tissue Mini Kit (Qiagen #74704) according to manufacturer's instructions.

## Library Preparation and RNA-Sequencing Analysis

Libraries were prepared using the SENSE mRNA-Seq Library Prep Kit V2 (Lexogen United States) according to the instructions of the manufacturer. Libraries were sequenced on an Illumina NovaSeq 6000 machine, at 10 M reads per liver sample, to provide sufficient reads to pass quality control (quality of reads and mapping quality percentage). Read counts were normalized and tested for differences using DEseq2 (Love et al., 2014). RNA sequencing results are detailed in **Supplementary Table 1**. Hierarchical clustering was performed using Euclidian distance on differentially expressed genes which were filtered with the following parameters: baseMean $> 5$, $p$adj $< 0.05$, and $|\log \text{fold change}| > 1$. Analysis was performed by Partek Genomics Suite software. Pathway analysis was performed using Metascape (Zhou et al., 2019). Significant pathways were determined if $p < 0.05$ (for details see **Supplementary Table 2**). Liver populations analysis was performed using CIBERSORT (Newman et al., 2015) based on published single-cell RNA-sequencing data (Heng and Painter, 2008; Dobie et al., 2019; Xiong et al., 2019). The gene signatures of liver subpopulations used for this analysis and the results table are detailed in **Supplementary Tables 3, 4**, respectively. The RNA-seq data that support the findings of this study have been deposited in the Gene Expression Omnibus under accession number GSE156450.

## CD8+ CD4+ Depletion

WT C57Bl/6 and *Per1*$^{-/-}$*Per2*$^{-/-}$ mice, $n = 7$–8 per genotype, were injected intraperitoneally with anti-mouse antibodies for CD8 (Clone 53–6.7) and CD4 (Clone GK1.5) or IgG2a control (Clone 2A3; 300 μg per injection per mouse for all antibodies; Bio X Cell), 2 days prior to the injection of MC38 cells to the portal vein as described above, and every 5 days to maintain full depletion until the endpoint. 50 μl blood were drawn to validate depletion of CD8+ and CD4+ cells by flow cytometry. At day 21, mice were sacrificed, body weights were measured, livers were fixed with 4% PFA, and macrometastases were quantified by visual inspection.

## Orthotropic Injection to the Colon

*Per2*$^{-/-}$ or WT C57Bl/6 male mice, $n = 10$–14 per genotype, were injected at the age of 16 weeks, under anesthesia, with 200,000 MC38 cells suspended in 20 μl PBS into the colon wall (submucosal). The injection was performed under endoscopic guidance. Body weights were measured using an analytical balance, the appearance of tumors was monitored by colonoscopy at days 10 and 14. At day 14, mice were sacrificed, colons were harvested, and tumor volumes were measured using a digital caliper and calculated using the formula: $V = (W^2 \times L)/2$.

## Orthotropic Injection to the Mammary Fat Pad

*Per2*$^{-/-}$ or WT C57Bl/6 female mice, $n = 11$–13 per genotype, were injected at the age of 8–11 weeks, under anesthesia, with 200,000 E0771 cells into the upper left mammary fat pad. Body weights were measured, and tumor volumes were measured using a digital caliper and calculated using the formula: $V = (W^2 \times L)/2$. Mice were sacrificed at day 32.

## Immunohistochemistry of Mouse Tissues

Livers were fixed in 4% Paraformaldehyde (PFA), processed and embedded in paraffin blocks, cut into 4–5 μm sections and immunostained as follows: Formalin-fixed, paraffin-embedded (FFPE) sections were deparaffinized, treated with 0.3% $H_2O_2$ and antigen retrieval was performed by microwave with citrate acid buffer (pH 6.0). Slides were blocked with 10% normal horse serum, and anti-SMA antibodies were used (Sigma #F3777). Visualization was achieved with 3, 30-diaminobenzidine (DAB) as a chromogen (#SK4100, Vector Labs Kit, CA, United States) / M.O.M Kit (Vector Labs). Counterstaining was performed with Mayer's Hematoxylin (MHS-16, Sigma-Aldrich, Rehovot, Israel). Images were taken with a Nikon Eclipse Ci microscope.

## Statistical Analysis

Statistical analysis and visualization were performed using R (Version 3.6.0, R Foundation for Statistical Computing, Vienna, Austria) and Prism 8.2.0. (GraphPad, United States). Statistical tests were performed as described in each Figure Legend.

# DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156450.

# ETHICS STATEMENT

The animal study was reviewed and approved by the Weizmann Institute of Science Institutional Animal Care and Use Committee.

# AUTHOR CONTRIBUTIONS

LS, SM, and CL designed and performed the experiments and analyses and wrote the manuscript. HL performed the experiments. AN wrote code for mathematical analysis of bioluminescence data and assisted with writing the manuscript. RS-S designed and supervised the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.587697/full#supplementary-material

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REVIEW
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# Organotypic Modeling of the Tumor Landscape

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Cancer is a complex disease and it is now clear that not only epithelial tumor cells play a role in carcinogenesis. The tumor microenvironment is composed of non-stromal cells, including endothelial cells, adipocytes, immune and nerve cells, and a stromal compartment composed of extracellular matrix, cancer-associated fibroblasts and mesenchymal cells. Tumorigenesis is a dynamic process with constant interactions occurring between the tumor cells and their surroundings. Even though all connections have not yet been discovered, it is now known that crosstalk between actors of the microenvironment drives cancer progression. Taking into account this complexity, it is important to develop relevant models to study carcinogenesis. Conventional 2D culture models fail to represent the entire tumor microenvironment properly and the use of animal models should be decreased with respect to the 3Rs rule. To this aim, *in vitro* organotypic models have been significantly developed these past few years. These models have different levels of complexity and allow the study of tumor cells alone or in interaction with the microenvironment actors during the multiple stages of carcinogenesis. This review depicts recent insights into organotypic modeling of the tumor and its microenvironment all throughout cancer progression. It offers an overview of the crosstalk between epithelial cancer cells and their microenvironment during the different phases of carcinogenesis, from the early cell autonomous events to the late metastatic stages. The advantages of 3D over classical 2D or *in vivo* models are presented as well as the most promising organotypic models. A particular focus is made on organotypic models used for studying cancer progression, from the less complex spheroids to the more sophisticated body-on-a-chip. Last but not least, we address the potential benefits of these models in personalized medicine which is undoubtedly a domain paving the path to new hopes in terms of cancer care and cure.

**Keywords: tumor microenvironment, cancer, 3D model, therapies, metastasis, tumor dissemination**

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## INTRODUCTION

Carcinogenesis is a complex multistep process, often described as somatic evolution. Typically, cancer progression involves the accumulation of genetic and/or epigenetic somatic modifications and exposition to environmental factors. Indeed, the development of many tumors is tightly linked with genotoxicity, chronic infections, dietary habits, or autoimmunity; which are all

underlined by inflammation. Early on, Fearon and Vogelstein (1990) described a sequence of defined genetic events driving the formation of colorectal cancers. Afterward, the seminal works of Hanahan and Weinberg (2000, 2011) contributed to shift cancer research from a reductionist point of view with a sole focus on the cancer cell itself to a more comprehensive view involving cues from the neighboring niche. Therefore, carcinogenesis is the fruit of the interplay between multiple cell autonomous and non-autonomous processes, defined as "Hallmarks of cancer," that include genomic instability, proliferative abnormality, stromal reprogramming, angiogenesis, immune suppression and tumor sustaining inflammation. In the following sections, we first define the tumor microenvironment (TME) and briefly depict its different components. We also summarize the recently described interactions between the TME actors and the tumor cells in the cancer progression cascade. In depth understanding of such interactions renders necessary the study of tumor cells within their microenvironment, as this is crucial for cancer progression. In this line of thought, we describe the most promising organotypic models used for modeling cancer progression stages from the initial tumor and its microenvironment to dissemination and metastasis.

# PART I—ROLE OF THE MICROENVIRONMENT IN TUMORAL PROGRESSION

The importance of the tumor microenvironment is embodied in the concept that cancer cells do not cause the disease alone, but rather corrupt recruited and neighboring normal cell types to serve as accessories to the crime (Hanahan and Coussens, 2012). In particular, interactions between cancer cells and their microenvironment represent a powerful relationship that influences disease initiation and progression and patient prognosis. For decades, the focus of cancer research has been almost exclusively on epithelial tumor cells. However, in the past few years, there has been a major shift toward the study of the TME, elucidating that tumor progression is dependent on an intricate network of interactions among cancer cells and their surroundings (McAllister and Weinberg, 2014; Taniguchi and Karin, 2018; Hinshaw and Shevde, 2019).

Tumors are unquestionably heterogenous entities, composed of phenotypically distinct cellular populations with different functions. This is illustrated by the clonal evolution theory (Nowell, 1976), TME heterogeneity (Junttila and de Sauvage, 2013) and hierarchal organization of cancer cell subpopulations that includes cancer stem cells (CSCs) and their progenies. Some studies have shown that CSCs are the driving force of tumor formation as they exhibit self-renewal and tumor−initiating capacities and phenotypic plasticity. Plasticity offers cancer cells the ability to switch from a differentiated state to an undifferentiated CSC-like state, responsible for long term tumor growth and drug resistance. Recently, observations of anatomically distinct niches of CSCs within tumors have emerged (reviewed in Plaks et al., 2015; Batlle and Clevers, 2017). These niches could have a role in preserving the plastic phenotype of CSCs and their protection from the immune system. Nonetheless, the heterogeneous tumor is a part of a larger society comprising many other actors that define the tumor microenvironment.

## Defining the Tumor Microenvironment

The tumor microenvironment, a diversified compartment of differentiated and progenitor cells, comprises all the non-malignant host cellular and non-cellular components of the tumor niche including, but not restricted to, endothelial cells, adipocytes, cells of the immune and nervous systems, and the stroma.

### Non-stromal Components

#### *Endothelial Cells*

The most well-known extrinsic modulator of cancer cell growth is neovascularization (Folkman, 1985). Early studies using mouse models show that the angiogenic switch increases the proliferation rate of cancer cells (Folkman et al., 1989). Angiogenesis is crucial to the ability of tumors to thrive and the vascular endothelium is an active participant in the formation of a growth-permissive tumor microenvironment. Vascularization is driven by the hypoxic center of the tumor where hyperproliferation results in increased oxygen demand. Consequently, low oxygen induces the expression of angiogenic proteins like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Papetti and Herman, 2002) that activate endothelial cells and attract them toward the tumor to form new vessels, allowing the delivery of nutrients and oxygen. Without angiogenesis, tumors are condemned to quiescence and cell death. Tumor vascularization requires the cooperation of different TME cells, mainly vascular endothelial cells that provide structural integrity to the newly formed vessels and pericytes that ensure their coverage and maturity (Weis and Cheresh, 2011). Endothelial cells also constitute routes to metastatic dissemination via angiogenesis and contribute to resistance to chemotherapies through an overexpression of drug efflux pumps thereby decreasing the tumor's access to the drug (Hida et al., 2013).

#### *Adipocytes*

Cancer-associated adipocytes (CAAs) support cancer growth mainly through secretion of adipokines like adipsin (Goto et al., 2019), chemerin (Lu et al., 2019) as well as proinflammatory cytokines (Dirat et al., 2011) and growth factors. CAAs also supply lipids for cancer cell membranes and organelles, induce metabolic reprogramming in cancer cells and provide proteases for cancer cell invasion (reviewed in Deng et al., 2016). Moreover, through the production of tumor-promoting cytokines and factors, they have been shown to confer resistance to hormone therapies, chemotherapies, radiotherapies and targeted therapies in breast cancer (Choi et al., 2018), and to contribute to tumor progression across a variety of obesity-associated cancers (Park et al., 2014) such as esophagus, gastric, liver, kidney, colorectal, pancreatic, breast, ovarian, prostate, and thyroid cancers. Adipocytes from white adipose tissue are recruited to tumors, can differentiate into pericytes and incorporate

into vessel walls contributing to angiogenesis and to tumor proliferation (Zhang et al., 2012).

### *Infiltrating Immune Cells*

Variations in immune profiles are linked to prognosis and therapeutic responses (Gentles et al., 2015). All adult solid tumors contain infiltrates of diverse immune cell subsets that influence pro-tumorigenic and antitumor phenotypes. Of all infiltrating myeloid immune subsets, tumor-associated macrophages (TAMs) best represent this paradigm. TAMs are abundant in all stages of tumor progression and can be polarized into inflammatory M1 or immuno-suppressive M2 macrophages, depending on microenvironment stimuli (Ruffell and Coussens Lisa, 2015). While a subset of TAMs has antitumoral effects, others stimulate cancer cell proliferation by secreting growth factors, produce proteolytic enzymes that digest the ECM to facilitate tumor cell dissemination, and provide a supportive niche for metastatic tumor cells (Mantovani and Allavena, 2015). Eosinophils, primitive actors of innate immunity, have been shown to infiltrate tumors and influence tumor progression. Activated eosinophils secrete IL-10 and IL-12, to inhibit cancer cells growth, or can mediate cell death by direct cytotoxicity (Gatault et al., 2015; Lucarini et al., 2017). However, they can also promote tumor growth by secreting growth factors such as epidermal growth factor (EGF) and transforming growth factor-β1 (TGF-β1) (Grisaru-Tal et al., 2020). As tumors grow, myeloid-derived suppressor cells (MDSCs) (Kumar et al., 2016), immunosuppressive precursors of macrophages and dendritic cells (DCs), promote tumor vascularization and disrupt major mechanisms of immunosurveillance, including tumoral antigen presentation, T cell activation and cytotoxicity (Lindau et al., 2013).

