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Extracellular matrix-mediated regulation of cancer stem cells and chemoresistance

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ABSTRACT

The identity of cancer stem cells (CSCs) remains an enigma, with the question outstanding of whether CSCs are fixed entities or plastic cell states in response to microenvironmental cues. Recent evidence highlights the power of the tumor microenvironment to dictate CSC functionality and spatiotemporal regulation that gives rise to tumor heterogeneity. This microenvironmental regulation of CSCs parallels that of normal tissues, whereby resident stem cells reside within specialized microenvironments or 'niches', which provide the cellular and molecular signals that wire every aspect of stem cell behavior and fate. The extracellular matrix (ECM), along with its sequestered growth factors, is a fundamental component of the stem cell niche. Pathological ECM remodeling is an established hallmark of cancer, with the ECM a key mediator of metastasis and drug resistance. In this review, we discuss the controversial identity of CSCs and new understanding of the impact of tumor microenvironment on CSC function and phenotype. We outline parallels between development, wound repair and cancer to discuss how changes in ECM dynamics influence stem cell function during normal physiological processes and pathological states, as well as the transition between the two in the form of precancerous lesions. We then explore examples illustrating the molecular circuits partnering cancer cells with stromal cells and how this communication involving ECM imparts a CSC phenotype and promotes chemoresistance. Understanding the mechanisms underlying CSC functionality and chemoresistance, along with mathematical modeling approaches and advancing technologies for targeting the undruggable proteome, should open opportunities for cancer breakthroughs in the future.

1. Introduction

The term 'cancer' is derived from the word, 'karkinos', recorded by Hippocrates around 460-370 B.C (Sudhakar, 2009). In the centuries since this date, cancer has managed to elude attempts to cure it completely or at least suppress it to the extent that it may be considered unequivocally as a chronic disease, rather than a primary cause of death (Richards et al., 2011; Bell and Ristovski-Slijepcevic, 2013; Global Burden of Disease Cancer Collaboration, 2017). Cancer, by the devastation it causes to individuals, their loved ones, and society, as well as its awe-inspiring complexities, continues to captivate the attention of researchers around the world. Historically, the cancer cell itself, with its oncogenic mutations, was the focus of cancer research (Cairns, 1975; Greenblatt et al., 1994; Balmain et al., 1993; Bishop, 1987) and led to the development of cytotoxic chemotherapies that exploited its known hyper-proliferative character (Gilman and Philips, 1946; Wintrobe and Huguley, 1948; Goodman et al., 1946; DeVita and Chu, 2008). However, it is now established that the environment in which cancer grows, referred to as the tumor microenvironment, plays a vital role in cancer initiation, progression, metastasis and drug resistance (Hanahan and Weinberg, 2011; Correia and Bissell, 2012; Lu et al., 2012). More specifically, research is now homing in on the cancer stem cell niche - a specialized subsection of the tumor microenvironment where cancer stem cells (CSCs; also known as cancer initiating cells or tumor-initiating cells) reside (Plaks et al., 2015; Borovski et al., 2011). Research efforts are underway to understand the cellular and molecular mechanisms underpinning how CSCs interact with their surroundings and co-opt stromal cells for adaptive advantage and survival.

The stem cell niche comprises a conglomeration of cells (Schofield, 1978; MacLean et al., 2014; Hsu et al., 2011; Calvi et al., 2003), signaling molecules (Fleming et al., 2008; Duncan et al., 2005; Zhao et al., 2017) and extracellular matrix (ECM) (Stier et al., 2005; Trappmann

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et al., 2012; Watt and Huck, 2013; Dos Santos et al., 2016; Bi et al., 2007; Rayagiri et al., 2018) that together provide instructional cues governing all aspects of stem cell behavior and function (Scadden, 2006; Watt et al., 2000). Akin to normal tissue resident stem cells, CSCs are thought to reside in niches (Plaks et al., 2015; Borovski et al., 2011). The ECM component of the niche is a three-dimensional structure composed of glycoproteins, collagens and proteoglycans that organizes cells within a tissue, conferring tissue architecture (Hynes and Naba, 2012). However, the function of the ECM extends beyond a mere physical support for cells as it also dynamically influences cell behavior and response (Hynes, 2009). For example, the ECM directly or indirectly regulates stem cell maintenance, proliferation, self-renewal, differentiation, and survival (Gattazzo et al., 2014). This regulation is achieved through ECM binding to cell surface receptors (Nakamura-Ishizu et al., 2012; Avigdor et al., 2004; Su et al., 2017), the liberation of ECM-bound growth factors (Kerever et al., 2009; Discher et al., 2009) and the cellular sensing of ECM-generated mechanical forces (Vining and Mooney, 2017; Totaro et al., 2017; Yui et al., 2018), which individually, or together, trigger intracellular signaling pathways that influence cell fate and behavior (Gattazzo et al., 2014). Furthermore, the ECM physically anchors stem cells to the niche where they remain in contact with the instructional cues regulating their existence (Plaks et al., 2015; Sneddon and Werb, 2007; Jones et al., 1995; Levesque et al., 1996). Another less studied role of the ECM that has recently been reviewed (Rainero, 2018) is its potential to be used by epithelial cells as a nutrient source for invasive migration, and survival under conditions such as glucose and growth factor starvation (Muranen et al., 2017; Rainero et al., 2015; Moreno-Layseca et al., 2019). For example, under nutrient depleted conditions, the ECM protein, laminin, along with its binding partner, the β4 integrin receptor, is endocytosed and subsequently degraded within matrix-attached human mammary epithelial cells, leading to elevated intracellular amino acid levels and increased mTORC1 signaling, promoting cell survival (Muranen et al., 2017). Although studies have begun to investigate the role of ECM on cellular metabolism in malignant and non-malignant epithelial cells, further research is also needed to understand the influence of the ECM on metabolic plasticity of CSCs specifically.

In this review, we discuss the controversial identity of CSCs and new understanding of the impact of tumor microenvironment on CSC function, heterogeneity and spatiotemporal dynamics within tumors. We outline parallels between development, wound repair and cancer to show how understanding ECM regulation of stem cell fate under normal physiological processes may be used to inform how aberrant changes in ECM architecture contribute to the development of precancerous lesions and progression to cancer. We then explore examples from a range of cancers to illustrate the molecular circuits partnering cancer cells with stromal cells and how this communication serves to confer or perpetuate a CSC phenotype and enable resistance to therapeutic agents. We particularly focus on how these CSC traits are imparted to cancer cells in response to ECM cues and ECM-associated growth factors released from stromal cells. To conclude, we discuss potential new treatment strategies for targeting CSC function and chemoresistance that take advantage of our understanding of CSC-stromal cell-ECM interactions and the evolutionary landscape of cancer. Lastly, we introduce the concept of emerging technologies that may inspire a new approach to targeting the cellular programs that stipulate the very behaviors that make a CSC just that – a cancer stem cell.

2. Cancer stem cells - what's in a name?

Cancer stem cells are defined by their capacity to undergo self-renewal, initiate tumors, repopulate tumors, and the presence of identifiable cell surface markers (Plaks et al., 2015; Prince et al., 2007). CSCs are also considered to be inherently resistant to chemotherapy and play key roles in driving relapse after treatment and initiation of metastasis at distal sites (Hermann et al., 2007; Kolev et al., 2017). Failures of CSC-targeting drugs in clinical trials (Garber, 2018a), and other emerging evidence, has prompted reassessment of the CSC model and the question of whether CSCs are hardwired entities or an imposed functional state (Garber, 2018a; Hermann and Sainz, 2018; Ball et al., 2017; Batlle and Clevers, 2017; Picco et al., 2017). Some studies have suggested that CSCs are discrete cell-types, and can be derived from their normal stem cell counterparts following oncogenic mutations (Lee et al., 2018; Alcantara Llaguno et al., 2009; Lapouge et al., 2011). Conversely, other studies have argued that all cancer cells are inherently plastic and have the capacity to function as CSCs either spontaneously (Chaffer et al., 2011) or on the proviso that appropriate microenvironmental cues or niche signals are available (Lenos et al., 2018; Vermeulen et al., 2010). Indeed, this concept of CSC-microenvironment dependency is rationalized when we consider the context of normal stem cell behavior and niche dynamics in the cornea, intestine and skin, whereby differentiated cells can transition to become a stem cell substitute under altered microenvironmental conditions associated with wound healing and tissue repair (Nasser et al., 2018; Blanpain and Fuchs, 2014; Donati et al., 2017).

To find a unanimous answer to the question of "what defines a cancer stem cell", an interdisciplinary effort will likely be needed. In this regard, integration of sophisticated mathematical and computational approaches with biological models holds promise for adding a new dimension of understanding to the processes underlying CSC identity, dynamics, and behavior. For example, a mathematical model of the breast CSC niche has been generated in conjunction with data from cell line and mouse xenograft experiments to predict population dynamics during cancer development and in response to therapies, including kinetics of interconversions between mesenchymal and epithelial states in breast CSCs (Sehl et al., 2015). This model was shown to faithfully predict inhibition of IL-6 and HER2 as the most effective combination to eliminate both mesenchymal and epithelial populations of breast CSCs, leading to a proposed clinical trial for HER2-positive breast cancer (Sehl et al., 2015). Given the complexity of the interacting components within the stem cell niche, including ECM, growth factors, cytokines, and intracellular signals, it has been highlighted that this creates a challenge as to which component or components to target to suppress or eradicate CSCs (Sehl et al., 2015). Mathematical models and stochastic simulation techniques have therefore been proposed as a way forward to not only understand CSC identity and tumor growth dynamics, but also as a means of predicting therapeutic response and resistance to therapies (Sehl et al., 2015; Sehl and Wicha, 2018; Bozic et al., 2013; Gupta et al., 2011; Scott et al., 2017; Pacheco et al., 2014; Nazari et al., 2018).

3. Microenvironment trumps cancer stem cell markers in tumor expansion

Historically, CSCs have been identified by dissociating a tumor, transplanting single cells into immunocompromised mice and then profiling the specific cell surface antigens or 'markers' of the single cells which gave rise to tumors that phenocopy the original tumor (Clevers, 2011). New research on colon cancer suggests there is a stark difference between CSCs that show tumor initiation ability and stem cell potential in xenograft transplantation assays, and those that drive tumor expansion. The CSCs driving tumor expansion in colon cancer xenograft models were concentrated towards the tumor edge and CSC clonal functionality was found to be dictated by cues from the microenvironment rather than any innate property (Lenos et al., 2018; Lamprecht et al., 2017). Therefore, the propensity for cancer cells to become clonogenic is liable to change over time depending on their locality (Lenos et al., 2018). However, whether these findings are specific to colon cancer or apply more broadly across other types of solid cancers remains to be investigated. In the case of colon cancer xenograft mouse models, cancer-associated fibroblasts (CAFs) surrounding the tumor and their secreted product, the ECM protein,

osteopontin, were demonstrated to drive clonogenic outgrowth *in vivo* (Lenos et al., 2018). As another explanation, it could be possible that clonogenicity and tumor expansion is also favored towards the outer tumor edges because of spatial restriction and cell density in the tumor core. For example, cancer cell population density has recently been shown to govern other processes, such as onset of metastasis, whereby breast cancer and fibrosarcoma cells at high density express IL-6 and IL-8 leading to synergistic paracrine JAK/STAT signaling and subsequent upregulated protein expression of matrix metalloproteinases. These matrix metalloproteinases, such as collagenase (MMP1), gelatinase (MMP2 and MMP9) and stromelysin (MMP3), degrade the ECM and promote cancer cell migration, thereby facilitating cancer cell escape from the primary tumor (Jayatilaka et al., 2018).

Importantly, it has been proposed that CSC functionality is distinct from CSC identity as inferred from CSC markers (Lenos et al., 2018). For example, using quantitative clonal tracing, no significant difference in the distribution of CSCs throughout the tumor of colon cancer xenograft mouse models was found according to the expression of the stem cell and CSC marker, $Lgr5^+$, or Wnt activity using TCF/LEF driven GFP reporter (TOP-GFP); yet the only functionally clonogenic CSCs were those positioned towards the periphery of the tumor (Lenos et al., 2018). Following chemotherapy treatment of any type of cancer, residual tumors are routinely found enriched with CSC marker-positive cells (Kolev et al., 2017; Li et al., 2008). In the colon cancer xenograft mouse model, administration of chemotherapy also led to a residual tumor population enriched with CSC marker-positive cells, however clonogenic outgrowth following chemotherapy treatment was again found to be driven by the microenvironment (Lenos et al., 2018). Overall, these findings implied that any cancer cell, irrespective of CSC marker status, which reaches the tumor periphery and is within the sphere of influence of CAFs, may become endowed with clonogenic properties enabling tumor regrowth and relapse (Lenos et al., 2018). Taken together, it may be proposed that, at least in the case of colon cancer, cancer cells with distinctive CSC markers drive tumor initiation and persist after chemotherapy, whereas any cancer cell may be responsible for tumor expansion, through acquiring clonogenic properties at the tumor-stroma interface. This mismatch between CSC marker status and clonogenic function may have therapeutic implications as the underlying mechanisms responsible presumably differ, introducing a potential pitfall in therapeutically targeting CSCs based on purported stem cell markers alone. Furthermore, it also highlights the need to better understand the precise mechanisms by which the surroundings of cancer cells govern the acquisition of CSC functional properties including tumor initiation, tumor expansion and drug resistance.

4. Microenvironment drives epithelial-mesenchymal transition (EMT) and cancer stem cell heterogeneity

The EMT program confers stem-like properties to cancer cells and has been linked to intratumoral heterogeneity and drug resistance (Shibue and Weinberg, 2017). The tumor microenvironment has been implicated in driving the spatial segregation of cells with variable EMT and CSC phenotypes within the tumor (Bocci et al., 2019). To illustrate this concept, breast cancer and ovarian cancer are two examples we will discuss. Breast CSCs with mesenchymal-like ($CD24^-$ $CD44^+$) phenotype are reported to reside at the tumor front and are mostly quiescent, whereas CSCs with epithelial ($ALDH^+$) or 'hybrid' EMT phenotype that co-express markers associated with epithelial and mesenchymal phenotypes are localized within the tumor interior (Bocci et al., 2019; Liu et al., 2014; Colacino et al., 2018). These spatiotemporal dynamics of EMT and CSC heterogeneity have recently been shown to be imparted by the tumor microenvironment *via* TGF-β diffusion gradient and juxtacrine, Notch-Jagged signaling induced by inflammatory cytokines, such as IL-6 (Bocci et al., 2019). For example, IL-6 stabilized the hybrid EMT phenotype and expanded the proportion of CSCs (Bocci et al., 2019). Knockdown of Jagged1 (ligand of Notch-Jagged signaling) from the triple negative breast cancer (TNBC) cell line with hybrid EMT phenotype, SUM149, significantly limited organoid formation, thereby confirming the role of Notch-Jagged signaling in supporting CSC phenotype (Bocci et al., 2019). Other signaling pathways have also been implicated in conferring mesenchymal and epithelial states to TNBC-CSCs. For example, yes-associated protein (YAP) signaling was upregulated in mesenchymal CSCs ($CD24^-$ $CD44^+$) whereas Wnt/β-Catenin signaling was upregulated in epithelial CSCs ($ALDH^+$) (Sulaiman et al., 2018). Dual inhibition of YAP and Wnt signaling was required to suppress tumor growth in TNBC, targeting TNBC-CSCs in both mesenchymal and epithelial states (Sulaiman et al., 2018).

In ovarian cancer, the isolation and identification of CSCs from primary human tumor samples has posed a challenge due to the lack of cancer specific markers, which has been attributed to heterogeneity of the disease (Lupia and Cavallaro, 2017). For example, different pools of ovarian CSCs have been isolated using various combinations of cell surface markers derived from other solid cancers ($CD133^+$, $CD44^+$/$CD24^+$/EpCAM$^+$/E-cadherin$^-$, $CD133^+$/$ALDH1^+$, $CD44^+$/$ALDH1^+$, $CD44^+$/$CD117^+$, $CD44^+$/$MyD88^+$) (Shah and Landen, 2013; Meirelles et al., 2012; Burgos-Ojeda et al., 2015; Alvero et al., 2009). However, these markers are unable to reliably enrich or purify CSCs from primary ovarian tumor samples indicating the need to identify CSC markers specific to ovarian cancer. For example, both CD133-positive and CD133-negative cell populations isolated from primary human ovarian cancer samples have been shown to give rise to tumors (Stewart et al., 2011). Furthermore, CD133 is expressed commonly in the epithelial cells of the normal human ovary and fallopian tube (Zhang et al., 2012), highlighting the dilemma in using this marker to identify ovarian CSCs. New research has identified and characterized subsets of cancer cells that occur commonly in high grade serous ovarian cancer and express different patterns of stem cell markers, implying stem cell/progenitor plasticity and adaptation to the dynamic tumor microenvironment (Gonzalez et al., 2018). As the ECM is a key component of the tumor microenvironment, the role of the ECM as a modulator of cancer cell plasticity and marker expression in ovarian cancer warrants investigation. Some subsets of cancer cells detected commonly in ovarian cancer were found to exhibit a transitional or 'hybrid' EMT phenotype (Gonzalez et al., 2018). Ovarian cancer cells displaying this transitional EMT phenotype express variable patterns of CSC markers (CD151, CD24, CD13, CD10, CD73, CD61, CD49f, CD90, CD44, CD133, endoglin, and ROR1) and stemness-associated signaling proteins (Sox-2, pSTAT3, pSTAT5, NFκB, pCREB, and β-Catenin) (Gonzalez et al., 2018). Comparison between epithelial, mesenchymal and transitional EMT cell subsets in ovarian cancer revealed differential expression of stem cell markers and proteins associated with different aspects of cancer progression including invasion, metastasis and drug resistance (Gonzalez et al., 2018; Jolly et al., 2018).

The ECM is also known to induce EMT programs in cancer cells (Venning et al., 2015), therefore understanding ECM-mediated regulation of the different cell subsets may provide insight into ECM proteins to target as a new therapeutic strategy. For example, β-Catenin was expressed more in the transitional EMT cell subset of ovarian cancer compared to the epithelial or mesenchymal cell subsets (Gonzalez et al., 2018). The Wnt/β-Catenin pathway has been found to induce a stem cell phenotype in ovarian cancer cells and is activated by an ECM complex (Condello et al., 2018). This complex formed between the extracellular matrix proteins tissue transglutaminase and fibronectin, and membrane receptor, integrin β1, was enriched in ovarian CSCs grown as spheroids *in vitro* and in human ovarian cancer tissue samples (Condello et al., 2018). In this context, the ovarian CSCs were defined by expression of the CSC marker combination, ALDH +/CD133+ (Condello et al., 2018). Transglutaminase regulates ovarian CSC-ECM interactions through complex stabilization with integrin β1 and interacts with the Wnt ligand receptor, Frizzled 7 (Condello et al., 2018). Disruption of the ECM complex using antibodies that block transglutaminase-fibronectin interactions inhibited ovarian

CSC spheroid formation and proliferation *in vitro*, as well as tumor initiating capacity in nude mice, *via* suppression of Wnt signaling (Condello et al., 2018). Consequently, this ECM complex has been proposed as a new stem cell target for ovarian cancer (Condello et al., 2018). Taken together, the above breast cancer and ovarian cancer examples demonstrate that the functional states and phenotypes of CSCs are a product of microenvironmental cues, with CSC heterogeneity a reflection of local variations in microenvironment mediators and signaling ligands.

5. The ECM-link between development, wound repair and cancer

During development, wound healing and normal tissue homeostasis, the ECM exists in a continuous state of remodeling that is tightly regulated (Cox and Erler, 2011). However, abnormal ECM dynamics are a hallmark of cancer and involve imbalances in ECM synthesis, deposition, post-translational modification, and degradation (Batlle and Clevers, 2017; Bonnans et al., 2014). Parallels are increasingly drawn between development, wound healing and cancer (Rognoni and Watt, 2018; Rybinski et al., 2014; Ge et al., 2017). Understanding ECM-stem cell interactions during these normal and pathological processes, as well as the transition from normal to pathological states, may provide insight into potential drug targets to prevent or treat cancer.