The other major subset of tumor infiltrating immune cells is of lymphoid origin and includes T lymphocytes and natural killer (NK) cells. T lymphocytes can be grouped into 3 major subtypes: (i) $T_H$ lymphocytes divided mainly in two lineages: pro-inflammatory $T_{H1}$ and anti-inflammatory $T_{H2}$; (ii) Regulatory T cells ($T_{reg}$), primarily pro-tumorigenic *via* their immunosuppressive activity; and (iii) cytotoxic T cells ($T_C$) that destroy tumor cells through granzyme and perforin mediated apoptosis (Fridman et al., 2012; Lindau et al., 2013). A third lineage of effector $T_H$ cells, characterized by IL-17 secretion, called $T_{H17}$ cells, acts as double-edged sword in anti-tumor immunity and tumorigenesis (Alizadeh et al., 2013).

### *Nerve Cells*

Peripheral nerves are a common feature of the TME and emerging regulators of cancer progression. Innervated tumors are aggressive, have high proliferative indices and an increased risk of recurrence and metastasis (Magnon et al., 2015). Cancer cells can grow around nerves and invade them in a process called perineural invasion, which represents yet another route for dissemination (reviewed in Jobling et al., 2015). Recently, Zahalka et al. (2017) have shown that adrenergic nerves promote angiogenesis by activating the angiogenic switch in endothelial cells. Moreover, many studies described the formation of new nerve endings within tumors, showing that they stimulate their own innervation, a process termed axonogenesis, by expressing neurotrophic factors (Wang et al., 2014; Huang et al., 2015) or releasing exosomes containing axonal guidance molecules (Madeo et al., 2018). In return, nerves provide the tumor with neurotransmitters that enhance cancer cell growth.

## Stromal Components

In healthy tissues, the stroma constitutes the main barrier against tumorigenesis. However, transformed cancer cells can direct stromal reprogramming to support tumor growth and progression.

The stroma is composed of the extracellular matrix (ECM) and specialized connective tissue cells, including fibroblasts, and mesenchymal stem cells.

### *The Extracellular Matrix*

The ECM constitutes the scaffold of tissues and organs, providing the essential signals to maintain tissue architecture and to regulate cell growth and apoptosis. It is a complex network of glycoproteins, proteoglycans, glycosaminoglycans and other macromolecules. About 300 different proteins have been classified as ECM proteins, in what is called the matrisome (Hynes and Naba, 2012). The ECM undergoes constant remodeling by different actors, mainly enzymes such as collagenases and matrix metalloproteases (MMPs) and by fibroblasts. ECM stiffening, induced by increased collagen deposition and crosslinking, disrupts tissue morphogenesis contributing to malignant progression, but also facilitates metastasis and infiltration of immune cells in tumor sites (Bonnans et al., 2014).

### *Cancer-Associated Fibroblasts*

Fibroblasts are widely distributed in all tissues. They constitute a multifunctional cell type residing in the ECM, shaping it by secreting collagens and fibrous macromolecules but also degrading it by releasing proteolytic enzymes, like MMPs.

Fibroblasts are known to modulate immune response by recruiting leucocyte infiltration and regulating inflammation *via* the secretion of growth factors, cytokines and chemokines and to play an important role in maintaining tissue homeostasis (Buckley et al., 2001). During wound healing or fibrosis, another type of specialized fibroblasts called myofibroblasts is present in the tissue (Tomasek et al., 2002). Tumors, for long considered as wounds that do not heal, are associated with a stroma similar to that observed in wound healing called the activated stroma, where fibroblasts resemble myofibroblasts and are called cancer-associated fibroblasts (CAFs). The activated stroma supports cancer progression (Hanahan and Coussens, 2012). Importantly, as for cancer cells, it has been described that CAF population is highly heterogeneous with tumor-promoting or tumor-suppressing CAFs and personalized anticancer therapies targeting CAFs could be of great interest (reviewed in Liu et al., 2019; Mhaidly and Mechta-Grigoriou, 2020).

### *Mesenchymal Stem Cells*

The definition and characteristics of mesenchymal stem cells (MSCs) have been a matter of debate for a long time, and their characterization is an active field of research

(Nombela-Arrieta et al., 2011). It is now established that MSCs are multipotent progenitor cells originating from the bone marrow that can migrate systemically through blood vessels and differentiate into osteoblasts, chondrocytes, or adipocytes. To date, the primary functions of MSCs within the TME are to regulate the immune response by the release of immunomodulatory cytokines and to promote tissue regeneration. Owing to their multipotent and cell fusion properties, they can also be at the origin of vascular cells, contributing to angiogenesis, of myofibroblasts and more rarely of cancer cells themselves.

## Crosstalk Between Tumor Cells and Components of the TME in Cancer Progression

The tumor microenvironment plays a critical role in determining tumor fate, and stromal reprogramming has been recognized to be critical for carcinogenesis (Mantovani et al., 2008). Rudolf Virchow first proposed the possibility of a link between chronic inflammation and tumorigenesis in the nineteenth century after the observation of infiltrating leukocytes within tumors. This is now considered a hallmark of cancer. Cancer progression is associated with an ever-evolving tissue interface of direct epithelial–stromal interactions that regulate cancer cell metastasis and disease progression. This section describes the complex crosstalk between the actors of the TME and the cancer cells that take place during the different stages of cancer progression from the early cell autonomous events to the late metastatic stages (**Figure 1**).

### Primary Tumor Progression

Cancer cells reprogram the tumor-infiltrating stromal and immune cells, which facilitates primary tumor growth and progression. Therefore, it is important to decipher the reciprocal crosstalk between cancer cells and their heterotypic microenvironment.

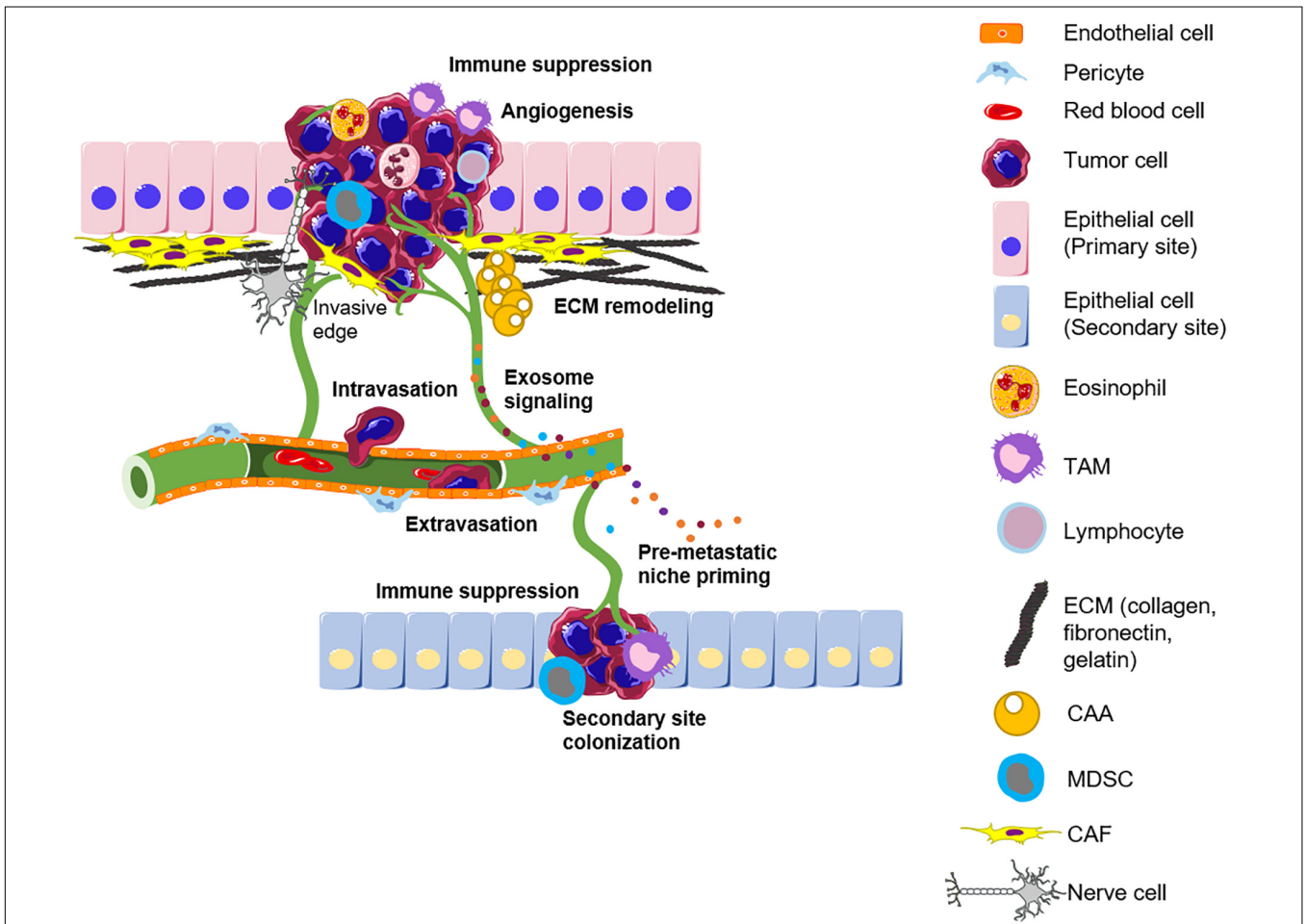


**FIGURE 1 |** The tumor microenvironment influences the different stages of cancer progression. The primary tumor is infiltrated by different immune subsets and surrounded by a remodeled matrix. Angiogenesis ensures tumor growth by supplying nutrients and also provides a route for metastasis. Intravasation of tumor cells into blood vessels allows their shuttling to a novel site. The secondary site is primed by exosomes secreted by tumor cells and the different actors of the TME to allow the successful seeding of incoming tumor cells. TAM, Tumor-associated macrophage; ECM, Extracellular matrix; CAA, Cancer-associated adipocytes; MDSC, Myeloid-derived suppressor cells; CAF, Cancer-associated fibroblast; TME, Tumor microenvironment.

Epithelial cancer progression is influenced by the cells' contact with immune cells and a carcinogen-exposed stroma (Barcellos-Hoff and Ravani, 2000), by an overexpression of metalloproteases (Fukuda et al., 2011) which create a suitable environment for invasion, or by the stimulation with altered stromal cells like CAFs. In the skin, epigenetic modifications of fibroblasts are induced by ultraviolet exposure, leading to the production of inflammatory cytokines and matrix-remodeling enzymes that together influence the formation of epithelial tumors (Hu et al., 2012). CAFs accumulate in the TME along with tumor growth (Kalluri, 2016) and are activated by cytokines and growth factors of the TME, such as TGF-β (Taniguchi et al., 2020) and Fibroblast Growth Factor (FGF). In their turn, CAFs provide growth factors like VEGF to enhance angiogenesis and vascular permeability (Fukumura et al., 1998). Furthermore, TAMs can support many aspects of tumoral progression. They can secrete mediators that enhance tumor cell survival and proliferation such as growth factors and cytokines [epidermal growth factor (EGF), interleukin 6 (IL-6), and tumor necrosis factor (TNF) (Noy and Pollard Jeffrey, 2014)].

Another crucial step for cancer progression is immune evasion. This is supported mainly by the action of MDSCs. These cells infiltrate the developing tumors and inhibit the mechanisms of immune editing of cytotoxic immune cells, all the while promoting tumor vascularization (Talmadge and Gabrilovich, 2013). TAMs can also promote cancer immune escape by displaying immunosuppressive functions (Noy and Pollard Jeffrey, 2014). Other myeloid cells including neutrophils, monocytes, and eosinophils infiltrate the tumor and promote tumor growth by inhibiting antitumor immunity. Neutrophils can even induce genotoxic damages (Wilson et al., 2015) or recruit tumor-promoting $T_{H17}$ lymphocytes (Ortiz et al., 2015). Additionally, invasion of the basement membrane underlying the epithelium by the tumor cells is a basic step for the upcoming dissemination. For this, CAFs have a physical impact on tumors that results in increased ECM stiffness around tumor cells and consequent mechanical stress. TAMs are also capable of driving invasive phenotypes (Condeelis and Pollard, 2006). In breast cancer, they facilitate invasion of tumor cells by sustaining a signaling paracrine loop involving CSF-1 and EGF (Goswami et al., 2005), and by the secretion of proteases (Gocheva et al., 2010). Thus, once the tumor cells evade the host immune system and gain the ability to invade the surrounding tissue, metastatic dissemination of cancer cells can take place.

## Metastatic Dissemination

Metastasis is the leading cause of mortality among cancer patients (Mehlen and Puisieux, 2006). In epithelial tumors, metastasis begins with the cellular invasion of the basement membrane and the subsequent migration of cancer cells into the blood stream. One of the initial steps for primary tumor invasion is epithelial-mesenchymal transition (EMT). Under the influence of various signals, mainly TGF-β, cells gradually lose their epithelial traits while gaining mesenchymal ones that confer migratory capacities (Mani et al., 2008). CAFs participate in a TGF-β and platelet-derived growth factor (PDGF) signaling crosstalk with tumor cells to support EMT and the acquisition of an invasive phenotype (van Zijl et al., 2009). EMT can also enable the acquisition of CSC traits (Mani et al., 2008), suggesting that not only it causes cancer cells to disseminate from the primary tumor but also can provide these cells with the self-renewal properties needed for their subsequent implantation at secondary sites. Although CSCs are not be the only cells responsible for metastasis, the CSC-generated hierarchy of stem-like and differentiated tumor cells is able to initiate metastatic growth (Merlos-Suárez et al., 2011). However, EMT is not the only mechanism used by epithelial cells for migration. Epithelial cancer cells can migrate as single cells, as loosely attached cords or as highly organized collective entities (reviewed in Friedl et al., 2012). During early stages of cancer migration, CAFs increase the production of collagen in the underlying stroma and the fibers become aligned, giving rise to a stiffer ECM hence allowing the migration of cancer cells away from the primary tumor (Conklin et al., 2011). This is largely mediated by CAFs secreted factors that stiffen the ECM, namely enzymes of the Lysyl Oxidase (LOX) family (Kalluri and Zeisberg, 2006).

During metastasis, cancer cells cross the endothelial barrier during a step called **intravasation** to enter the blood stream, and by **extravasation** to exit from circulation into distant tissues, processes that involve different receptors, a plethora of signaling pathways, and interactions with the actors of the surrounding microenvironment (Reymond et al., 2013). Intravasation seems to require the cooperative work of a triad consisting of macrophages that localize to blood vessels where they help tumor cells intravasate into the blood stream (Harney et al., 2015). However, despite the help of macrophages, only 0.01% of cells that intravasate form detectable metastases (Chambers et al., 2002). Cancer cells in the blood stream can be shielded by platelets from NK-mediated cytotoxicity (Palumbo et al., 2005), and platelet binding enhances cancer cell adhesion to vessel wall and subsequent extravasation (Zhang et al., 2011; Schumacher et al., 2013). Inflammation also modulates endothelial crossings through TNF-induced vascular permeabilization, cyclooxygenase 2 (COX2)-dependent prostaglandin production and MMP-mediated tissue remodeling.

## Secondary Organ Colonization

Docking of cells in organs to form secondary tumors is not a random process. Organ tropism has been first described by Stephen Paget in 1886 as the "seed-and-soil" theory, in which he suggests that metastasis is not the fruit of hazard but tumors have clear organ preferences for secondary colonization. Paget's theory gave the basis for the description of the premetastatic niche: the primary tumor executes preparative events, preceding detectable metastasis, that render the secondary milieu less hostile for colonization by cancer cells. Studies of the premetastatic niche are still in their infancy but some traits and events are now clearer. Settlement of tumor cells at distant sites is dependent on tumor-secreted cytokines and extracellular vesicles, like exosomes, that enable the premetastatic microenvironment to support their colonization (Liu and Cao, 2016). These tumor-secreted factors communicate to both hematopoietic and mesenchymal stem cell compartments. It has been shown that bone marrow-derived VEGFR1+ cells are already present in premetastatic sites

before tumor cell arrival, suggesting the communication between primary and secondary sites (Kaplan et al., 2005). Seeding is also facilitated by the LOX-mediated fibronectin upregulation in resident fibroblasts and recruitment of myeloid cells (Erler et al., 2009). Neutrophils may also be involved in the priming of metastatic sites. Neutrophils accumulate in premetastatic livers of mice bearing colorectal tumors (Wang et al., 2017) and their accumulation has been shown to be required for pancreatic cancer metastasis (Steele et al., 2016). Recently, it was also shown that omentum resident macrophages are required for ovarian cancer metastasis (Etzerodt et al., 2020). Neutrophils also serve as an energy source to fuel metastatic tumor cells. In a breast cancer model, infiltrating neutrophils are induced to store lipids upon interaction with resident mesenchymal cells in the lung so that when disseminated tumor cells (DTCs) arrive, neutrophils transfer their stored lipids to DTCs for their survival and proliferation (Li et al., 2020).

Colonization of secondary tissues requires the same elements as growth of the primary tumor namely, sufficient nutrients and oxygenation. One important step for metastatic tumor cell survival is the reversal to an epithelial phenotype *via* mesenchymal–epithelial transition (MET) to regain the ability of proliferation and differentiation. Once tumor cells colonize the secondary site, genetic instability inherent in neoplastic cells continues to operate at each cell division, and these cells continue the remodeling of the site, just as described above.

Accordingly, the crosstalk between cancer cells and their microenvironment provides valuable insights into cancer formation, progression and spread. Hence, it is necessary to study cancer as a whole process by modeling the interactions between tumor cells and their microenvironment to improve development of new therapies against cancer progression and metastasis.

# PART II—ORGANOTYPIC *IN VITRO* MODELS

## Advantages of 3D Models Over 2D Models and Animal Experiments

Cancer research has long been based on two-dimensional (2D) cell culture, mainly in order to earn the right of passage to *in vivo* experiments. Conventional 2D cell cultures allowed the study of many mechanisms that drive tumor growth and the evaluation of optimal drug doses and toxicities. However, currently available cell lines fail to represent the genetic background across the range of human cancers (Huang A. et al., 2020) and may adapt to growth in culture, rather than mimic the behavior of the tumor in a complex microenvironment. Because they also lack all elements of the tumor stroma and surrounding tissue, they fail to mimic the complexity of the tumor microenvironment (Gillet et al., 2011). Owing to this, a large gap exists between the knowledge obtained in these models compared to *in vivo* cancer models because results of 2D experiments rarely predict therapeutic response in animals. This can be explained by the fact that cells cultured in 2D do not have the same architecture as cells *in vivo* that are arranged in three-dimensional (3D) structures unattached to planar surfaces. Furthermore, cultured monolayers lack the capacity to mimic *in vivo* tumoral hypoxia and exhibit a very different metabolism. Consequently, cells in monolayer cultures proliferate at unnaturally rapid rates (Langhans, 2018), differ in gene/protein expression compared to *in vivo* models, and alter their dynamic processes such as cell division and migration (Duval et al., 2017).

Even though *in vivo* experiments have the advantage of being physiologically relevant in contrast to cells cultured out of their bodily context, they have many flaws (Day et al., 2015). Aside from being long, expensive and ethically questionable, the use of human cancer cells in mouse models mostly requires the use of immunocompromised mice that lack, to varying extents, the immune components, thus limiting the advantages of these approaches in modeling tumoral progression and response to drugs. Indeed, the inflammatory immune cell component is lacking in immunocompromised mice. Although the engrafted tumors may exhibit a stromal response with the growth of endothelial cells and fibroblasts, these stromal cells originate from mice and therefore the implication of human TME could not be extrapolated. Moreover, it has recently been shown that patient-derived xenografts (PDXs) present genomic instability with continuously changing copy number alterations landscapes, and so their passaging causes a drift from the original tumor (Ben-David et al., 2017). As such, mouse co-clinical trials using PDXs have shown very little progress beyond proof of concept due to logistical issues (Clohessy and Pandolfi, 2015).

Even with strong supporting preclinical evidence, many targeted therapies produce modest clinical results, a fact now highlighted by the tremendous National Lung Matrix Trial that assessed personalized medicine in non–small cell lung cancer (NSCLC) (Middleton et al., 2020). The results have been fairly disappointing with a response rate of only 10% with some abandons due to lack of treatment efficacy. Genetically engineered mouse models of NSCLC, used for preclinical studies, have mutational burdens more than 100-fold lower than that of human disease (McFadden et al., 2016) arguing for the use of more appropriate preclinical models that integrate the immune and stromal landscapes beyond the genetic aberrations.

Another issue resides in the ability to translate results of immunotherapy from bench to clinic because of the high failure rate observed in human clinical trials after promising results obtained in mouse models. Even the durable clinical benefits observed with immune checkpoint blockers (ICBs) in some tumor types have been seen in a minority of patients (Cardin et al., 2014; Herbst et al., 2014; Hammel et al., 2016). Given the complexity of the tumor microenvironment, it is imperative to create models that include different immune cell types the administered compound may interact with.

Efforts have been made these last few years to "humanize" the mouse's immune system by grafting human hematopoietic stem cells in mice or by transgenic expression of Human Leucocyte Antigen (HLA) (reviewed in Shultz et al., 2012; De La Rochere et al., 2018). However, the high cost of recipient mice, scarcity of human bone marrow acquisitions, engraftment variability, and laborious technical demands represent high inconveniences in a preclinical setting. Hence, optimal mouse studies are very

cumbersome for simultaneous evaluation of numerous drugs and may be inefficient due to the different metabolic processing of drugs between humans and mice. Thus, high-throughput *in vitro* screening systems are essential precursors to *in vivo* evaluations. Developing 3D organotypic models that recapitulate physiological functions would allow further replacement and reduction of animal models as recommended by the 3Rs rule[1].

*In vitro* 3D cultures recapitulate much better the architecture of tissues and capture the complexity of solid tumors than 2D counterparts, all the while allowing the modeling of different stages of the carcinogenic process (Yamada and Cukierman, 2007; Tanner and Gottesman, 2015). The concentric arrangement of cells in 3D cultures resembles initial avascular stages of solid tumors *in vivo* and non-vascularized micro-metastatic foci. More sophisticated 3D cultures also include different elements of the TME; allowing their use to study cellular interactions within tumors and to model stages of cancer progression. Additionally, genome-wide screens performed on 3D cultures showed improved detection of cancer genes and pathways compared with those performed in 2D (Han et al., 2020). Thus, increased biologically relevant behavior and characteristics could be acquired from genetic editing in organoids, cocultures, and 3D growth models. Moreover, the coalition between biologists, bioengineers and physicians inspired many strategies to reproduce *ex vivo* the complexity of biological systems. These approaches mimic organ topography, mechanical forces of tumor cells, matrix stiffness, functionality, and complexity much better than 2D or even 3D culture systems (van Duinen et al., 2015).

In the following section, we will describe the existing *in vitro* organotypic models for cell culture (**Figure 2**).

## Overview of *in vitro* Organotypic Cellular Models

### Multicellular Spheroids

Multicellular spheroids (MCSs) or 3D cellular aggregates represent the bridge that fills the gap between 2D cultures and more elaborate 3D techniques. They are fairly representative of the *in vivo* situations because of their heterogeneity as they are composed of proliferating, non-proliferating, well-oxygenated, hypoxic and necrotic cells. Other features of MCSs like cell-cell signaling and interactions, the presence of different cellular layers, the genetic expression profiles, and drug resistance patterns are similar to characteristics of the natural cellular conditions. Currently, there exists many techniques for MCS production such as the forced floating methods in non-adherent plates, hanging drop method, the use of scaffolds and matrices, or even more sophisticated methods using microfluidic systems (reviewed in Ferreira et al., 2018).

MCS can be used for tumoral modeling by either forming homogenous cultures using solely cancer cells, or by more sophisticated cultures using cancer cells with components of the TME like fibroblasts, endothelial cells (Andrique et al., 2019) or immune cells, hence forming heterotypic spheroids. Encapsulating MCS in biomimetic hydrogel scaffolds offers biophysical and biochemical cues that simulate the behavior of extracellular matrix, essential for regulating cancer cell behavior (Li and Kumacheva, 2018).

### Organoids

The term organoid, meaning resembling an organ, was first used in 1946 by Smith and Cochrane to describe a case of cystic teratoma. Ever since, it has been inaccurately used to describe some cell structures and aggregates, but the actual definition is now clearer: an organoid is a collection of organ-specific cell types that develops from stem cells, that possesses a minima of specific organ functions, and self-organizes to mimic the architecture of the organ itself (reviewed in Lancaster and Knoblich, 2014). Early pioneering works of Mina Bissell showed that primary epithelial cells derived from mouse mammary glands could self-organize into glandular structures and secrete milk proteins (Lee et al., 1984). These advances were followed by the works of Clevers' lab, that described the generation of intestinal crypt organoids from Lgr5+ stem cells (Sato et al., 2009).

Now, it is recognized that organoids can be generated using two types of stem cells: **pluripotent stem cells (PSCs)** which can be embryonic or induced pluripotent stem cells or **adult stem cells (ASCs)** that reside in adult tissues and are tissue-specific, cultured under specific growth factor cocktails that allow their long-term expansion by mimicking the organ stem cell niche. To date, organoids have been developed for many organs including intestine (Spence et al., 2011), kidney (Takasato et al., 2015), brain (Lancaster et al., 2017), liver (Camp et al., 2017), stomach (McCracken et al., 2014), pancreas (Hohwieler et al., 2017), ovary (Kessler et al., 2015), and lung (Dye et al., 2015) among others. These organoids have been used for multiple approaches such as high-throughput drug screening efficacy and toxicity, host-microbe interactions and infectious diseases (Bartfeld et al., 2015; Leslie et al., 2015; Garcez et al., 2016), and disease modeling (reviewed in Dutta et al., 2017) in particular tumor development, which will be later discussed in detail.

Epithelial organoids recapitulate many aspects of organ development and disease and represent many opportunities for cancer modeling and anticancer drug testing. However, it is important to note the existence of some drawbacks and limitations. Organoids lack the native organ microenvironment: the stromal compartment, immune cells and vascularization, and they are mostly cultured in poorly defined animal matrices. Although, novel synthetic analogous ECM may constitute a better alternative as they are controllable and permit fine tuning of matrix constituents (Gjorevski et al., 2016).

### 3D-Tissues

The recreation of simple tissues has been described in a cell sheet engineering method using cells grown to confluence on culture dishes grafted with a temperature-responsive polymer, poly-(N-isopropylacrylamide). This technique allows cell growth at 37°C and cell harvest at room temperature as intact cell sheets and subsequently the stacking of different sheets to generate heterotypic thin 3D tissue analogs. Using this technique vascularized tissues (Asakawa et al., 2010) and liver tissue-like structures (Kim et al., 2012) were obtained.