The ECM is involved in the determination of cell fate, from directing development of organs in embryonic life to cellular reprogramming during wound repair. For example, ECM changes have been reported to drive intestinal regeneration by reprogramming intestinal epithelial cells to transiently adopt a primitive state, which is reminiscent of foetal development (Yui et al., 2018). Yui et al. demonstrated that in mice with dextran sulfate sodium-induced colitis, the regenerating epithelial cells showed increased expression of foetal intestinal markers (Sca1, Ly6a) and reduced expression of both intestinal stem cell markers (Lgr5, Olfm4) and secretory cell lineage markers (Yui et al., 2018). Importantly, despite reduced expression of intestinal stem cell markers, $Sca1^+$ cells still formed organoids *in vitro*, demonstrating preserved stem cell capacity (Yui et al., 2018). This change in cell fate and transcriptional program was driven by enhanced local deposition of collagen I that increased focal adhesion kinase (FAK)/Src signaling and downstream activation of yes-associated protein and transcriptional coactivator with PDZ-binding motif (YAP/TAZ) (Yui et al., 2018). Furthermore, the same cellular reprogramming could only be replicated *in vitro* when collagen I matrix was supplemented with the Wnt pathway activating ligand, Wnt3a (Yui et al., 2018). This supports a cooperation between the Wnt pathway and ECM mechanical induction of downstream YAP/TAZ activity, and aligns with results from other studies (Sulaiman et al., 2018; Azzolin et al., 2012; Totaro et al., 2018). Interestingly, proliferating epithelial organoids derived from single $Lgr5^+$ cells can fuse together to form tubes in floating collagen I gels in the absence of supplemented Wnt3a (Sachs et al., 2017). Consequently, it was suggested that distinctions need to be drawn between different contexts (initial formation *versus* maintenance of established organoids) and that a specific timeframe or cell-state may influence microenvironment-induced cell fate transitions (Huels and Medema, 2018). This notion aligns with our earlier discussion on CSC identity and the need to delineate mechanisms governing CSC function across the cancer spectrum: from cancer initiation to tumor expansion.

The signaling pathways and transcriptional programs used by stem cells to generate and regenerate tissues are some of the same signals utilized by cancer cells (Ge et al., 2017). During wound repair in the skin, specific stem cells differentiate into lineages outside of their usual scope, referred to as stem cell lineage infidelity, enabling these stem cells to assume the role of those stem cells that have been lost during wounding (Ge et al., 2017). Whilst stem cell lineage infidelity is transient during wound repair and involves transcriptional rewiring, the difference in cancer is this lineage infidelity becomes permanently instated (Ge et al., 2017). Interestingly, patients with a history of the chronic blistering skin condition, epidermolysis bullosa, particularly the recessive dystrophic form of the disease, have a high risk of development of cutaneous squamous cell carcinoma (SCC) (Fine et al., 2009). Mutations in specific ECM proteins that normally anchor the epidermal layer to the dermis leads to impaired skin integrity and fragility, and chronic wounds, which are hallmarks of epidermolysis bullosa (Bruckner-Tuderman and Has, 2014). For example, recessive dystrophic epidermolysis bullosa involves mutations in *COL7A1*, an integral component of the anchoring fibrils (Varki et al., 2007). Recessive dystrophic epidermolysis bullosa would serve as an ideal model to study how the dermal ECM architecture influences transcriptional rewiring during wound repair and how the ECM may contribute to aberrant transcriptional rewiring in cancer. Indeed, the correlation between epidermolysis bullosa and subsequent development of SCC has already led to investigations of the links between altered ECM dynamics, wound repair and cancer (Yuen and Jonkman, 2011; Watt and Fujiwara, 2011; Mittapalli et al., 2016; Nyström and Bruckner-Tuderman, 2018; Ng et al., 2012).

To study how precancerous changes in ECM from tissue damage drive SCC development, Mittapalli et al. (2016) employed a collagen VII hypomorphic mouse model that phenocopied human dystrophic epidermolysis bullosa. When this mouse model was treated with carcinogens it developed invasive tumors resembling SCC from dystrophic epidermolysis bullosa, whereas wild-type mice only formed benign epithelial tumors, known as papillomas (Mittapalli et al., 2016). Chronic injury and inflammation of the skin in epidermolysis bullosa results in changes in ECM architecture and deposition that contribute to progressive tissue fibrosis (Mittapalli et al., 2016). TGF-β is a major mediator of this tissue fibrosis, whereby downstream signaling (pSMAD2/3, JAK1/STAT3 and RhoA/ROCK) stimulates fibroblast-to-myofibroblast conversion, ECM deposition and remodeling (Mittapalli et al. 2016; Nyström and Bruckner-Tuderman, 2018). Stiffening of the dermis in recessive dystrophic epidermolysis bullosa activates mechanosignaling in SCC tumor cells *via* integrin β1/pFAK/pAKT, promoting invasion and survival (Mittapalli et al. 2016). Knowledge of how ECM regulates cell behavior and stem cell fate under normal physiological processes of embryological development and wound repair, will likely provide insight for understanding how aberrant changes in ECM architecture contribute to cancer development and progression.

Changes in ECM composition and architecture in patients with recessive dystrophic epidermolysis bullosa have been suggested to create a favourable microenvironment for tumor development and progression (Mittapalli et al., 2016; Ng et al., 2012). Loss of collagen VII from dermal fibroblasts in recessive dystrophic epidermolysis bullosa reprograms these fibroblasts to display a gene expression profile resembling that of CAFs (Ng et al., 2012). These gene expression changes include increased expression of type XII collagen, thrombospondin-1, and Wnt-5A, with the functional outcome of enhanced tumor cell invasion *in vitro* and tumor growth *in vivo* (Ng et al., 2012). Furthermore, comparative transcriptome profiling revealed progressive changes in fibroblast ECM gene expression from normal skin to sporadic UV induced-SCC and recessive dystrophic epidermolysis bullosa, through to recessive dystrophic epidermolysis bullosa-SCC (Ng et al., 2012). For example, types V and XII collagens, integrin subunits alpha 3 and alpha 6, and thrombospondin-1 were upregulated in both sporadic UV induced-SCC and recessive dystrophic epidermolysis bullosa fibroblasts, and further upregulated in recessive dystrophic epidermolysis bullosa-SCC fibroblasts (Ng et al., 2012). It has been hypothesized that the increased mutation load and aggressiveness of recessive dystrophic epidermolysis bullosa-SCC compared to sporadic UV induced-SCC is a product of the damaged tissue microenvironment that creates a selective pressure for the epidermal cells bearing these mutations (Nyström and Bruckner-Tuderman, 2018). Transcriptome profiling of myofibroblasts from mouse skin has demonstrated the existence of distinct myofibroblast subpopulations during wound repair, fibrosis and aging, and recently, macrophage-myofibroblast interactions have been found

to dictate functional myofibroblast heterogeneity during wound repair (Shook et al., 2018). For example, CD301b-expressing macrophages directly stimulate the proliferation of adipocyte precursor-myofibroblasts (Shook et al., 2018).

Loss of collagen VII in recessive dystrophic epidermolysis bullosa also leads to impaired innate immunity, as the function of this collagen protein in the lymphoid extracellular matrix is to sequester innate immune activators in the spleen and lymph node (Nyström et al., 2018). Consequently, patients with recessive dystrophic epidermolysis bullosa are prone to increased commensal bacterial colonization of the skin beyond that typically associated with large open wounds (Nyström et al., 2018). This bacterial load in patients with epidermolysis bullosa contributes to chronic infections and development of SCC (Nyström et al., 2018; Hoste et al., 2015). The induction of tumor formation from bacteria-induced tissue inflammation has been shown to involve toll-like receptor-5 signaling in leukocytes, with associated increase in high-mobility group box 1, which can exhibit oncogenic activity (Hoste et al., 2015). Taken together, injury-induced changes in the dermal architecture, along with inflammation, and shared similarities in fibroblast gene expression with SCC, create a microenvironment in recessive dystrophic epidermolysis bullosa skin that is conducive to cancer-initiating changes in keratinocytes and SCC progression (Mittapalli et al., 2016; Ng et al., 2012; Hoste et al., 2015; Föll et al., 2018).

The metabolic state of dermal fibroblasts is intimately connected to regulation of skin ECM homeostasis and fibrosis, whereby the choice of fuel source dictates the generation of an anabolic or catabolic fibroblast phenotype (Zhao et al., 2019). Using a combination of primary human dermal fibroblast *in vitro* and murine skin fibrosis model, Zhao et al. (2019) demonstrated that glycolysis promotes fibroblast ECM production whereas fatty acid oxidation promotes ECM degradation (Zhao et al., 2019). Induction of the fatty acid oxidation pathway by peroxisome proliferator-activated receptor signaling reprogrammed fibroblasts toward a catabolic phenotype involving increased internalization of collagen-1 by the fatty acid transporter, CD36, and subsequent lysosomal degradation (Zhao et al., 2019). Conversely, inhibition of glycolysis in cultured primary human dermal fibroblasts reduced extracellular protein expression of fibronectin, collagen-1 and plasminogen activator inhibitor-1 (PAI-1) (Zhao et al., 2019). Furthermore, Bertero et al. demonstrated that increased ECM stiffness of the tumor niche instructs metabolic reprogramming and cross-talk between cancer cells and CAFs in SCC and other cancers that is responsible for sustaining tumor growth and metastasis *in vivo* (Bertero et al., 2019). Stiffening of the ECM provides biophysical cues, activating the YAP/TAZ mechanosignaling pathway in both cancer cells and CAFs, which in turn upregulates transcription of both glutaminase, an enzyme that converts glutamine to glutamate, and the amino acid transporter, SLC1A3, which mediates the flux of aspartate/glutamate across the plasma membrane (Bertero et al., 2019). Cancer cell-derived glutamate is taken up by CAFs and either used to generate glutathione for cell contractility and protection against ROS, or channelled into the tricarboxylic acid cycle to produce aspartate (Bertero et al., 2019). This aspartate is taken up by the cancer cell *via* the SLC1A3 transporter to fuel nucleotide biosynthesis for sustained cell proliferation (Bertero et al., 2019). Whilst the recent studies by Zhao et al. (2019) and Bertero et al. (2019) did not investigate metabolic plasticity in CSC populations specifically, the dynamic metabolic circuitry that links ECM, fibroblasts and cancer cells conceivably also plays fundamental roles in regulating CSC heterogeneity and function. Indeed, the interaction between tumor microenvironment and metabolic plasticity in CSCs has been considered in recent reviews (Sancho et al., 2016; Ahmed et al., 2018; Peiris-Pagès et al., 2016). Collectively, the studies by Zhao et al. (2019) and Bertero et al. (2019) showed how metabolic fuel choice tunes fibroblast-derived ECM output whereas biophysical cues conferred by the desmoplastic tumor microenvironment rewires cell metabolic phenotypes, promoting cancer progression. Future studies that investigate how immune and metabolic crosstalk between CSCs and fibroblasts modulates ECM dynamics along the disease continuum from normal skin, to epidermolysis bullosa, through to SCC, may unveil important new drug targets for wound repair, fibrosis, as well as the treatment and prevention of SCC.