[1]https://www.nc3rs.org.uk/the-3rs

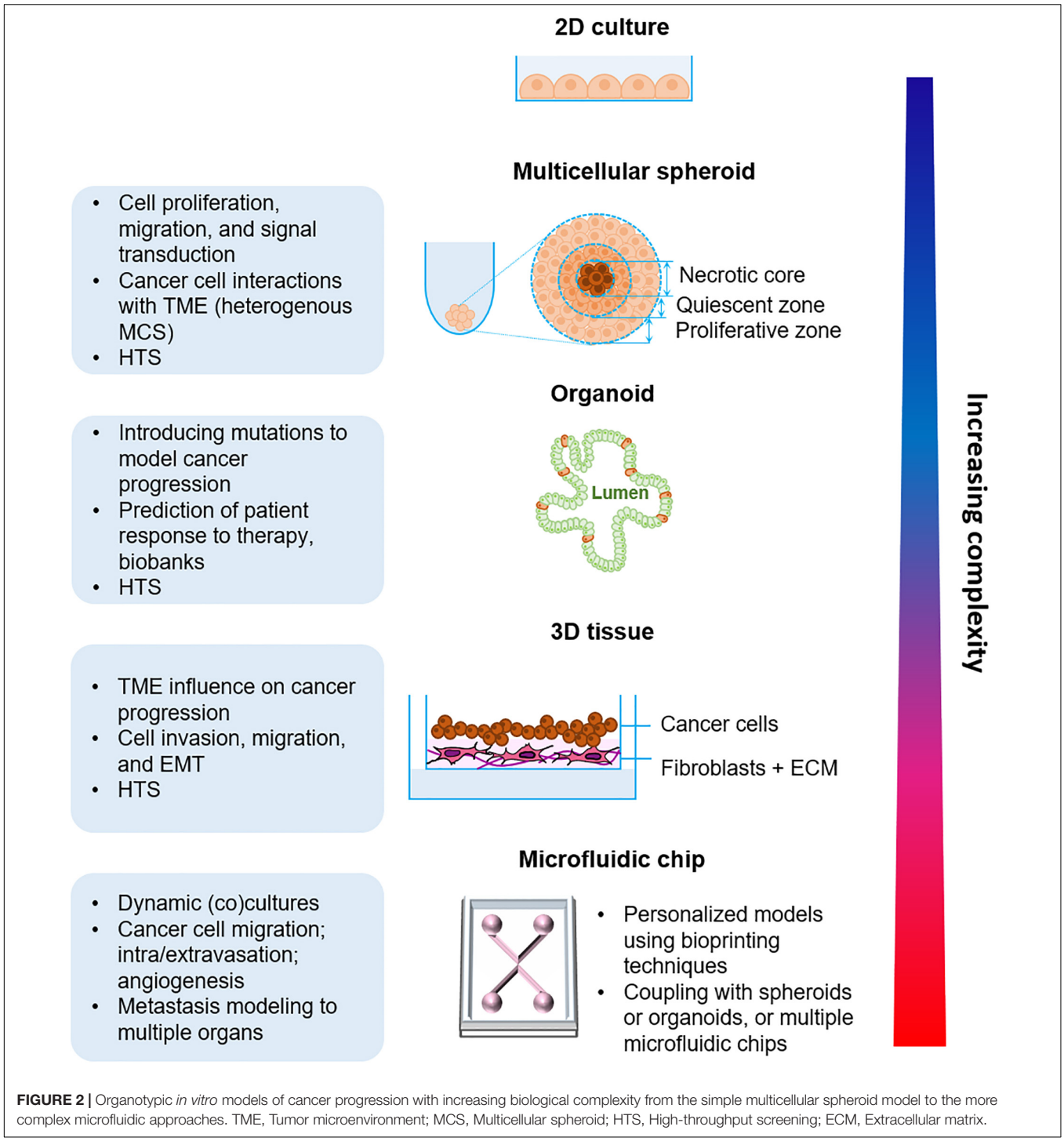


**FIGURE 2 |** Organotypic *in vitro* models of cancer progression with increasing biological complexity from the simple multicellular spheroid model to the more complex microfluidic approaches. TME, Tumor microenvironment; MCS, Multicellular spheroid; HTS, High-throughput screening; ECM, Extracellular matrix.

Organotypic epithelial raft cultures, originally developed to study keratinocytes (Fuchs, 1990), represent an interesting approach to study epithelial cancer cell behavior, notably cancer cell invasion. These cultures are mechanically supported by semipermeable inserts and are either submerged in medium or maintained at an air–liquid interface. Epithelial tissues can be constructed in stages by first embedding stromal cells, mainly fibroblasts, for several days followed by seeding of epithelial cells on top (Kalabis et al., 2012), or also embed immune cells within the layers to obtain an integral tissue (Huang et al., 2017). These cultures generate a stratified tissue resembling the epithelium seen *in vivo* with a proliferating basal layer and differentiating supra-basal layers. The use of 3D-tissue revealed some advantages compared to organoids when the access to the epithelial cells' apical surfaces is needed, for example to study host-pathogen interactions. To illustrate this using a colonic 3D-tissue, Martin and colleagues have shown that infection with genotoxin-producing *Salmonella* enterica synergises with the

loss of APC to promote genomic instability and carcinogenesis (Martin et al., 2019). Although it should be noted that an elegant recent study has described the possibility to revert organoid polarity allowing access to the apical surfaces of the cells (Co et al., 2019). Miniaturized 3D tissues can be used to facilitate high-throughput drug screening (Dutta et al., 2017).

### Microfluidic Approaches

The static nature of nutrients and metabolites in 3D cultures isn't representative of the physiological conditions due to the lack of fluid shear stress and hydrostatic pressure that can greatly influence cell behavior (Polacheck et al., 2011). Microfluidic systems, based on the progress in synthetic biology, have enabled the development of *in vitro* assays that facilitate the study of cellular behavior under a spatiotemporally controlled microenvironment in which molecular, biophysical and cellular components can be tuned according to physiologically relevant parameters. These microfluidic cell culture systems, known under the term organ-on-a-chip, are usually made of continuously perfused hollow microchannels populated by living cells (reviewed in Bhatia and Ingber, 2014). To date, many organs have been successfully modeled in microfluidic devices. One of the first models was lung alveoli that responded to bacterial infection and inflammation (Huh et al., 2010), but also that reflected drug toxicity (Huh et al., 2012). Many studies followed that assessed nephrotoxicity in human kidney tubules-on-a-chip (Jang et al., 2013), liver function (Beckwitt et al., 2018), and more recently, a simulation of a body-on-a-chip multi-organ system (McAleer et al., 2019) to assess drug efficiency and toxicity.

## PART III—ORGANOTYPIC MODELS OF CANCER PROGRESSION AND DRUG RESPONSE

Understanding the key aspects of tumoral progression is of utmost importance for the development of novel successful anticancer strategies. Organotypic modeling of these aspects alongside the interactions between the different actors of the TME would allow a better comprehension of the mechanisms that mediate tumoral progression and a first solid step toward preclinical drug screening in physiologically relevant situations. In this section, we describe how the previously mentioned organotypic models have been applied to study the different steps of tumor growth and metastasis (**Table 1**).

## Cancer Modeling Using Organotypic Models

### Tumor Growth *in situ*—Interactions of Cancer Cells With the TME Elements

Many *in vitro* organotypic models have been used to study tumor initiation and growth and to identify how parenchymal cells (endothelial, epithelial, immune, nerve and stromal cells) and components (ECM, secreted factors) of the TME influence the growth *in situ* of different cancer types.

Modeling cancer initiation using organoid is highly attractive owing to the relative ease of genetic manipulation of cells. Using CRISPR-Cas9 genome editing, tumor suppressors have been identified (Michels et al., 2020), as well as the consequences of mutations in the DNA repair deficiency genes (Drost et al., 2017) or mutations that drive cancer progression (Fumagalli et al., 2017) have been elucidated. Such approaches allow the introduction of defined mutations to transform normal organoids and induce tumor growth, upon xenotransplantation. Matano and colleagues model human colon adenocarcinoma by introducing canonical colorectal cancer (CRC) driver mutations into primary human colon organoid cultures (Matano et al., 2015), revealing that mutations in *APC*, *SMAD4*, *TP53*, and *KRAS* simultaneously are sufficient to model colonic adenomas but not tumorigenesis, perhaps due to the lack of TME components within the organoids. Similarly engineered CRC organoids with *APC* and *KRAS* mutations formed dysplasia and could invade submucosa (Takeda et al., 2019), and transformed mammary organoids formed tumors upon xenotransplantation (Dekkers et al., 2020). Thus, deconstructing carcinogenesis into single genetic elements by engineering cancer genes in untransformed human organoids is a powerful tool for investigating how individual genetic aberrations contribute to the acquisition of cancer phenotypes.

Nevertheless, the genetic alterations driving cancer initiation are supplemented by the interactions of cancer cells with their microenvironment to ensure successful cancer progression. A refined cancer 3D-tissue model using cancer-associated genetic modifications and a stromal department showed the neoplastic transformation of normal epithelia which became invasive (Ridky et al., 2010). Indeed, many tumors are characterized by a prominent stromal compartment that modulates tissue architecture, due to extensive ECM remodeling mainly mediated by CAFs. Adding stromal fibroblasts to prostate organoids facilitated their branching (Richards et al., 2019), while the addition of CAFs to lung squamous carcinoma spheroids recapitulated the pathological changes of tumorigenesis, from invasion and hyperplasia to dysplasia (Chen et al., 2018). Additionally, CAFs were shown to enhance invasion and migration of breast cancer cells in a 3D microfluidic device (Nguyen et al., 2018; Truong et al., 2019).

Furthermore, the coculture of pancreatic stellate cells, a resident mesenchymal cell population that differentiates into CAFs, with pancreatic cancer patient-derived organoids (PDOs) (Öhlund et al., 2017) or with spheroids (Ware et al., 2016) produced a highly desmoplastic stroma, typical of pancreatic carcinomas. Equally investigating the role of the TME in CRC initiation using organoids, Roulis and colleagues performed single-cell RNA sequencing of the murine intestinal mesenchymal niche and found a population of fibroblasts in intestinal crypts that orchestrate intestinal tumorigenesis by exerting paracrine control over tumor initiating stem cells (Roulis et al., 2020).

Other key elements of the TME which significantly affect cancer cell behavior are immune cells. Tumor-immune system interactions have been widely studied by culturing immune cells recovered from patients together with established cancer cell lines in conventional monolayer cultures. However, these approaches fail to account for critical aspects of the TME. Indeed,

**TABLE 1 |** Organotypic models used to study cancer progression stages and drug response.

| | Primary tumor growth | TME-tumor cells interactions | Invasion and migration | Angiogenesis and intravasation | Extravasation and secondary organ colonization | Drug response |
|---|---|---|---|---|---|---|
| **Multicellular spheroids** | **Tumor growth**<br>Ovarian cancer (Yin et al., 2016)<br>Bladder cancer (Namekawa et al., 2020) | **CAF-mediated interactions**<br>Lung cancer (Chen et al., 2018)<br>Pancreatic cancer (Ware et al., 2016) | **Invasion**<br>Breast cancer (Avgustinova et al., 2016)<br>Colon cancer (Nam et al., 2018)<br>Colorectal cancer (Libanje et al., 2019) | **Vessel sprouting and intravasation**<br>Colon cancer (Ehsan et al., 2014) | **Niche activation and colonization**<br>Breast cancer (del Pozo Martin et al., 2015) | **High-throughput toxicology assay**<br>Breast cancer (Lee et al., 2008) |
| **Organoids** | **Introduction of carcinogenesis driver mutations**<br>Colorectal cancer (Matano et al., 2015; Takeda et al., 2019)<br>Breast cancer (Dekkers et al., 2020) | **Stromal interactions**<br>Pancreatic cancer (Öhlund et al., 2017)<br>Intestinal cancer (Roulis et al., 2020)<br>**Immune cells-mediated interactions**<br>Colorectal cancer (Dijkstra et al., 2018)<br>Different tumor types and stages (Neal et al., 2018) | **EMT**<br>Breast cancer (Jung et al., 2019)<br>**Invasion and migration**<br>Breast cancer (Zhang et al., 2019; Georgess et al., 2020) | **Angiogenesis**<br>Breast cancer (Wörsdörfer et al., 2019) | **Extravasation**<br>Breast cancer (Fernández-Periáñez et al., 2013)<br>B Cell Lymphoma (Jia et al., 2020) | **Tumor genetic profiling and response to chemotherapy**<br>Rectal cancer (Ganesh et al., 2019)<br>Pancreatic cancer (Tiriac et al., 2018)<br>Colorectal cancer (van de Wetering et al., 2015; Fujii et al., 2016; Ooft et al., 2019)<br>Gastrointestinal cancers (Vlachogiannis et al., 2018)<br>Renal cancer (Calandrini et al., 2020) |
| **3D-tissues** | **Neoplastic transformation**<br>Multiple epithelia (Ridky et al., 2010)<br>Colon cancer (Chen H. J. et al., 2016) | **ECM influence**<br>Glioblastoma (Sood et al., 2019) | **Invasion**<br>Multiple epithelia (Ridky et al., 2010)<br>Glioblastoma (Koh et al., 2018) | **Angiogenic response**<br>Breast cancer (Mazio et al., 2018) | **Colonization**<br>Breast cancer (Xiong et al., 2015) | **High-throughput drug screening**<br>Hepatocarcinoma (Chen et al., 2010)<br>Breast cancer (Brancato et al., 2018) |
| **Microfluidic approaches** | **Tumor growth**<br>Breast cancer (Nashimoto et al., 2020) | **CAF-mediated interactions**<br>Breast cancer (Pelon et al., 2020; Truong et al., 2019)<br>Melanoma (Jenkins et al., 2018) | **Invasion and migration**<br>Breast cancer (Chen et al., 2018; Truong et al., 2019)<br>**Migration**<br>Lung cancer (Hsu et al., 2011)<br>Breast cancer (Li et al., 2017) | **Angiogenesis**<br>Microvessels formation and endothelial functions (Zheng et al., 2012)<br>**Angiogenic growth and intravasation**<br>Breast cancer (Zervantonakis et al., 2012; Tang et al., 2017; Sano et al., 2018; Shirure et al., 2018) | **Extravasation**<br>Breast cancer (Jeon et al., 2015; Chen M. B. et al., 2016, 2017)<br>**Metastasis**<br>Breast cancer (Bersini et al., 2014) | **Response to chemotherapy**<br>Lung cancer (Hassell et al., 2017)<br>Breast cancer (Choi et al., 2015) |

microfluidic devices customized with human tumor spheroids containing immune cells recapitulate some features of response or resistance to immune checkpoint blockade in melanoma (Jenkins et al., 2018), but without features of the stromal compartment. The recent promise of therapies manipulating tumor-infiltrating immune cells created a particular exigency for human cancer models that recapitulate this TME diversity. In an effort to integrate an immune competent microenvironment to organoid cultures, a platform to induce and analyze tumor-specific T-cell responses to epithelial cancers was established (Dijkstra et al., 2018). Enrichment of functional tumor-reactive T lymphocytes from CRC or non-small cell lung cancer (NSCLC) patients was successfully established by cocultures of peripheral blood lymphocytes with autologous tumor organoids. These tumor-reactive T cells efficiently recognize and kill autologous tumor organoids, while leaving healthy organoids unharmed. Moreover, a recent study presents organoid modeling that preserves primary tumor epithelium with its endogenous immune and non-immune stromal elements (Neal et al., 2018).

## Cancer Progression: EMT, Cancer Cell Migration and Invasion

The metastatic cascade initiates with invasion and migration of tumor cells away from the primary tumor. Invasion through the basement membrane is considered a differentiating step between neoplasia and malignant tumors. Because cancer cell contractility and matrix stiffness are critical parameters for invasion, accurate invasion models should include tunable matrix parameters (Wisdom et al., 2018). This is possible using organotypic 3D tissues, where virtually any component can be readily modulated. The stromal compartment can be enriched not only with fibroblasts but with myofibroblasts, endothelial cells or inflammatory cells (reviewed in Coleman, 2014).

To study the basis of cancer invasion, significant efforts have been made to recapitulate tumor–stroma interactions. Multicellular spheroids combined with ECM containing fibroblasts showed enhanced invasion (Avgustinova et al., 2016). However, the tumor and its environment being highly dynamic, microfluidic approaches are more fitted to study tumor cell migration. Indeed, the use of such approaches unveiled the contributions of different cell types to tumor cell migration and invasiveness. A 3D microfluidic coculture system containing side-by-side tumor and stroma regions showed that CAFs enhanced the migration and invasiveness of cancer cells (Truong et al., 2019; Pelon et al., 2020). TGFβ secreted by cancer cells was shown to stimulate fibroblasts to transform into myofibroblasts, which then produced soluble factors that fed back to increase the migration speed of the cancer cells (Hsu et al., 2011). Likewise, the cytokines secreted by macrophages cocultured with cancer cells in a microfluidic device, increased cancer cell migration speed and persistence in a MMP-dependent fashion (Li et al., 2017).

### Angiogenesis and Cancer Cell Intravasation

Over the last decade, biomimetic 3D vascular models have been developed, contributing to the understanding of angiogenic processes. Rings of tissue from human umbilical arteries embedded into a 3D matrix were able to sprout in response to tumor-derived proangiogenic factors (Seano et al., 2013). However, vascular organotypic models should not be static as shear forces and blood flow are important for the vascularization process. So, microfluidic approaches have been developed in which endothelial cells are seeded into a channel within ECM to form a primitive vasculature that can be stimulated by angiogenic factors (Zheng et al., 2012; Nguyen et al., 2018), or with an incorporated layer of human bone marrow stromal cells around the channels to recapitulate perivascular barrier function (Alimperti et al., 2017). These microfluidic chips can also be used to trigger vasculogenesis; in that case, instead of seeding endothelial cells beside the matrix, endothelial cells, fibroblasts (Jeon et al., 2014) and tumor cells (Chen M. B. et al., 2017) are loaded within the matrix. Moreover, the ability of organoid-on-a-chip to mimic perfusable blood vessels may address an important issue of organoid use: the lack of nutrient supply. To surmount this, a tumoroid-on-a-chip was developed. It was created in a microfluidic device consisting of three interconnected chambers that enable the self-assembly of endothelial cells into a 3D network of blood vessels and their angiogenic growth toward the organoid-like structures from breast cancer patients (Shirure et al., 2018). However, in such approaches, endothelial cells may not always be free to interact with tumor cells because of the artificial membranes used in the organ-on-a-chip devices. To address this issue, endothelial cells were modified to produce 'reset' vascular endothelial cells (R-VECs) that grew into 3D branching vessels capable of transporting human blood in microfluidic chambers and when transplanted into mice (Palikuqi et al., 2020). These R-VECs adapted their growth upon their coculture with either normal colon organoids or patient-derived colorectal organoids. They arborized normal colon organoids and helped sustain their proliferation while they erratically infiltrated tumor-derived organoids, thus providing a novel physiological platform to study vasculogenesis and angiogenesis.

Entry of tumor cells into the blood stream is a critical step in cancer metastasis. Using microfluidic devices, interactions between invasive cancer cells and endothelial cells have been studied. It was shown that treatment of the endothelium with TNF or coculture with macrophages resulted in rapid and increased numbers of tumor cell–endothelial cell attachment events (Zervantonakis et al., 2012). The secretion of cytokines and chemokines by cancer cells increases the permeability of the endothelial barrier, allowing tumor cells to intravasate and extravasate (Reymond et al., 2013). This feature was modeled using a perfused microfluidic platform containing a vascular compartment with breast cancer cells and their associated endothelial cells separated via a micropillar array interface that allows direct communication of tumor and endothelial cells. The permeability of the vessels was greatly increased in response to the presence of tumor cells or tumor cell-conditioned medium (Tang et al., 2017). Moreover, a tissue-engineered model containing a realistic microvessel in coculture with mammary tumor organoids allowed real-time monitoring of tumor cell-vessel interactions. Using this model, it was shown that tumor cells can reshape, destroy, or intravasate into blood vessels (Silvestri et al., 2020).

### Extravasation and Secondary Site Colonization

Cancer cells within vessels must extravasate to colonize new sites. This process is different from intravasation, because the vasculature to be breached is healthier and cancer cells experience fluid shear stresses due to blood flow. After extravasation, cancer cells have one final task to complete: colonization of secondary sites. Extravasation of tumor cells has been shown to occur *via* endothelial apoptosis *in vitro* (Heyder et al., 2002) but *via* necroptosis *in vivo* (Strilic et al., 2016). Thus, accurate modeling of the extravasation and colonization steps requires tissue-specific cell types, microenvironmental cues, and vascularization. Breast cancer cells extravasated through a vascular network into a bone-mimicking microenvironment generated by culturing osteo-differentiated MSCs within a hydrogel, or within a microfluidic device (Jeon et al., 2015; Sano et al., 2018). It was shown that extravasation rates were much higher to the bone microenvironment than to stromal matrices alone. Another similar model showed that β1 integrin expression is required for cancer cells to be able to invade through the endothelial basement membrane (Chen M. B. et al., 2016). Increased complexity and clinical relevance can be incorporated into organ-on-a-chip models, as devices have been developed to mimic interactions between circulating tumor cells (CTCs), endothelium and bone microenvironments as a model of metastasis to bone (Bersini et al., 2014).

## Therapeutic Applications of Organotypic Models

Although the demand for anticancer drugs is constantly increasing, their development is slow and fastidious. Monolayer cultured cells are the most widely used *in vitro* models

despite their inability to accurately reflect drug's metabolism and pharmacokinetics in the human body. For years, cell-based drug discovery was based on monolayer cultures of authenticated cell lines (Smith et al., 2010; Barretina et al., 2012), but in this blooming era of precision medicine (Prasad et al., 2016), organotypic models represent great promise for anticancer drug discovery.

In line with this, using an organ-on-a-chip approach, a human lung cancer chip has been developed to study tumor growth patterns and drug response (Hassell et al., 2017). When lung cancer cells were cultured within a physiological-like microenvironment composed of lung endothelial cells, normal lung alveolar epithelium and ECM, they presented rampant growth and resistance to tyrosine kinase inhibitors (TKI) similar to NSCLC patient's response, while they failed to do so in static conventional culture. Likewise, McAleer and colleagues designed a modulable five-chamber multi-organ system to monitor drug effects and simultaneously examine anticancer drug efficacy and off-target toxicity (McAleer et al., 2019). In two models incorporating an array of cancer and healthy human cell types, the system provided insight into the efficacy and toxicity of diclofenac, imatinib, and tamoxifen.

Beyond engineered organoids, organoids derived from patient biopsies or resected tumors, called patient-derived organoids (PDOs) have been successfully cultured with a high success rate and indefinite expansion. These contain tumor cells and stromal cells, thus providing a more realistic microenvironment and they seem to retain the tissue identity of the patient (Tiriac et al., 2018; Ganesh et al., 2019), indicating their great potential for personalized medicine approaches. Recent studies suggest that PDOs mirror clinical responses of individual patients to therapy within a clinically meaningful timeframe and even predict patient response to chemotherapy (Ooft et al., 2019; Pasch et al., 2019). Indeed, PDOs derived from glioblastoma samples were used to test responses to standard of care therapy as well as targeted treatments, like chimeric antigen receptor T (CAR-T) cell immunotherapy in a clinically relevant timescale (Jacob et al., 2020). These PDO properties laid the foundation of what is now known as organoid biobanks (van de Wetering et al., 2015; Calandrini et al., 2020) used for applications such as drug testing, cytological analyses, and xenografting.

With the significant need for biomarker identification of drug response, PDOs could also be considered as a tool for biomarker discovery by analyzing secreted factors such as extracellular vesicles (Huang L. et al., 2020) in contrast with PDX models, due to the presence of contaminating host factors. Although molecular diagnostic testing is now routinely used to determine the choice of targeted therapies for the treatment of cancer patients, patients in advanced stages who have exhausted standard clinical care approaches lack personalized therapeutics and will endure the arduous regimen of chemotherapy and see little or no benefit. Even if the use of functional testing in guiding personalized medicine in still in its infancy, the use of metastatic cancer site derived PDOs to evaluate drug response has proven its efficacy by recapitulating patient response (Weeber et al., 2015; Fujii et al., 2016; Pauli et al., 2017; Vlachogiannis et al., 2018). These evaluation platforms could be of great interest in orienting the treatment of advanced cancer patients.

## Shortcomings and Future Directives of Organotypic Models in Translational and Preclinical Settings

The use of organotypic models for cancer modeling is a blooming area of research, however, there are still limitations to their use (Puca et al., 2018; Fujii and Sato, 2020). As an example, studying angiogenesis is rudimentary when it comes to organotypic models. Indeed, the use of vasculature is very basic and organotypic models with other surrounding tissue types are necessary to model more physiological situations. It would be of great interest to model angiogenesis and neovascularization within a transformed organoid. Additionally, complexifying organotypic models by engineering organoids surrounded by muscle, an immune system, and containing a neuronal network along with functional vasculature is something to look forward to in the near future.

When it comes to preclinical studies, organotypic models face many caveats. Spheroid-based 3D models must be used with caution when it comes to clinical relevance. Because they are generated from non-primary tumor cell lines (Friedrich et al., 2009), their use should be restricted to signaling pathways, mechanistic studies and first-line HTS drug screens. More sophisticated models like organoids could be used to validate drug candidates. Stem cell-derived organoids are important for modeling epithelial tumors. However, the lack of standardization and quality control of stem cell culture are an obstacle for their use in clinical studies. The use of pluripotent stem cells for organoid generation can be hampered by the presence of contaminating progenitors that can yield undesired cell types and a small population of undifferentiated PSCs can give rise to tumors that out-compete organ reconstitution *in vivo* (Fowler et al., 2020). Furthermore, due to different culture methods, organoids may present undesired phenotypic variabilities. Interestingly, the recent development of microwell arrays in a matrix-free solid manner allowed the high-throughput assessment of homogenous organoids in 3D culture (Brandenberg et al., 2020). The most exciting aspect for organoid use in clinics is the implementation of PDOs for personalized medicine but this requires that pure PDO cultures can be established, which is not always the case. For example, prostate cancer organoids can only be generated from metastases because normal prostate epithelial cells overgrow cancer cells (Gao et al., 2014). Additionally, the majority of organoids derived from intrapulmonary tumors were overgrown by normal airway organoids (Dijkstra et al., 2020), hampering their use for preclinical studies. Nonetheless, evidence of divergence from primary tumors emerged over time with a decreased abundance of populations from the TME coupled with lower expression of immune-related genes in PDOs (Jacob et al., 2020). Future studies are needed to improve this issue and to maintain the immune compartment, notably for relevant testing of immunotherapies in PDO biobanks.