6. Drug resistance mechanisms and cross-talk between stromal cells, ECM and cancer stem cells

Given the role of ECM in driving transient changes in cell fate during tissue regeneration, it is not surprising that abnormal ECM remodeling is also fundamental in driving changes in cell fate and behavior in cancer. Tissue polarity, tissue architecture and signaling pathway activation, determined by specific basement membrane-cell receptor interactions, have been shown to regulate cell response to apoptotic assaults (Weaver et al., 2002). For example, binding of the ECM protein, laminin, to β4 integrin receptors on mammary epithelial cells, was pinpointed as the driving mediator of resistance to apoptosis (Weaver et al., 2002). The laminin-β4 integrin interaction protected the mammary epithelial cells from apoptosis by driving apico-basal polarity, forming organized 3D structures, and activating nuclear factor kappa B signaling (Weaver et al., 2002). Both normal and malignant cancer cells were afforded protection against apoptosis-inducing stimuli provided polarity and proper integrin interactions were established (Weaver et al., 2002). In addition, the instructional cues conferred by the ECM have been shown to override the cancer phenotype that has been encrypted by oncogenic mutations, thereby persuading cancer cells to behave like normal cells and coaxing tumor masses to revert to phenotypically normal looking structures (Weaver et al., 2002, 1997; Nelson and Bissell, 2006). Given the dualistic power of the ECM to either suppress and revert malignancy, or to promote cancer initiation and chemoresistance, understanding the cellular and molecular mechanisms that decide these outcomes opens new therapeutic opportunities.

To date, many drug resistance mechanisms involving the ECM have been identified across cancer types and these mechanisms have been classified into a range of categories including physical barriers to treatment (hypoxia, pH, and interstitial fluid pressure) and cell-adhesion-associated drug resistance (ECM organization, mechanosignaling and pro-survival signaling pathways) (Holle et al., 2016). Whilst many studies have explored the influence of ECM-mediated chemoresistance on cancer as a whole ‘organ’, far fewer studies have examined the effect of the ECM on inherent stem cell or CSC subpopulations specifically. The growing number of purported stem cells and CSCs identified by cell surface markers has made the ability to dissect the effect of ECM on these cells increasingly possible, thereby filling this gap in the literature. Furthermore, sophisticated tools for tracking stem cells, together with models that allow native ECM tissue constructs to be preserved, enables ECM-stem cell interactions to be studied in unprecedented detail. For example, multiple lineages of stem cells can now be traced concurrently using a ‘confetti’ mouse model (Schepers et al., 2012; Wuidart et al., 2016), and intact ECM can be visualized from decellularized tissues using tissue clearing (clarity)/IsDOT techniques (Mayorca-Guiliani et al., 2017).

In this next section of our review, we will highlight recent advances in our understanding of how the ECM and ECM-associated growth factors released from stromal cells confer CSC phenotype and chemoresistance. Whilst we have not presented a conclusive list of these interactions, we have provided in-depth, illustrative examples from a range of cancers (skin cancer, breast cancer and glioblastoma multiforme). Our aim was to convey the complexity and diversity of the mechanisms and cellular cross-talk leveraged by CSCs to survive therapeutic assaults. It is this complexity and diversity that presents challenges for therapeutic targeting, particularly given the known evolutionary adaptability of cancer. Addressing these challenges will form the focus of our final section on treatment strategy and emerging

therapies.

6.1. The link between nuclear factor-erythroid 2-related factor 2 (NRF2) and ECM remodeling in cancer-associated fibroblast activation

The skin is subject to a barrage of assaults over time, including ultraviolet light, which generates reactive oxygen species (ROS) and induces associated DNA damage, predisposing the skin to malignant transformation (Hiebert et al., 2018). A key mediator responsible for co-ordinating the antioxidant defence response in skin cells is the transcription factor, NRF2 (Schäfer et al., 2012). NRF2 protein levels are negatively regulated through ubiquitination-proteasomal degradation by E3 ubiquitin ligase complexes, including the KEAP1-CUL3-RBX1 complex (Rojo de et al., 2018). Conversely, NRF2 is activated by ROS and electrophiles that modify critical cysteine thiols on KEAP1 and NRF2 (Ma, 2013). NRF2 protein levels increase in response to ROS and electrophiles. To counter this oxidative stress, NRF2 translocates to the nucleus where it binds to antioxidant response elements to direct transcription of enzymes integral for the detoxification of ROS, carcinogens or foreign substances (*e.g.* glutathione S-transferase and NAD(P)H:quinone oxidoreductase 1) (Rojo de et al., 2018; Ma, 2013; Kaspar et al., 2009). Discovery of the antioxidant role of NRF2 naturally led to its consideration as a therapeutic candidate for cancer prevention. However, more recent research has exposed sinister consequences of NRF2 activation in promoting tumor growth and drug resistance of CSCs, thereby prompting reconsideration of its therapeutic potential as a cancer preventative (Rojo de et al., 2018).

Skin fibroblasts with sustained activation of NRF2 secrete an altered cocktail of ECM and ECM-associated proteins that reprograms neighbouring fibroblasts to undergo cellular senescence and phenotypic conversion to CAFs (see Fig. 1) (Hiebert et al., 2018). The ECM has also recently been shown to direct phenotypic heterogeneity of lung fibroblasts, whereby ECM composition, stiffness and TGF-β growth factor signaling govern FAP^{Hi} reactive fibroblast and αSMA^{Hi} myofibroblast subpopulations (Avery et al., 2018). The matrisome changes mediated by NRF2 activation in skin fibroblasts included upregulated protein expression of Abi3bp, Adam23, Angptl2, Ecm1, Gpc1, Nid1, PAI-1, TGF-βr2 and Wnt5a and downregulated protein expression of collagens (Col3a1, Col5a1, Col1a1), Eln, Htra3 and Postn (Hiebert et al., 2018). Of these ECM proteins, PAI-1, was further validated as a direct gene target of NRF2 transcriptional activity and a key mediator of fibroblast reprogramming (Hiebert et al., 2018). The reprogrammed senescent fibroblasts demonstrated expedited wound healing capabilities by stimulating keratinocyte proliferation (Hiebert et al., 2018). Importantly, these fibroblasts were also given the title of CAFs as they were found to increase tumor growth compared to their wild-type counterparts and possessed a gene expression profile representative of CAFs in other tumors (Hiebert et al., 2018). ECM deposited by NRF2-activated fibroblasts was responsible for CAF activation and CAFs are known, in general, to play important roles in regulating plasticity (Lau and Yuen, 2016; Chen et al., 2014a) and drug resistance (Su et al., 2018) of CSCs (see Fig. 1). Furthermore, some of the ECM-related components deposited by NRF2-activated fibroblasts (*e.g.* Wnt5a) have been shown to confer stem-like traits in other types of squamous cell carcinoma (Qin et al., 2015).

Regulation of fibroblast senescence by NRF2 is controversial as activation of NRF2 has been shown to both delay and promote fibroblast senescence (Hiebert et al., 2018; Kapeta et al., 2010). In addition, NRF2 inhibition and ablation has also been reported to induce premature senescence (Volonte et al., 2013; Jódar et al., 2011). In the case of matrisome-mediated fibroblast senescence, NRF2 was activated yet ROS and DNA damage levels were reduced (Hiebert et al., 2018). This outcome was not anticipated as detoxification of ROS is usually considered protective against DNA damage and subsequent onset of senescence (Hiebert et al., 2018). Fibroblast senescence has also been linked to NRF2 inhibition and increased ROS levels (Hiebert et al., 2018; Villeneuve et al., 2009). These differences in NRF2 regulation of fibroblast senescence may be explained by variations in oxidative stress status (Villeneuve et al., 2009), competition between replicative senescence and cellular senescence processes, heterogeneity in fibroblast type, and differences between *in vitro* and *in vivo* models (Hiebert et al., 2018). The finding that NRF2-activated fibroblasts reprogram other fibroblasts *via* matrisome changes has been validated in a mouse model with NRF2 activated specifically in skin fibroblasts (Hiebert et al., 2018). Therefore, this transgenic mouse model has the benefit of cell-specificity, providing the ability to dissect the influence of NRF2 activation on fibroblasts alone (Hiebert et al., 2018). Furthermore, using an *in vivo* approach is advantageous as it may include other potentially contributing or competing cellular and molecular factors that may not have been accounted for in earlier *in vitro* studies. Overall, further research is warranted to better understand the context-dependency of NRF2 on fibroblast senescence and relevance of NRF2 to cancer development and progression.