Microfluidics require refined technical innovations to enable scaling up for HTS. Integration of organotypic models, spheroids, organoids or PDOs, with simulated physiology in microfluidic platforms could represent one of the most relevant *in vitro* models. Two very exciting studies recently reported a near complete body-on-a-chip system. One described an eight organ-chip model linked via vascular endothelial-lined compartments: gut, liver, heart, kidney, lung, heart, brain, blood-brain barrier, and skin (Novak et al., 2020). Using the same approach, intravenously administered cisplatin *via* an arteriovenous reservoir, provided clinically relevant results when compared to *in vivo* behavior (Herland et al., 2020). In this regard, the microfluidic field is still maturing, with a need for regulatory guidelines among the scientific community, specifically for the validation of organ-on-a-chip technology for pharmacological drug testing.

## CONCLUDING REMARKS

Understanding tumors, now considered as heterogenous abnormal organs, is insufficient if the tumor cells are studied individually. Methods that are more inclusive are needed that integrate the cellular, genomic, microenvironmental and spatial features of cancers to be able to understand and overcome their numerous resistance mechanisms. Increasing the complexity of the used models lead to the development of many organotypic cancer models that are physiologically relevant and allow in-depth understanding of the interactions that take place within a tumor. Moreover, future studies are needed to standardize organoid culture methods across the scientific community, as this is very heterogenous at the moment. It is also needed to enhance such cultures by adding stromal and immune compartments to organoid culture to better mimic the tumor microenvironment. This is important because patient-derived organoids represent a very promising approach for personalized medicine, as they retain patient and tumor identity and mirror drug response, thus allowing the use of tailored medicine and avoiding the use of unnecessary treatments. Such organoids, cryo-preserved and collected to form biobanks, should they be available to the scientific community, may replace conventional drug screening assays because they fit the requirements of automated high-throughput screenings. More sophisticated organotypic models, fruits of the collaboration between biologists and engineers, could represent the future of cancer research. Multi-organoid systems also referred to as "body-on-a-chip" will enable the development of biologically complex systems, where organoids derived from different tissues are brought together and allowed to integrate, mimicking organ function and allowing disease modeling.

## AUTHOR CONTRIBUTIONS

OM conceived the review outline. MH wrote the manuscript and made the figures with support from OM. CN and CV contributed to the final manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Targeting NR4A Nuclear Receptors to Control Stromal Cell Inflammation, Metabolism, Angiogenesis, and Tumorigenesis

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The NR4A1–NR4A3 (Nur77, Nurr1, and Nor-1) subfamily of nuclear receptors is a group of immediate early genes induced by a pleiotropy of stimuli including peptide hormones, growth factors, cytokines, inflammatory, and physiological stimuli, and cellular stress. NR4A receptors function as potent sensors of changes in the cellular microenvironment to control physiological and pathological processes through genomic and non-genomic actions. NR4A receptors control metabolism and cardiovascular and neurological functions and mediate immune cell homeostasis in inflammation and cancer. This receptor subfamily is increasingly recognized as an important molecular connection between chronic inflammation, altered immune cell responses, and cancer development. In this review, we examine how transcriptome analysis identified NR4A1/NR4A2 receptors as transcriptional regulators in mesenchymal stromal cell (MSC) migration, cell cycle progression, and cytokine production to control local immune responses. In chronic inflammatory conditions, such as rheumatoid arthritis, NR4A receptors have been shown to modify the activity of MSC and fibroblast-like stromal cells to regulate synovial tissue hyperplasia, pathological angiogenesis, and cartilage turnover *in vivo*. Additionally, as NR4A1 has been observed as a major transcriptional regulator in tumor–stromal communication controlling tumorigenesis, we discuss how advances in the pharmacological control of these receptors lead to important new mechanistic insights into understanding the role of the tumor microenvironment in health and disease.

**Keywords: NR4A nuclear receptors, inflammatory homeostasis, fibroblast-like and mesenchymal stromal cells, angiogenesis, cancer immunity and tumorigenesis, NR4A control of tumor-stromal communication**

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## NR4A SUBFAMILY OF NUCLEAR RECEPTORS

The NR4A1–NR4A3 (Nur77/NGFI-β/TR3, Nurr1/TINUR/NOT, and Nor-1/MINOR/CHN) subfamily of nuclear receptors is a group of immediate early genes induced by a pleiotropy of stimuli including peptide hormones, growth factors, cytokines, inflammatory and physiological stimuli, and cellular stress. NR4A receptors function as potent sensors of changes in the cellular microenvironment to control physiological and pathological processes through genomic and non-genomic actions. NR4A receptors control metabolism, vascular homeostasis, and cardiovascular and neurological functions and mediate immune cell homeostasis in inflammation and cancer. The majority of NR4A activity is due to direct activation or repression of transcriptional target

expression. A growing body of information demonstrates direct non-genomic roles of NR4As through posttranslational modifications and interactions with binding partners (recently reviewed by Herring et al., 2019). NR4A receptors bind directly as monomers or homodimers to promoter regions of target genes that contain the NBRE response element (AAAGGTCA). Furthermore, NR4A receptors can dimerize with retinoid X receptor (RXR) nuclear receptors and bind to a DR5 (direct repeat with 5-bp length spacer) motif, providing a mechanism for NR4A receptors to regulate distinct target genes *in vivo*. Similar to NR4A receptors, RXR is also a member of the nuclear receptor subfamily controlling pathways associated with cell development, differentiation, metabolism, and cell death (Evans and Mangelsdorf, 2014).

Expression levels, subcellular localization, and the cellular context/environment can influence NR4A tissue-specific functional roles as observed in several cancers and diseases characterized by chronic inflammation (Zhao and Bruemmer, 2010; Mohan et al., 2012; Murphy and Crean, 2015). The cross talk of the NR4A1–NR4A3 subfamily members with various oncogene and tumor suppressor pathways is well-established (Safe et al., 2014; Beard et al., 2015; Wan et al., 2020). Extensive numbers of studies indicate that NR4A genes can act paradoxically either as oncogenes or as tumor suppressors. For example, they serve as oncogenes in lung cancer, melanoma, and colorectal cancer (CRC) but display tumor suppressor roles in acute myeloid leukemia (AML), breast cancer, and metastatic ovarian cancer. The NR4A1–NR4A3 cross talk with pro-tumorigenic or tumor-suppressive signaling pathways underwrites a dichotomous and context-dependent role(s) for members of this receptor subfamily (reviewed by Beard et al., 2015, and Wan et al., 2020).

Disordered stromal cell function, including altered signaling and secretion of paracrine factors, has been linked to disease progression of cancer and inflammatory disorders (Spaeth et al., 2008; Nissen et al., 2019). The molecular cross talk between cancer cells and their surrounding stroma and tumor microenvironment (TME) has a crucial role in the regulation of tumorigenesis and disease advancement. Evidence on the association between NR4A1–NR4A3 functional activities, their influence on the tumor–stromal cell environment, and the impact on disease pathogenesis is emerging. Innovative studies are providing compelling support that NR4A orphan receptors, by changing the local cellular microenvironment, can mediate inflammatory responses, promote angiogenesis, control cellular metabolism and survival, and alter cancer cell immunity to influence the course and outcome of disease. The purpose of this review is to discuss how recent advances in NR4A nuclear receptor biology are leading to important new mechanistic insights into understanding their role in stromal cells and the TME in health and disease.

## TME AND TUMOR STROMA

The TME is composed of non-malignant host cellular and non-cellular components, including cells of the immune system, blood cells, endothelial cells, fat cells, and the stroma. The tumor stroma is an essential component of the TME and is composed of cellular and non-cellular connective tissues that support functional tissues, including fibroblasts, mesenchymal stromal cells (MSCs), osteoblasts, chondrocytes, and the extracellular matrix (ECM). While specialized cells of the TME, such as endothelial cells, pericytes, adipocytes, and immune cells, can be included as constituents of the stromal compartment, these cells are more accurately defined as non-stromal cells within the TME (Valkenburg et al., 2018).

It is well-established that the TME plays a pivotal role in tumor progression, and together with cells of the stroma, this milieu plays a major role in maladapting and promoting tumor survival and progression. As outlined, the TME consists of a complex system of heterogeneous cancer cells alongside local host cells and non-stromal components of the TME including adipocytes, pericytes, and immune cells, which also contribute to the tumor–stromal communication (de Groot et al., 2017). Within this environment are fluxes in multiple signaling molecules such as cytokines and adenosine, alterations in metabolic states, regions of hypoxia, acidosis, fibrosis, structural/architectural breakdown from factors such as matrix metalloproteinases (MMPs), and enhanced angiogenesis concurrent with inadequate perfusion, leading to poor nutrient supply (Liotta and Kohn, 2001; De Palma et al., 2017; Binnewies et al., 2018; Chandler et al., 2019). This complex environment is not only a consequence of cancer progression but also a pivotal factor in aiding its progression (Liotta and Kohn, 2001; Binnewies et al., 2018).

The tumor stroma, like its normal counterpart, is essential for tissue structure and remodeling. It is a vital component of the TME involved in tumorigenesis, cancer progression, and metastasis (Valkenburg et al., 2018). In addition to the main components of the stroma, including MSCs, osteoblasts, and the ECM, the tumor stroma also has a high abundance of cancer-associated fibroblasts (CAFs). While stromal cells display antitumor activities in normal conditions, in the background of such altered and harsh environments, stromal cells respond and undergo phenotypic changes and in turn play a pivotal role in influencing tumor progression (de Groot et al., 2017). One of the most notable maladaptive responses is the activation of the hypoxia inducible factor-1α (HIF-1α)-dependent gene transcription in hypoxic tumors, leading to metabolic changes and increased oxygen supply, which aid tumor survival and progression, leading to worse outcomes for patients (Semenza, 2010). Predictably, several of these tumor-associated alterations, or factors which promote or maintain the complexity of these transformed systems, are targets for cancer therapy design, for example, targeting tumor angiogenesis, reprogramming cellular metabolism, and adjusting inflammatory responses leading to altered immune cell populations, with the goal of correcting the environment, which aids in the survival of cells that promote tumorigenesis (Semenza, 2010; Naing et al., 2018; Wang et al., 2018). As we outline herein, leading reports elucidating the novel regulatory role of the NR4A1–NR4A3 receptors implicate these receptors as important mediators controlling these crucial cellular processes leading to changes in the TME and cancer progression.

## (I) NR4A1–NR4A3 RECEPTORS, THE LOCAL INFLAMMATORY RESPONSE, AND TUMOR STROMAL CELLS

Tumor–stromal communication plays an important role in not only cancer initiation but also the proliferation and metastasis of these cells into distant organs, potentially causing cancer recurrence. Inflammatory cytokines and growth factors released by MSC, CAFs, and tumor-associated macrophages (TAMs) initiate a signaling cascade and create a microenvironment conducive to tumorigenesis (Guo and Deng, 2018; Leuning et al., 2018). In 2014, Zhou and colleagues revealed a distinct mechanism by which the microenvironment stimulates breast cancer cell invasion and metastasis. With a genome-wide cDNA screen, it was identified that inflammation-induced NR4A1 (NR4A2 and NR4A3 also respond) is a critical factor for the activation of TGF-β/SMAD-mediated breast cancer cell migration, invasion, and metastasis *in vitro* and *in vivo*. Loss of NR4A1 inhibits TGF-β-induced epithelial-to-mesenchymal transition (EMT), revealing a novel mechanism by which the microenvironment stimulates breast cancer cell invasion and metastasis (Zhou et al., 2014).

Furthermore, a recent study proposes a far-reaching model (using serial single-cell RNA sequencing) of paracrine signaling mediated by the activity of cyclooxygenase (COX-2) enzyme activity and production of the proinflammatory mediator, prostaglandin E2 (PGE2), by prolactin receptor ($PLR^+$)-expressing tumor cells (Zheng et al., 2019). In this *in vivo* model of tumorigenesis, stromal cell expression and activity of all three NR4A receptors are activated by the enhanced secretion of PGE2 by neighboring tumor cells. NR4A receptors rapidly heterodimerize with the RXR, and a stromal cell NR4A–RXR complex induces the transcription, production, and secretion of the peptide hormone prolactin. The secreted hormone feeds back to neighboring tumor cells, increasing their proliferation, leading to tumorigenesis. Importantly, it was found that induction of stromal NR4A expression by PGE2 selectively stimulates expression of NR4A receptors but not the RXR family (Zheng et al., 2019). COX-2, prolactin, and prolactin receptor show consistent differential expression in tumor and stromal compartments across several human cancers. The observed cellular paracrine cross talk may be important factors in the efficacy of the anti-inflammatory COX-2 inhibitors in cancer suppression.

The COX-2/PGE2/NR4A signaling findings complement previous studies where NR4A has been shown to be a regulator of stromal and immune cell functions (Murphy and Crean, 2015) and linked to the expression of prolactin expression in inflammatory joint disease (McCoy et al., 2015). Further, in rheumatoid arthritis (RA), psoriasis, and colon cancer, the NR4A subfamily has previously been singled out as a downstream effector of prostaglandin (PGE) signaling. PGE2 potently induces NR4A2 expression levels via a cAMP/PKA-dependent pathway (Holla et al., 2011; McMorrow and Murphy, 2011). Studies by Holla et al. (2011) suggest that the molecular cross talk between PGE2 and NR4A2 is central to controlling CRC survival mediated through the regulation of apoptosis by blocking cleavage of caspase-3, with NR4A2 playing a central role as a point of transcriptional integration coupling eicosanoid and metabolic pathways.