6.2. The link between NRF2 and ECM-associated growth factors in cancer stem cell chemoresistance

Activation of NRF2 confers drug resistance to CSCs in SCC *via* upregulation of the glutathione pathway (Oshimori et al., 2015). The mechanism responsible for activating the glutathione pathway involves TGF-β binding to its membrane receptor, TGF-βR2, followed by upregulation of p21 that competes with binding of the proteasomal protein, KEAP1, to NRF2, thereby stabilizing the transcription factor NRF2 (Oshimori et al., 2015). Activated NRF2 in turn switches on the glutathione pathway in the CSCs by initiating the transcription of glutathione metabolism genes (*e.g.* glutathione S-transferase A family genes 1–5 and glutathione peroxidase 2) (Oshimori et al., 2015). The glutathione regulatory pathway is typically employed by normal, healthy cells to inactivate harmful ROS within the cell (Tanaka et al., 2002). This use of the glutathione pathway by TGF-β responsive CSCs is another prime example illustrating the irony of how cancer cells skilfully hijack normally 'protective' mechanisms for their own advantage. In addition, the TGF-β-responsive SCC-CSCs were found to be slower-cycling or quiescent, which also contributes to chemoresistance as cytotoxic chemotherapy induces apoptosis by causing DNA damage in rapidly dividing cells (Oshimori et al., 2015; Brown et al., 2017). Differential response of SCC-CSCs to TGF-β contributes to heterogeneity in CSC properties. The SCC-CSCs that do not respond to TGF-β are more proliferative and enhance tumor expansion and differentiation (Oshimori et al., 2015). In contrast, SCC-CSCs that are responsive to TGF-β are more invasive, slower cycling and more resistant to oxidative stress, conferring greater potential for metastasis as well as longevity to the tumor by protecting against ROS and cytotoxic chemotherapy (Oshimori et al., 2015).

6.3. Hidden circuitry between fibroblasts and cancer stem cells activated by NRF2 and ECM remodeling

Improved understanding of the effect of NRF2 activation on fibroblasts and CSCs in SCC has revealed an apparent circuitry involving immune cells from the perivasculature, ECM proteins (*e.g.* PAI-1) and ECM-associated growth factors (*e.g.* TGF-β). Collectively, this cross-talk between CSCs and stromal cells promotes tumor progression and chemoresistance of SCC-CSCs (see Fig. 1). In SCC, CSCs reside at the interface between the cancer and stroma (Oshimori et al., 2015; Brown et al., 2017). The source of concentrated TGF-β ligands governing CSC heterogeneity is the immune cell residents of the perivasculature contiguous with the cancer-stroma interface (see Fig. 1) (Oshimori et al., 2015). The perivasculature conveniently serves as a potential portal for metastasis of those TGF-β-responsive SCC-CSCs with invasive features (Oshimori et al., 2015). TGF-β is secreted from immune cells, including monocytic myeloid cells (Oshimori et al., 2015), in a latent complex

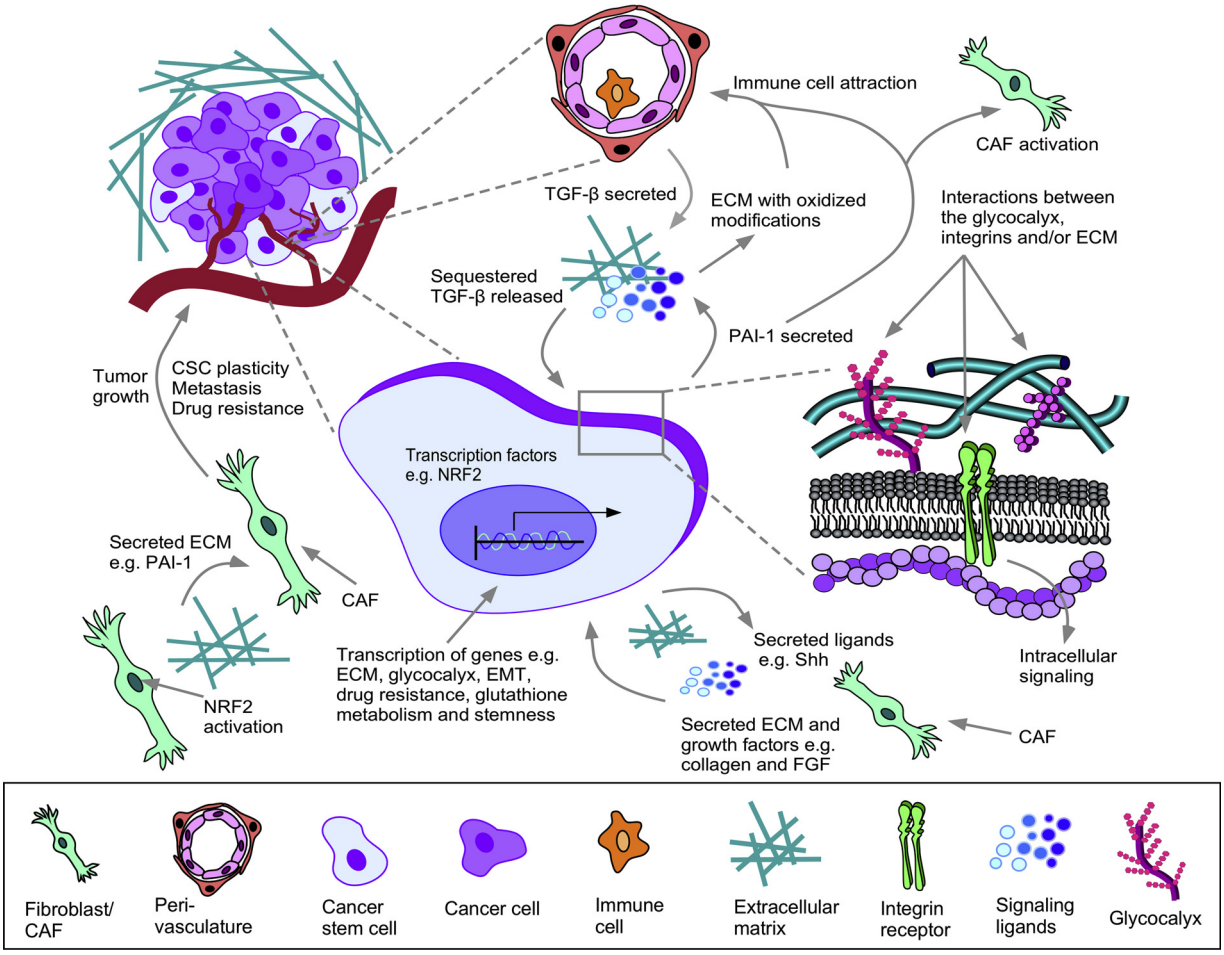

Fig. 1. Interplay between cancer cells and stromal cells in the tumor microenvironment confer cancer stem cell phenotype and chemoresistance.
A schematic of the cancer stem cell niche showing the molecular and cellular circuits partnering cancer cells with stromal cells that lead to the acquisition of cancer stem cell phenotype (CSC), function and chemoresistant properties. Fibroblasts are reprogrammed to a cancer-associated fibroblast (CAF) phenotype by external signals such as extracellular matrix (ECM) secreted from other fibroblasts or secreted ligands from cancer cells. These CAFs in turn can secrete ECM and growth factors which ultimately induce transcriptional changes in CSCs. Dynamic interactions can occur between immune cells and CSCs, whereby immune cells secrete growth factors like TGF-β that confer chemoresistance and stimulate CSCs to secrete ECM that attract immune cells, leading to the development of a feed-forward cycle. Furthermore, oxidized ECM may also stimulate immune cell attraction and contribute to the feed-forward cycle. The glycocalyx and integrin receptors of cancer cells interact with the ECM, co-operating to induce mechanosignaling that generates a stem-like, chemoresistant phenotype.

and sequestered into the ECM (Murphy-Ullrich and Poczatek, 2000). For TGF-β to bind to its receptor and induce cell signaling, it must first be activated and liberated from the ECM. Multiple mechanisms are available to 'activate' or release TGF-β from the ECM, with key mediators including integrins, thrombospondin-1, proteases and ROS (Murphy-Ullrich and Poczatek, 2000). TGF-β itself induces ROS production and this reciprocity leads to tissue fibrosis (Liu and Desai, 2015). Additionally, the ECM composition and mechanical properties of the ECM have been proposed to regulate the availability of TGF-β (Hinz, 2015). Interestingly, oxidative modifications of the ECM have been found to encourage a "second wave" of inflammation through activation of β2-integrins on macrophages that mediate macrophage migration (Yakubenko et al., 2018). This supports another role for ROS besides the release of TGF-β from ECM. The ECM oxidative modifications involve adducts between ECM proteins and products of phospholipid oxidation, such as 2-(ω-carboxyethyl)pyrrole generated from the polyunsaturated fatty acid, docosahexaenoate (Yakubenko et al., 2018). These oxidative modifications arise during the early stages or "first wave" of inflammation because of recruited neutrophils secreting peroxidases that generate ROS (Yakubenko et al., 2018). In addition, immune cell attraction can also be stimulated by specific ECM components themselves (for example, PAI-1) (Hallmann et al., 2015; Honjo et al., 2017). Therefore, oxidative changes to the ECM created by the "first wave" of inflammation, as well as ECM components (*e.g.* PAI-1) released from NRF2-activated fibroblasts, may initiate a feed-forward mechanism whereby ECM changes attract more immune cells, which then release more latent TGF-β (see Fig. 1). In this way, ECM could be viewed as a regulator of TGF-β activity through increasing the availability of TGF-β *via* immune cell attraction. To add further complexity, TGF-β signaling itself can induce downstream PAI-1 expression (Omori et al., 2016; Samarakoon et al., 2009). Therefore TGF-β responsive SCC-CSCs may also contribute to phenotypic conversion of skin fibroblasts into CAFs by secreting PAI-1 (see Fig. 1). Furthermore, TGF-β can activate a CAF phenotype in culture and NRF2-activated fibroblasts show upregulated expression of the TGF-βR2 receptor (Hiebert et al., 2018), implying another potential feedback loop. TGF-β-induced intracellular PAI-1 is responsible for keeping hematopoietic stem cells in their niche (Yahata et al., 2017); whether a similar mechanism operates to retain SCC-CSCs in their niche is unknown. Taken together, complex ECM- and NRF2-meditated circuitries connect CSCs, fibroblasts, CAFs and immune cells. Comprehensive understanding of the dynamic and reciprocal cellular and molecular interactions involved in these circuitries will be required for developing therapies to inhibit tumor growth and overcome drug resistance in those CSCs responsible for tumor relapse in

SCC.