Chronic inflammation can generate an immunosuppressive microenvironment that allows advantages for cancer formation and progression. MSCs, and their secreted paracrine factors, can modulate inflammatory and immune responses (Fontaine et al., 2016). The immunosuppressive environment has been shown to be PGE2 regulated in several cancers (Wang and Dubois, 2018). Recent investigations into the adaptation of the leukemic mesenchymal microenvironment reveal a novel COX-2/PGE2-NR4A/WNT signaling axis, correlating chronic inflammation with changes in cellular metabolism, leading to reduced immune surveillance (Wu et al., 2018). Reduced secretion of prostaglandins by the mesenchymal inhibition of COX-2 led to decreased expression of NR4A receptors and regulatory T-cell (Treg) genes, FOXP3 and CTLA4, in the MSC-cocultured $CD34^+$ cells. The significance of these findings highlights that upregulated NR4A-WNT/β-catenin signaling functions to attenuate antileukemic immunity by upregulating Tregs and blocking the production of leukemia-reactive $CD8^+$ cytotoxic T lymphocytes.

Tregs, which prevent overt immune responses and autoimmunity, have been shown to accumulate aberrantly in some types of TMEs to suppress antitumor immunity and to sustain the establishment of an immunosuppressive environment. Impeding Treg-mediated immune tolerance is central when considering cancer immunotherapy. Mice lacking *NR4A1* and *NR4A2* genes, specifically in Treg cells, show resistance to tumor growth in transplantation models without exhibiting any serious systemic autoimmunity (Hibino et al., 2018). Treatment with a chemotherapeutic agent, camptothecin, together with a COX-2 inhibitor was found to inhibit induction and transcriptional activity of NR4A factors, and they synergistically display antitumor effects *in vivo* (Hibino et al., 2018). Thus, genetic inactivation or pharmacologic inhibition of NR4A receptors can unleash effector activities of $CD8^+$ cytotoxic T cells and stimulate potent antitumor immune responses within the TME. Chen et al. (2019) further ascertained that an NFAT–NR4A axis controls the expression of several inhibitory receptors and that treatment of tumor-bearing mice with CAR-T cells, lacking all three NR4A receptors, results in tumor regression and prolonged survival. Collectively, these studies indicate translational and therapeutic implications in the development of effective TME anticancer therapies by modulating NR4A receptor function in tumor-infiltrating T cells.

It is thought that MSCs exhibit a gain of function which allows them to preferentially migrate toward tumor sites as they would to a wound (Spaeth et al., 2008). The molecular mechanisms behind this movement are still not fully elucidated but are becoming more recognized as important, owing to the fact that MSCs within the TME are often linked with poorer prognosis, augmenting angiogenesis, tumor formation, and metastasis (Karnoub et al., 2007). In breast cancer, for example, many studies have used the MDA-MB-231 cell line to examine the plausible chemotactic factors responsible for this movement

(Dwyer et al., 2007; Lin et al., 2008). This MDA-MB-231 cell line models the most invasive and migratory triple-negative (TN) form of breast cancer (Lanning et al., 2017). Signaling molecules including SDF-1α, TGF-β, IL-6, PDGF, MCP-1, and cyclophilin B were identified as trophic factors associated with MSC recruitment, but it is proposed that they are not working alone and that additional signaling pathways, transcriptional mechanisms, and regulatory elements need to be ascertained to fully understand the complexity of the molecular processes involved. The NR4A family is associated with the migration of MSCs and their movement into tumor sites. In a study examining the difference in gene profiles between migratory and non-migratory MSCs, it materialized that, of the 12 genes which displayed differential expression, both *NR4A1* and *NR4A2* genes showed the highest expression change in the migratory variety in response to SDF-1α and PDGF, signifying a potential regulatory role for these transcription regulatory factors (Maijenburg et al., 2012). Enhanced *NR4A1* and *NR4A2* expression levels in MSCs lead to increased cytokine and growth factor production, suggesting that these receptors regulate migratory MSCs with the capacity to specifically modulate the local immune response.

## (II) NR4A196NR4A3 RECEPTORS, THE TME, AND STROMAL CELL HYPERPLASIA

One of the first indicators that normal cells may be switching to a cancerous phenotype is the initiation of hyperplasia and changes in metabolic activity, where the tissue of an organ is enlarged due to an abnormal increase in cell reproduction and proliferation (Dupont et al., 1993). Recent studies discerning the associations with stromal and non-stromal cells of the TME and NR4A1–NR4A3-dependent changes on cell density, metabolic activity, cell hyperplasia, survival, and invasion are described herein.

In breast cancer, the density of the breast tissue, "how many" stromal cells it has, has often been associated with increased cancer risk and poorer prognosis. A functional role for NR4A2 has been suggested by a study examining the mechanisms behind obesity-associated breast cancer risk (Ghosh et al., 2010). In human adipose stromal cells (ASCs), NR4A2 is established to be a key regulator of *aromatase* gene transcription. The function of *aromatase* is the enzymatic conversion of androgen to estrogen, which catalyzes the final and rate-limiting step in estrogen biosynthesis. This increase in *aromatase* activity and subsequently estrogen levels in adipose tissue is proposed to be one of the main causal factors in the substantial increased likelihood that women classified as obese will develop breast cancer (Bulun et al., 2005). Ghosh et al. hypothesized that with increased breast cell density, there is a concomitant decrease in the tumor-suppressive function of BRCA1 and NR4A receptors. When NR4A2 expression and activity are depleted in isolated human ASCs, there is a substantial increase in *aromatase* gene expression and, therefore, estrogen biosynthesis, increasing the likelihood of breast cancer occurrence (Ghosh et al., 2010).

As a major component in the breast cancer microenvironment, ASCs are capable of secreting large quantities of metabolic substrates, such as fatty acids, to establish a metabolic TME. NR4A1 has been identified as a regulator for fatty acid uptake in breast cancer, leading to restrained fatty acid metabolism and inhibiting breast cancer progression both *in vitro* and *in vivo*. This study reveals that NR4A1 binds and recruits a corepressor molecule to the promoter regions of CD36 (also known as FA translocase) and fatty acid-binding protein 4 (FABP4), leading to transcriptional suppression, which hampers fatty acid uptake, leading to the inhibition of cell proliferation and impeding tumor cell growth *in vivo* (Yang et al., 2020). In contrast, in a study of melanoma, NR4A1 contributes to the metabolic adaptation of melanoma cells by regulating fatty acid uptake and oxidation (Li et al., 2018). The NR4A1-dependent metabolic adaptation protects melanoma cells undergoing loss of attachment (LOA) to the ECM and supports the survival of circulating tumor cells in the circulation. As highlighted, eicosanoid signaling and fatty acid metabolism through PGE2-mediated upregulation of NR4A2 receptors in colon cancer cells have been established (Holla et al., 2011). The studies propose that COX-2-derived PGE2 potentially regulates an adaptive shift in metabolism and NR4A2 activity central to this process. NR4A2 transcriptional activity increases fatty acid oxidation by inducing the expression and activity of several enzymes central to the fatty acid metabolic pathway. Collectively, these studies indicate that alteration of NR4A activity may fine-tune an adaptive shift to energy utilization via fatty acid oxidation, a metabolic adjustment that is observed in several types of cancer.

The role of NR4A1 in the TME has been further recognized with *in vivo* studies of tumor metastatic spreading models in wild-type ($NR4A1^{+/+}$) mice and $NR4A1^{-/-}$ mice (Hanna et al., 2015; Li et al., 2017). Expression of host NR4A1 was identified as a critical factor in antitumor metastasis because, in the absence of NR4A1, metastatic spreading was greatly accelerated (Li et al., 2017). Furthermore, this study reveals two potential key mechanisms by which the absence of NR4A1 expression facilitates cancer cell invasion and metastasis. Lack of NR4A1 in TME macrophages promotes inflammatory cytokine TNF-α production, which stimulates cancer cells to undergo EMT and promotes invasive properties. In addition, lack of NR4A1 results in reduced levels of colony-stimulating factor-1 receptor (CSF-1R) expression, which decreases the migratory capacity of inflammatory cells and subsequently hinders cell chemotaxis and tumor infiltration. These results unveil a novel function of NR4A1 in regulating tumor invasion and metastasis, which is consistent with a previous study reporting that NR4A1-deficient mice specifically lack "patrolling monocytes," resulting in increased cancer lung metastasis *in vivo* (Hanna et al., 2015).

NR4A1 protein expression is also decreased in the mouse basal-like mammary tumors during the tumor progression process and in a large proportion of human TN breast cancer (TNBC) tumors. The low expression of NR4A1 protein in human TNBC samples is associated with advanced tumor stage, lymph node metastasis, and disease recurrence. Expression of NR4A1 in TNBC MDA-MB-231 cells significantly inhibits the proliferation, viability, migration, and invasion of these cells in culture and *in vivo* and the growth and metastasis of these cell-derived tumors in mice (Wu et al., 2017). These results demonstrate that NR4A1 functions to inhibit the initiation and growth of the MDA-MB-231 cell-derived tumors in mice by reducing their proliferation rate.

Constitutive migration and TGFβ-induced migration of breast cancer cells are reliant on nuclear and extranuclear expression of NR4A1, respectively, and it has been shown that selective NR4A1 antagonists inhibit both pathways by decreasing the NR4A1-dependent expression of β1-integrin, inhibit TGFβ-induced nuclear export of NR4A1, and induced EMT (Hedrick et al., 2016). It has been recognized in CRC tissues that aberrant NR4A1 expression in cancer tissues and cells of the TME acts to promote cell growth and survival by serving as an important mediator of the WNT/β-catenin and AP-1 signaling pathways (Wu et al., 2011). Moreover, the receptor can promote metastasis and invasion through controlling the MMP9/E-cadherin axis (Wang et al., 2014) and β1-integrin (Hedrick et al., 2017) and forming a feedforward loop with β-catenin under hypoxic conditions (To et al., 2014). These studies propose a novel molecular basis for understanding the biological properties of tumorigenesis and reveal that selective targeting of NR4A1 functional activity may represent a mechanism for altering cancer metastasis *in vivo*.

## (III) NR4A EXPRESSION AND ACTIVITY IN TUMOR ANGIOGENESIS

Angiogenesis is one of the key factors associated with cancer progression, with studies finding that the balancing levels of pro- and anti-angiogenic factors, including vascular endothelial growth factor (VEGF) levels, in tissue reflect the aggressiveness with which tumor cells spread. Additional cellular players involved in angiogenesis include CAFs (Kalluri, 2016; Santi et al., 2018). VEGF has been found to potently activate the expression and functional activity of all three NR4A members, and in NR4A1$^{-/-}$ knockout mice, tumor growth, angiogenesis, and microvessel permeability are almost completely inhibited, further purporting a critical mode for these orphan receptors in tumorigenesis (Zeng et al., 2006). Recent studies strengthen this significant body of work, further demonstrating that NR4A1 is essential for VEGF-A-induced pathological angiogenesis, tumor growth, and metastasis *in vivo* (Ye et al., 2019; Chen et al., 2020). Extending previous findings that tumor growth was inhibited in NR4A1 knockout mouse models, metastasis of colorectal tumor was completely inhibited in NR4A$^{-/-}$ mice. Tumor masses were increased by ∼70% and decreased by ∼40% in transgenic EC-NR4A1-S mice and EC-NR4A1-DN mice, in which the full length and a dominant negative mutant of NR4A1 were induced and specifically expressed in the mouse endothelium. In human disease, NR4A1 is highly expressed in the vasculature and tumor cells of human melanoma and CRC tissues, but not in normal tissues. Furthermore, tumor angiogenesis and modulation of genes associated with angiogenesis were critically reduced in tumor tissues treated with NR4A1 shRNAs and selective minigenes (Ye et al., 2019; Chen et al., 2020). Silencing endothelial NR4A1 inhibits the proliferation and migration of tumor cells, indicating that the NR4A1 receptor functions as a regulator of tumor growth and metastasis *in vivo*. Together, these studies demonstrate that NR4A1 is a prospective therapeutic target with translational potential for several human cancers by targeting the vasculature within the TME.

## (IV AND V) CAFS, INTRA-TUMORAL FIBROSIS, AND NR4A RECEPTORS

CAFs are the dominant cell type within the stroma of tumors. They orchestrate paracrine pro-tumorigenic signaling with adjacent tumor cells, thus exacerbating the hallmarks of cancer and accelerating tumor malignancy. In breast cancer, up to 80% of the normal fibroblasts in breast tissue acquire the CAF phenotype during cancer progression (Kalluri, 2016; Santi et al., 2018). Molecular insights into the transcriptional programs that enable the oncogenic function of CAFs are emerging (Chan et al., 2018). The complete human NR profile in CAFs from clinical cutaneous squamous cell carcinoma (SCC) biopsies has recently been accomplished (Chan et al., 2018). Interestingly, both NR4A2 and NR4A3 are significantly upregulated in microdissected CAFs ($n = 10$). A highly similar NR4A2/NR4A3 profile was observed between the microdissected CAFs and explanted CAFs, indicating that the NR profile of SCC CAFs is retained during *in vitro* culture. Pharmacological targeting of specific driver NRs in CAFs diminished SCC invasiveness, proliferation, drug resistance, energy metabolism, and oxidative stress status.