6.4. The link between cancer-associated fibroblast recruitment and chemoresistance

Triple negative breast cancer cells recruit CAFs from the tumor milieu to confer stem-like characteristics and chemoresistance upon themselves (Cazet et al., 2018). A paracrine signaling dialogue has been proposed between TNBC cells and CAFs using single-cell sequencing technology with *in vitro* cellular and *in vivo* mouse models, whereby the release of hedgehog ligands from TNBC cells has been shown to activate CAFs (Cazet et al., 2018). The activated CAFs provide a supportive niche for the induction of a stem-like, chemoresistant phenotype in cancer cells by remodeling the ECM and secreting signaling ligands (see Fig. 1) (Cazet et al., 2018). The breast cancer CSCs were located at the tumor-stroma interface (Cazet et al., 2018), which is similar to that seen in colon cancer (Lenos et al., 2018) and SCC (Oshimori et al., 2015; Brown et al., 2017), thereby highlighting the importance of the interaction between CSCs and components of the stroma for CSC regulation.

Hedgehog-activated CAFs remodel the ECM through increased deposition of fibrillar collagen and potentially by altering other ECM components, as indicated by upregulated gene expression of metalloproteinases (Mmp3, Mmp13, Mmp15, Adamts3, Adamts18) and ECM glycoproteins (Rspo3, Lama5, Edil3, Thbs4) (Cazet et al., 2018). The CAF-mediated increase in collagen density and fiber linearity at the tumor-stroma interface was accompanied by increased epithelial activation of FAK and CSC phenotype, as evidenced by both increased clonogenic capacity and expression of CSC markers (Id3, Itgb3 (CD61), mouse-Krt6 (CK6) and human-ALDH1) (Cazet et al., 2018). In support of these findings, other studies have implicated FAK in the maintenance of breast CSCs and breast cancer development (Kolev et al., 2017; Luo et al., 2009). For example, inhibition of FAK activity in TNBC xenograft mouse models preferentially reduced the CSC load in tumors, inhibited metastasis and delayed relapse following cessation of chemotherapy (Kolev et al., 2017). Mechanistically, FAK inhibition was shown to block activation of the Wnt pathway effector, β-Catenin (Kolev et al., 2017). Therefore, the dependency of TNBC-CSC self-renewal on FAK signaling is explained by cross-talk with the Wnt/β-Catenin pathway (Kolev et al., 2017) – a developmental pathway that regulates normal stem cells and is often deregulated in cancer (Clevers, 2006). Since cytotoxic chemotherapy has an apparent tendency to preferentially eliminate non-CSCs (cancer cells without stem cell markers), whereas FAK inhibitors preferentially target CSCs, the rationale has been given to combine these therapies to target both cancer cell subpopulations (Kolev et al., 2017). The addition of FAK inhibitors to chemotherapy was shown *in vivo* in the TNBC xenograft mouse model to reduce tumor growth and offered a more sustained response, exceeding the effect of chemotherapy alone (Kolev et al., 2017).

Hedgehog activation of CAFs stimulates an increased release of fibroblast growth factor-5 (FGF5) (Cazet et al., 2018). In response, FGF signaling in TNBC cells contributes to both chemoresistance and CSC plasticity, as shown by elevated sphere-forming capacity and expression of CSC markers (*e.g.* Sox10 and Id3) (Cazet et al., 2018). The exact mechanism underlying how FGF5 leads to drug resistance in TNBC-CSCs is unclear. FGF signaling has been implicated in the emergence of chemoresistance in other cancers such as cervical cancer (Song et al., 2017), small cell lung cancer (Pardo et al., 2006) and acute myeloid leukemia (Karajannis et al., 2006), however most of these studies have focused on the actions of fibroblast growth factor-2 (FGF2). Both FGF2 and FGF5 share common FGF receptors (Ornitz and Itoh, 2015), therefore, some of the detailed mechanisms for FGF2 may be relevant to FGF5-mediated drug resistance in TNBC. For example, in oestrogen-positive breast cancer and cervical cancer, FGF2-FGFR signaling mediates drug resistance by driving downregulation of the pro-apoptotic protein, Bim, *via* activation of downstream MEK/ERK signaling (Song et al., 2017; Jang et al., 2017; Shee et al., 2018) In small cell lung cancer, FGF2-FGFR signaling is coupled to MEK/ERK activation and upregulation of the anti-apoptotic proteins, Bcl-X_L and XIAP, by a multiprotein complex comprised of B-Raf, PKCε and S6K2 (Pardo et al., 2006).

Whilst FGF5 signaling and FAK signaling can act independently to confer stemness to TNBC cells, co-operation between the two signaling pathways and details of downstream effectors that lead to upregulation of the transcription factors involved in CSC plasticity are not known (Cazet et al., 2018). However, co-operation between these two signaling pathways may occur as FGF2, FGF receptor-1, FAK signaling along with β3 integrin have been shown to co-operate to promote metastasis in TNBC (Brown et al., 2016). Taken together, although targeting FGF and FAK separately to overcome drug resistance in TNBC is feasible, targeting hedgehog signaling in CAFs has the advantage of inhibiting the effects of both downstream FGF and FAK activation.

6.5. Spatiotemporal regulation of JNK signaling and chemotherapy-induced ECM changes confer chemoresistance

Cytotoxic chemotherapy has been the long-standing cornerstone of cancer medicine, however more research is needed to understand the effect it has on ECM dynamics and the ensuing development of acquired chemoresistance in CSCs. Both inhibition and activation of c-Jun N-terminal kinase (JNK) signaling has been reported to promote chemoresistance in breast cancer cells (Ashenden et al., 2017; Insua-Rodríguez et al., 2018). JNK signaling is known to be highly context-dependent, giving rise to pleiotropic tumor-suppressive and tumor-supportive effects (Insua-Rodríguez et al., 2018). The paradoxical effects of JNK signaling on chemoresistance in breast cancer may be explained by molecular differences between breast cancer subtypes, differences in genetic mutation profile and differences in chemotherapy (Ashenden et al., 2017; Insua-Rodríguez et al., 2018). Activation of the JNK pathway in breast cancer cells induces a gene expression program linked to stem cell properties, wound healing and the ECM. The JNK signature is enriched more in basal-like breast cancer compared to luminal and HER2 subtypes (Insua-Rodríguez et al., 2018). JNK signaling directly upregulates transcription of the ECM proteins, osteopontin and tenascin C, which serve functional roles in breast cancer metastasis and chemoresistance (Insua-Rodríguez et al., 2018). Paclitaxel treatment has also been shown to increase JNK signaling activity in breast cancer cells, thereby contributing to stem cell properties and chemoresistance in both primary and metastatic tumors (Insua-Rodríguez et al., 2018). This link between JNK signature and diverse therapy resistance signatures in breast cancer has been supported by gene set enrichment analysis data as well as overall patient survival data (Insua-Rodríguez et al., 2018). The notion that chemotherapy can induce ECM changes leading to drug resistance has been observed across other cancers, such as ovarian cancer (Ricciardelli et al., 2013) and multiple myeloma (Bandari et al., 2018). For example, carboplatin cytotoxic chemotherapy was demonstrated *in vitro*, with the support of patient clinical data, to stimulate deposition of the ECM protein, hyaluranon, which binds to its receptor, CD44, on ovarian cancer cells and causes chemoresistance by upregulating ABC drug transporters that pump chemotherapy out of cancer cells (Ricciardelli et al., 2013). In another example, anti-myeloma chemotherapy stimulates enhanced secretion of chemoexosomes with high cargo of heparanase from myeloma cells (Bandari et al., 2018). This heparanase remodels the ECM by degrading heparan sulfate, increases shedding of syndecan-1 proteoglycan from the surface of myeloma cells and stimulates ERK signaling in myeloma cells (Bandari et al., 2018). In addition, the chemoexosomes stimulate enhanced secretion of TNF-α and cytokines from macrophages (Bandari et al., 2018). Together these chemotherapy-induced changes are suggested to likely facilitate chemoresistance (Bandari et al., 2018).

JNK signaling is differentially activated amongst cancer cells within primary and metastatic breast tumors (Insua-Rodríguez et al., 2018).

JNK activity and associated stem cell properties were most pronounced during early stages of metastatic colonization and reduced with metastatic tumor expansion (Insua-Rodríguez et al., 2018). Whilst this spatiotemporal variation in JNK signaling and associated stem cell properties is supported by other studies showing enrichment of CSCs in early metastatic lesions (Lawson et al., 2015), it does suggest that fundamentally different processes are operating across the stages of metastatic tumor progression. Therefore, it is interesting to speculate that metastatic tumor growth at some point may become driven by cancer cells at the tumor periphery that acquire clonogenic properties because of microenvironmental cues, as demonstrated in colon cancer (Lenos et al., 2018). However, the point in which JNK signaling quietens and switches in favour of other processes driving growth is unclear and requires further investigation. These ideas challenge us to delve deeper into the cellular and molecular mechanisms underpinning CSC properties and to perhaps redefine surrogate markers of CSC activity.

6.6. The extracellular matrix and pericellular matrix join 'forces' to confer stemness and chemoresistance

Neural stem cells residing within the subventricular zone have been pinpointed as the cells of origin of human glioblastoma (Lee et al., 2018). These neural stem cells carry low-level glioblastoma driver mutations and accumulate further mutations as they migrate away from the subventricular zone during disease progression (Lee et al., 2018). The ECM protein, tenascin C, is highly expressed in the subventricular zone during development and regulates neural stem cell self-renewal, differentiation, as well as growth factor signaling (Garcion et al., 2004). Tenascin C is also upregulated in glioblastoma multiforme, which increases the stiffness of the brain tumor (Miroshnikova et al., 2016). This increase in tissue stiffness has been proposed to drive chemoresistance and relapse in glioblastoma by expanding the CSC subpopulation or inducing EMT to generate a stem-like phenotype (Barnes et al., 2018).