Tumors have often been described as "wounds that do not heal," and as such, it is not unforeseen that fibrosis not only is a major consequence of progressing tumors but also plays a causal role in their progression (Chandler et al., 2019; Chen and Song, 2019). It is CAFs within the stroma that are the main protagonists of such fibrosis in cancers, while other cells, such as immune cells, may also contribute in secreting fibrotic activators (Yamauchi et al., 2018). To date, the specific cell origin of these CAFs remains unknown, with the likelihood that they are derived from diverse cell types within the stroma (Cirri and Chiarugi, 2011; Kalluri, 2016). Interestingly, several factors involved in fibrosis are similarly implicated in multiple stages of cancer progression. Numerous ECM proteins, including fibronectin, are secreted by CAFs and have been shown to enhance tumor aggression, invasion, and metastasis. TGFβ, a major pro-fibrotic factor, has been shown to drive the differentiation of fibroblasts to CAFs, which aid in tumor aggression and invasiveness (Kalluri, 2016). Additionally, TGFβ receptor activation is responsible for the secretion of multiple MMPs within tumors, which play pivotal roles in tissue breakdown enhancing the metastatic capabilities (Hawinkels et al., 2014). Of note, activation of NR4A1 in breast cancer enhances TGFβ3 signaling, potentiating its oncogenic activities, by inducing SMAD7 degradation. Moreover, NR4A1 was shown to enhance TGFβ-induced EMT, a process later shown to be dependent on NR4A1 nuclear export. These studies confirm a pivotal role for NR4A1 in mediating TGFβ/p38-dependent induction of β-catenin in TNBC migration and invasion (Hedrick et al., 2016, 2017; Hedrick and Safe, 2017).

In a separate analysis, inflammatory cytokines, IL-1β, and TNF-α potently induce NR4A2 expression, which, in the presence of TGFβ, potentiates SMAD activation of fibrosis and cancer development (Zhou et al., 2014; Palumbo-Zerr et al., 2015). These responses are significantly amplified when NR4A is overexpressed, leading to TGFβ/SMAD-induced EMT and invasion by interacting with and promoting

AXIN2-RNF12/ARKADIA-induced SMAD7 degradation to enhance the expression of activated TGFβ type I receptor (TβRI). These observations suggest that both NR4A1 and NR4A2 are important mediators in responding to inflammatory stimuli by activating TGFβ signaling and underline the need for further identification of NR4A1–NR4A3-specific functions in controlling intra-tumoral fibrotic pathways and the impact on EMT.

The functional activity of NR4A3 in breast and lung cancer progression has been examined, and a notable association between this NR4A family member and the tumor suppressor p53 was determined (Fedorova et al., 2019). *NR4A3* gene expression is a direct transcriptional target of p53, suggesting that the NR4A3 functional activity and tumor suppressor role in cancer progression is activated by p53. As part of this study, patient survival analysis, using a publicly available clinical data repository, was conducted, establishing that high levels of NR4A3 expression positively correlate with increased survival rates for patients with breast and lung cancers. Thus, these studies reveal that *NR4A3* is a novel transcriptional target of p53, which triggers apoptosis and has a tumor-suppressive role in breast and lung cancers (Fedorova et al., 2019). Whether NR4A3 is a *bona fide* tumor suppressor needs further elucidation. However, several lines of evidence support this notion. In *NR4A1*/*NR4A3* double-knockout mice, it was reported that loss of these two genes can result in the development of AML, due to uncontrolled expansion of myeloid progenitor cells (Boudreaux et al., 2012, 2019).

## (VI) NR4A1-NR4A3 RECEPTORS IN RHEUMATIC DISEASES

NR4A1–NR4A3 expression is elevated in synovial tissue MSC and fibroblast-like stromal cells (FLSs), macrophage, endothelium, cartilage, and PGE2-stimulated chondrocytes from patients with RA, psoriatic arthritis, or osteoarthritis, making NR4A receptors attractive targets in rheumatic and skin diseases (McMorrow and Murphy, 2011; Marzaioli et al., 2012; Shi et al., 2017; Xiong et al., 2020). Stromal cell hyperplasia and development of a "tumor-like pannus" are characteristics of chronic inflammatory diseases including RA and psoriatic arthritis (Bottini and Firestein, 2013). Expression of NR4A1/NR4A2 subfamily members are significantly upregulated in the pannus tissue and are mediators of cytokine, growth factor, and prostanoid (PGE2) action contributing to the hyperplastic and invasive phenotype of FLSs that leads to the modulation of MMP production and cartilage homeostasis (Mix et al., 2012).

## POTENTIAL FOR TARGETING NR4A RECEPTORS

NR4A receptors' expression and function in stromal cells and their influence on non-stromal cells of the TME are emerging as a promising area for the promotion of novel therapeutic targets in the treatment and prevention of human cancer progression. Developments on mechanisms of NR4A silencing and/or strategies for their activation are leading to new therapeutic interventions (Lee et al., 2014; Safe et al., 2014; Boudreaux et al., 2019). Over the last decade, multiple agents have been identified to modulate NR4A1–NR4A3 expression and functional activity *in vitro* and *in vivo* (Safe et al., 2014, 2016). While previously it was understood that NR4A receptors were not "druggable" targets given their bulky and inhospitable ligand binding domain (de Vera et al., 2019), endogenous mediators, including fatty acids, have been shown to act as potent modulators (de Vera et al., 2016, 2019). These modulating agents, both agonists and antagonists, have been examined extensively, with their binding capabilities and exact mechanisms of action being rationalized systematically (Safe et al., 2014, 2016; Munoz-Tello et al., 2020). More recently, Conneely et al. have made important therapeutic innovations in AML with the identification of several drugs that modulate NR4A1/NR4A3 expression and function (Boudreaux et al., 2019). Both NR4A1 and NR4A3 are silenced in AML and display tumor-suppressing qualities suppressing MYC activity. NR4A1/NR4A3 silencing in AML is mediated through the blockade of transcription elongation, while treatment with dihydroergotamine (DHE) rescued NR4A silencing and allowed their tumor-suppressing ability to reactivate with concomitant slowing of AML progression *in vivo* (Boudreaux et al., 2019).

Thus, recent findings (summarized in **Figure 1**) support the targeting of NR4A receptors as potential specific therapeutic targets in enhancing the efficacy of cancer treatments and include (i) pursuing NR4A receptors (downstream of COX-2/PGE2 activity) as key regulators of prolactin production and signaling by stromal cells to reduce cancer cell proliferation and tumorigenesis; (ii) modulating NR4A2 transcriptional activity as a regulator of fatty acid utilization in CRC and *aromatase* transcription and production of estrogen in TNBC; (iii) controlling NR4A1–NR4A3 as regulators of angiogenesis, with the functional activity of NR4A1 in mediating VEGF-A-induced pathological/tumor angiogenesis facilitating cell proliferation and cancer cell migration; (iv) CAF activity, migration of MSCs and immune cells into tumor sites, and their role as mediators of EMT and cell metastasis; and (v) further analyzing the molecular interactions of this subfamily with the ECM, TGFβ/SMAD signaling, and control by altered p53 activity, which could also prove extremely beneficial to elucidating the NR4A1–NR4A3-dependent molecular mechanism(s) controlling cell survival, hyperplasia, and death and altering immune tolerance against cancer. Modulation of NR4A1–NR4A3 receptors as therapeutic targets in cancer biology reveals a novel category of mechanism-based therapies, and future efforts, due to their dichotomous responses, should involve meticulous evaluation of the cancer cell type targeted and moreover the method(s) of drug delivery, given the tissue- and cell-specific functional roles that NR4A receptors display in controlling cancer biology.

## SUMMARY

While it is well-established that the initiation and development of cancer is a disease brought about by mutations in the cancer cells themselves, there is no doubt that the TME and stromal microenvironment are critically important for

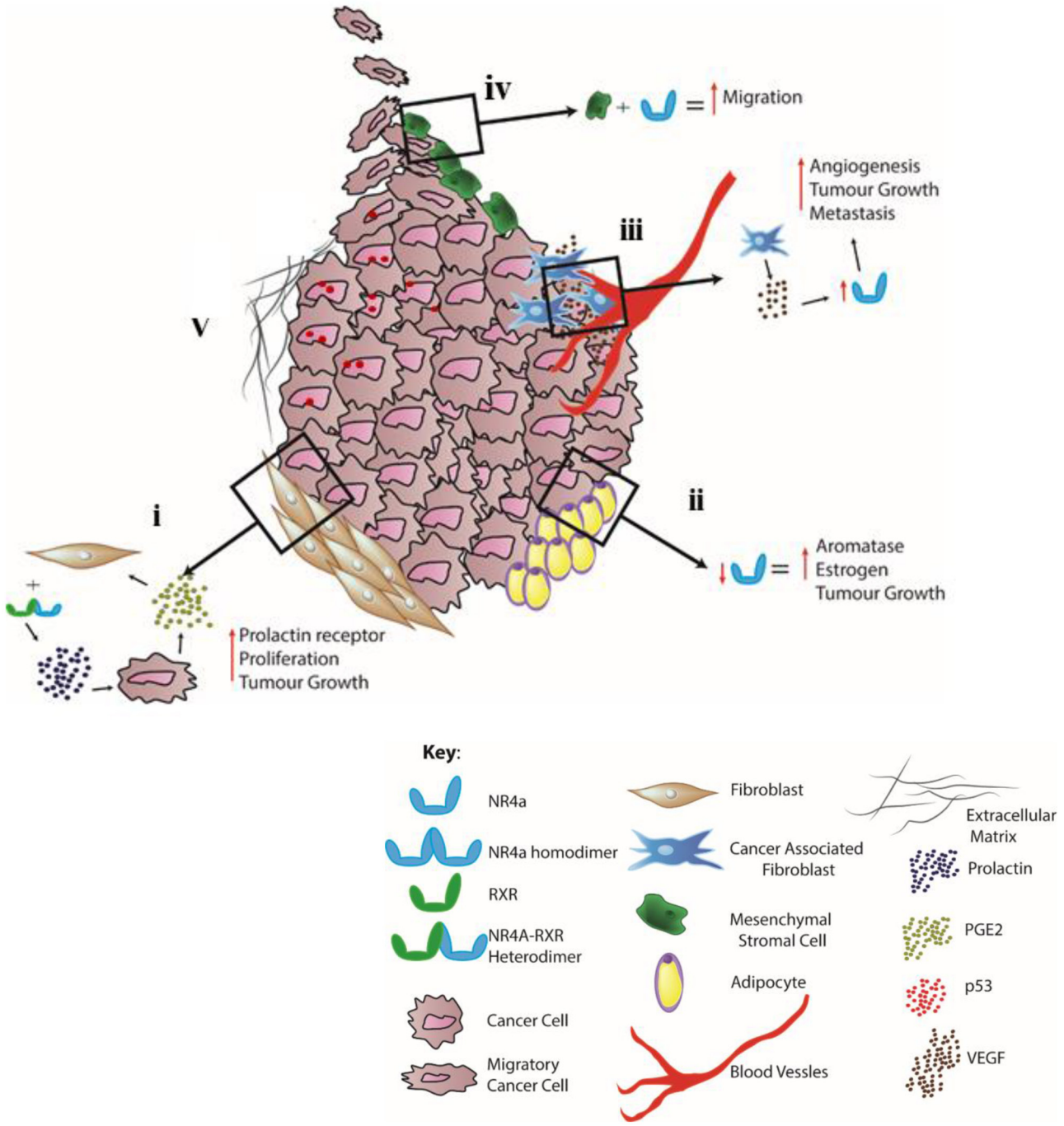


**FIGURE 1 |** Distinct cell-specific functions controlled by NR4A1–NR4A3 nuclear receptors in the TME–stromal microenvironment. **(i)** Stromal fibroblasts: NR4A receptor dimerization with RXR receptors (downstream of pro-inflammatory COX-2/PGE2 activity) functions as potent regulators of prolactin production/signaling by stromal cells. **(ii)** Adipocytes: NR4A activity promotes an adaptive shift to energy utilization via fatty acid oxidation with NR4A2 regulating *aromatase* transcription and production of estrogen in TNBC. **(iii)** Endothelium and CAFs: NR4A receptors regulate VEGF-A-induced pathological/tumor angiogenesis, CAF activity, and tumor metastasis. **(iv)** MSC: NR4A1 functions in mediating MSC proliferation and cytokine and growth factor production, leading to cell migration resulting in altered immune (T regulatory, T cytotoxic, and macrophage) cell function. **(v)** ECM: cross talk between NR4As and the stroma, including interaction with the ECM, TGFβ/SMAD signaling, and p53 activity.

disease progression and tissue response to therapy. Recent developments discerning NR4A1–NR4A3 receptor function in cells within the tumor–stromal environment highlight the distinct functional role(s) that these receptors perform in modulating pro-inflammatory signaling, proliferation, hyperplasia, death/survival, migration, angiogenesis, and tumor immune surveillance. The described NR4A studies uncover the fundamental molecular mechanisms controlling cell-specific activity that facilitates tumor–stromal cell communication within the TME. With the development of mechanism-based NR4A biologics demonstrating efficacy at transforming tumor angiogenesis, growth, metastasis, and immune surveillance, these intracellular receptors may function as central receptors demonstrating translational capacity in cancer cell biology.

## AUTHOR CONTRIBUTIONS

EM conceived the theme of the review. EM and DC wrote the manuscript and finally approved the manuscript.

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