The pericellular matrix of cells, known as the glycocalyx, and the extracellular matrix are both comprised of glycoproteins and proteoglycans that function as fundamental structural components as well as regulators of cell behavior and response (Hynes, 2009; Sabri et al., 2000). The glycocalyx is comprised of an assortment of glycoconjugates (glycoproteins, proteoglycans and glycolipids) studded into the plasma membrane that project outwards forming a layer enveloping the cell exterior (Roseman, 2001). Glycoconjugates are susceptible to enzymatic modifications (glycosylations) which can alter their chemical structure, spatial occupancy and resultant biophysicochemical properties (Dall'Olio et al., 2012; Shental-Bechor and Levy, 2008; Daniotti et al., 2013). Much like the ECM, the composition of the glycocalyx is dynamic and changes in its expression signature influence stem cell fate and behaviors during development, wound repair and cancer (Dall'Olio et al., 2012; Rouhanifard et al., 2018; Huang et al., 2014; Lanctot et al., 2007). We are now beginning to understand mechanistically how interactions between the glycocalyx, extracellular matrix and integrins converge to cause changes in intracellular signaling and gene transcription that ultimately affect cancer phenotype, survival, metastasis and treatment response (see Fig. 1) (Barnes et al., 2018; Woods et al., 2017; Paszek et al., 2014).

Glioblastoma multiforme, the most aggressive form of glioblastoma, is associated with a mesenchymal, stem-like, treatment-resistant phenotype (Barnes et al., 2018). This phenotype is promoted by a tension-driven circuitry whereby ECM stiffness and steric burden of the bulky glycocalyx enhance integrin-dependent/FAK-mechanosignaling - in turn, transcription of ECM (*e.g.* tenascin C), mesenchymal (*e.g.* vimentin, MET, Twist1 and WNT5a) and glycocalyx-related genes are upregulated (Barnes et al., 2018). The bulky glycocalyx is generated by increased expression of constituent glycoproteins (*e.g.* hyaluronan and CD44), which serves to regulate integrin clustering, focal adhesion assembly and integrin-dependent growth factor signaling involved in cell survival and metastasis (Paszek et al., 2014). The tension-driven circuitry is further potentiated by increased expressed of lectins (*e.g.* galectin-1) that bind to glycoconjugates of both the glycocalyx and ECM and regulate cell-ECM interactions (Barnes et al., 2018; Liu and Rabinovich, 2005). Consequently, targeting the stiff ECM and bulky glycocalyx has been suggested as a therapeutic strategy for overcoming metastasis and chemoresistance in glioblastoma and other cancers (Barnes et al., 2018; Woods et al., 2017; Paszek et al., 2014).

In our review, a common theme among the mechanisms conferring stem cell properties and chemoresistance is the pleotropic effects or context-dependency of many of the mediators. For example, JNK signaling is heterogeneous in its distribution within a tumor and demonstrates divergent tumor suppressor/promotor effects depending on the context (Ashenden et al., 2017; Insua-Rodríguez et al., 2018), which is analogous to TGF-β and NRF2 signaling in SCC (Hiebert et al., 2018; Oshimori et al., 2015). These pleiotropic or paradoxical activities cause obvious problems for discerning precisely when and how to selectively target these mediators therapeutically. Whilst a comprehensive understanding of the regulatory mechanisms offers hope for harnessing the potential of such key mediators of stemness and chemoresistance; the ideal scenario is to identify a mediator or mechanism essential for cancer survival that explicitly eliminates or neutralizes all cancer cells without adverse effect on normal, healthy cells.

7. Cancer stem cell-targeting drugs in clinical trials

Given the role of CSCs in tumor progression, targeting these cells has been pursued as a promising key to treating cancer (Garber, 2018a; Saygin et al., 2019; Desai et al., 2019). Strategies for therapeutically targeting CSCs and their progress in clinical trials have recently been reviewed (Saygin et al., 2019; Desai et al., 2019). The CSC-targeting strategies currently under evaluation in clinical trials can be classified according to mechanism and include targeting developmental signaling pathways (*e.g.* hedgehog, notch and Wnt inhibitors), growth factor signaling (*e.g.* TGF-β inhibitors), CSC surface markers (*e.g.* anti-EpCAM, anti-CD123, anti-CD47), CSC niche (*e.g.* CXCR4 inhibitors, FAK inhibitors), CSC metabolism (*e.g.* Bcl2 inhibitors), drug efflux pumps (multi-drug resistance inhibitors) and transcription factors (*e.g.* NANOG inhibitors) (Saygin et al., 2019). A caveat of current anti-CSC strategies highlighted by Saygin et al. is that many target stemness-associated factors that share commonality with normal tissue resident stem cells, thereby raising concerns regarding size of therapeutic window (Saygin et al., 2019). To address this concern, identification of CSC-specific targets, optimized dosing relative to biological function and rationalized combinatorial therapies have been suggested as ameliorative strategies (Saygin et al., 2019). Furthermore, failure of CSC-targeting drugs in clinical trials may be partly because CSC heterogeneity, plasticity and functional behavior of CSCs, which are modulated by the tumor microenvironment, have not been adequately addressed. Targeting components of the tumor microenvironment that regulate CSC fate with stroma-directed therapies has the potential for broad use across CSC populations as tumors of different origin or genotype share common niche components (Saygin et al., 2019). An additional advantage of targeting stromal cells is that these cells are considered genetically stable and therefore may avoid some of the challenges of targeting CSCs themselves such as evolving mutational landscape (Saygin et al., 2019; Quail and Joyce, 2013) and plasticity between non-CSC and CSC states that contribute to the development of chemoresistance (Saygin et al., 2019).

8. Potential treatment strategies for targeting cancer stem cell function and chemoresistance

Although the goal is to cure cancer, until curative treatments are discovered, the next priority is to identify and target cancer cells functioning as CSCs, and to overcome or curb drug resistance - for which, new drug targets are needed. The ideal scenario is to develop

therapies with the ability to eradicate all cancer cells including chemoresistant 'CSCs', thereby nullifying the need to control tumor expansion. However, the research on colon cancer, discussed earlier in our review, suggests that even if resistant cancer cells remain after initial treatment, relapse or further tumor expansion may be suppressed providing interactions between cancer cells at the tumor edge and specific microenvironmental components can be disrupted. Therefore, a possible new treatment strategy would be to combine therapies that overcome drug resistance and metastasis with 'safety net' therapies targeting the cancer cell-microenvironment interactions responsible for tumor regrowth.

The traditional approach to treat metastatic cancer is to administer standard of care treatment(s) in repetitive cycles, at maximum-tolerated doses, following initial favourable response and to continue the same regimen unless the tumor progresses (Staňková et al., 2018). This approach, designed for maximal cancer cell killing, can unintentionally expedite the development of resistance mechanisms (Staňková et al., 2018). In opposition to the fixed approach, new theory based on mathematical modeling argues for adaptive therapeutic regimens, which change along with the evolving tumor responses that typically lead to resistance – promoting a proactive approach, rather than a reactive approach once treatment options have failed (Staňková et al., 2018; Gallaher et al., 2018). However, this approach will require ways of measuring the proportion of resistant cancer cells and the dynamic molecular mechanisms of resistance, which are still being deciphered (Staňková et al., 2018). The mathematical model given is based on 'game theory' whereby the clinician and cancer are the two 'players' (Staňková et al., 2018). The evolutionary game theory model has also been used to study interactions between different cell types and has been adapted to include multiple 'players' to account for tumor heterogeneity and tumor-stroma interactions (Archetti, 2013; Basanta et al., 2011). Given cancer is unable to predict changes in treatment, it cannot undergo evolutionary resistance changes until new therapies are initiated (Staňková et al., 2018). In this way, the clinician maintains the advantageous position of leader, steering and interceding predicted evolutionary changes by continuously modifying treatment and monitoring evolutionary dynamics of the cancer (Staňková et al., 2018). Conversely, the traditional fixed treatment approach allows the cancer to assume position of leader, developing resistance and progression that becomes difficult for the clinician to reclaim control (Staňková et al., 2018). Furthermore, the success of this traditional treatment approach in metastatic cancer is argued to be based on the unlikely scenario of tumor homogeneity and slow adaptive responses (Staňková et al., 2018).

Given the complexity of tumor heterogeneity and the dynamic microenvironment, mathematical and computational modeling has been proposed to bridge the limitations of *in vitro* and *in vivo* studies (*e.g.* cost, time, variables) to simulate the cellular and molecular mechanisms of drug resistance and to evaluate drug combinations (Sun et al., 2016). To illustrate the value of integrating mathematical models with biological models, this approach has been used to investigate how signaling variation amongst cancer cells within a tumor affects response to targeted therapies, and how this signaling heterogeneity itself is modulated by both spatial competition between cell subpopulations (Gallaher et al., 2018) and tumor microenvironment heterogeneity (Sun et al., 2016; Kim et al., 2018). The ability to understand and predict how signaling heterogeneity within tumors influences therapeutic response is important as targeted therapies are often plagued by the problem of pathway rewiring, whereby inhibition or bypass of one aberrant signaling pathway causes activation of alternative pro-survival pathways leading to treatment failure. This redundancy arises because oncogenic signaling proteins are not confined to isolated pathways – instead, they co-operate in protein complexes that are embedded within a larger web of integrated and complex signaling networks (Kim et al., 2018). As a proof-of-concept, Kim et al. pharmacologically manipulated the Ras/MAPK and PI3K/Akt signaling pathways and used a combination of mathematical models with *in vitro* lung cancer model to study, predict and validate spatiotemporal signaling and phenotypic responses of cancer cells (Kim et al., 2018). This study highlighted the need to further dissect and understand interactions between intratumoral heterogeneity (genetic and cell signaling) with microenvironmental heterogeneity to inform possible drug resistance mechanisms and choice of combination treatment strategies (Kim et al., 2018). In conjunction with modeling, single-cell profiling technology has been recognized as a powerful tool to characterize CSC heterogeneity for understanding dynamics of CSC driven tumor growth and treatment responses (Sehl and Wicha, 2018; Kim et al., 2018).

In another example, Sun et al. employed mathematical modeling to study targeted therapy-induced chemoresistance and metastasis in heterogeneous tumors comprising drug-sensitive and drug-resistant cells. This modeling also accounted for microenvironmental influence such as secretion of resistance-inducing growth factors (*e.g.* IGF and HGF) by drug-sensitive cancer cells and was validated using clinical patient survival and circulating tumor cell DNA data (Sun et al., 2016). Combination therapies are often used as a strategy to overcome chemoresistance to targeted therapies and, therefore, Sun et al. generated mathematical models to evaluate efficacy and cellular kinetics of various combinations of BRAF, MEK and PI3K inhibitors in melanoma with BRAF mutations (Sun et al., 2016). This model could predict distinct dose-dependent synergistic effects between dual BRAF and MEK inhibitor treatment compared to dual BRAF and PI3K inhibitor treatment, and suggested that optimized dosages of combination therapies may reduce chemoresistance (Sun et al., 2016). Taken together, the use of molecular techniques to identify new drug targets, mathematical modeling of tumor responses, and personalized treatment strategies (Staňková et al., 2018) offers promise for undermining the tumor evolution responsible for drug resistance and treatment failure.

9. Emerging technologies to target cancer stem cell chemoresistance and cellular programs

Proteolysis-targeting chimeras (PROTACs) and RNA-based therapeutics are two emerging technologies that have the potential to revolutionize cancer medicine as they have the capacity to target currently undruggable proteins or gene products, respectively. The 'undruggable' proteome, alternatively described as 'difficult to drug' or 'yet to be drugged', represents all the proteins that are currently known to play a role in disease but lack an associated pharmacological therapy (Dang et al., 2017). This portfolio of proteins is destined to expand as more disease-associated proteins are revealed. PROTACs and RNA-based therapeutics, at the protein or mRNA level, respectively, can degrade enzymes, kinases, steroid receptors, transcription factors, membrane-bound scaffolding proteins, and transmembrane proteins (Bisanz et al., 2005; Cromm et al., 2018; Burslem et al., 2018; Lieberman, 2018). Therefore, both PROTACs and RNA-based therapeutics are promising technologies as they have scope to target the range of mediators that determine CSC function and chemoresistance, including mediators that are difficult to target with traditional classes of therapy (Dang et al., 2017; Coleman and Crews, 2018; Neklesa et al., 2017; Chen et al., 2014b). For example, PROTAC-3 selectively degrades FAK, blocking its kinase-dependent signaling, as well as its scaffolding function that mediates kinase-independent signaling through signaling complexes at the plasma membrane (Cromm et al., 2018). The scaffolding function of FAK is neglected by traditional small molecule FAK inhibitors as they specifically target the central kinase domain of FAK (Cromm et al., 2018). Defactinib is a traditional small molecule FAK kinase inhibitor under clinical investigation, however it failed its initial clinical trial for targeting pleural mesothelioma stem cells (Cromm et al., 2018). PROTAC-3 demonstrated superior performance to defactinib in inhibiting FAK signaling and FAK-mediated invasion and migration of TNBC cells (Cromm et al., 2018). Addressing the scaffolding function of FAK is important as it has a demonstrated role in

CSC regulation as well as cancer hallmarks such as invasion and migration (Fan et al., 2013). For example, a MMTV-PyMT mouse model of human breast cancer with a knock-in mutation in the FAK gene at a site related to its scaffolding function (P878 A/P881 A mutation) was used to confirm that FAK scaffolding activity mediates EMT and stemness of mammary CSCs *in vivo* (Fan et al., 2013). In these mutant mice, disruption of FAK scaffolding-mediated phosphorylation of endophilin A2 resulted in reduced cell surface expression of membrane-type 1 matrix metalloproteinase – an ECM-degrading enzyme that facilitates cell invasion and migration (Fan et al., 2013; Wu et al., 2005). This reduced metalloproteinase expression lead to increased mammary cancer cell expression of E-cadherin, as well as reduced mammary CSC self-renewal as demonstrated by tumor sphere formation assays *in vitro* and limiting dilution cell transplantation assays *in vivo* (Fan et al., 2013). Overall, disrupting FAK scaffolding activity was found to inhibit both metastasis and mammary tumor growth (Fan et al., 2013), thereby supporting the benefit of PROTAC-mediated FAK degradation with its ability to target FAK scaffolding function unlike traditional small molecule FAK kinase inhibitors. However, although FAK deletion in mammary epithelial cells has been shown to suppress mammary tumorigenesis by reducing the pool of mammary cancer stem/progenitor cells and impairing their self-renewal (Luo et al., 2009), FAK deletion has also been proposed to deplete the pool of normal mammary stem cells (Fan et al., 2013). Therefore, despite the benefit of complete degradation of mediators of CSC regulation by PROTACs and RNA-based therapies, choosing targets with selectivity towards CSCs and minimal effect on normal stem cell activity will optimize the outcome of these promising emerging platforms.

The PROTAC technology capitalizes on the ubiquitin-proteosome system that is present within mammalian cells and is the protein disposal system used by the cell to degrade proteins and control protein homeostasis (Coleman and Crews, 2018). In a multiple-step process, a target protein is flagged for degradation by the proteasome following the sequential addition of a chain of ubiquitin molecules by an E3 ubiquitin ligase complex (Coleman and Crews, 2018). PROTACS induce the degradation of specific target proteins by facilitating interaction between the protein of interest and the ubiquitin-proteosome system (Coleman and Crews, 2018). In this respect, PROTACS act as molecular adaptor units - one end of the molecule has a unique ligand tailored to the target protein and the other end, a ligand suitable for recruiting a ubiquitin E3 ligase (Coleman and Crews, 2018). RNA-based therapeutics include small-interfering RNAs (siRNAs), microRNAs (miRNAs), short-hairpin RNAs (shRNAs), antisense oligonucleotides (ASOs), RNA aptamers and catalytic RNAs (Ribozymes) (Dowdy, 2017; Burnett and Rossi, 2012). In general, these RNA-based therapeutics act by disrupting RNA processing or by binding target sequences of mRNA to either repress translation or cause cleavage and degradation of the target mRNA (Burnett and Rossi, 2012; Deng et al., 2014; Chen et al., 2018). This leads to either reduced production of the corresponding target protein or complete prevention of gene expression, referred to as 'gene silencing' (Burnett and Rossi, 2012; Deng et al., 2014; Chen et al., 2018). RNA-based therapeutics also encompass single guide RNAs (sgRNAs) used for CRISPR-Cas. However, unlike other RNA-based therapeutics which act at the RNA level, CRISPR-Cas involves sgRNAs binding to target sequences on chromosomal DNA for genomic DNA editing, such as adding or deleting genes or correcting mutations (Lieberman, 2018; Kaczmarek et al., 2017). Since CRISPR-Cas modifies the genome, this raises major ethical concerns (Lieberman, 2018) and provides an argument against their application for clinical use.

The advantages and limitations of PROTACs and RNA-based therapeutics have been the focus of recent reviews (Lieberman, 2018; Chen et al., 2018; Kaczmarek et al., 2017; Lai and Crews, 2016; Churcher, 2018; Gu et al., 2018). In brief, both PROTACs and RNA-based therapeutics have advantages over traditional small molecular inhibitors. For example, PROTACs and RNA-based therapeutics have catalytic action, whereas traditional small molecular inhibitors competitively bind to receptors and therefore require sustained systemic exposure and relatively high drug concentrations to block receptors, increasing risk of systemic toxicity. In addition, PROTACs and RNA-based therapeutics offer versatility to target proteins that have not been possible with traditional small molecular inhibitors, such as proteins with scaffolding functions (Coleman and Crews, 2018; Neklesa et al., 2017). Furthermore, PROTACs and RNA-based therapeutics can overcome the problem of drug resistance from acquired mutations in receptors – such mutations may negate the efficacy of traditional small molecular inhibitors by preventing them from binding to their target receptor, or alternatively, the mutations may cause conformational changes at the target binding site that switch inhibitor activity from antagonist to agonist (Burslem et al., 2018; Salami et al., 2018). For example, under selective pressure from smoothened inhibitors such as sonidegib and vismodegib, the smoothened receptor is known to undergo mutations in the drug binding pocket that confer chemoresistance (Sharpe et al., 2015; Danial et al., 2016; Pricl et al., 2015). As a way forward, PROTACs and RNA-based therapeutics can degrade the ligand or the receptor despite the presence of the receptor mutation (Neklesa et al., 2017; Salami et al., 2018). A limitation of PROTACs and RNA-based therapeutics is bioavailability or drug delivery challenges (Neklesa et al., 2017). Advancements in drug delivery and nanotechnology, such as liposome formulations and nanoparticles, are now addressing some of these shortfalls in administration and bioavailability (Chen et al., 2018). For example, the first RNA interference drug to be approved for therapeutic use by the US Food and Drug Administration is a double-stranded siRNA oligonucleotide that is encapsulated in a lipid nanoparticle (Garber, 2018b). In addition, ARV-110, a PROTAC designed to degrade the androgen receptor, has demonstrated efficacy for the treatment of enzalutamide-resistant prostate cancer in preclinical studies (Neklesa et al., 2018) and is now on the cusp of transitioning into clinical trials as an orally bioavailable formulation for metastatic castration-resistant prostate cancer.

Furthermore, other new ways to target previously pharmacologically intractable targets have recently been reported. For example, thalidomide analogs have been found to target a wider range of zinc finger transcription factors than previously known and proof-of-concept studies have opened the possibility of using zinc finger library screens for designing chemically modified thalidomide analogs to selectively degrade specific zinc finger transcription factors (Sievers et al., 2018). In addition, the 'difficult to drug' oncogenic pathway of RAS/RAF/MEK/ERK has been targeted with a SHP2 phosphatase inhibitor as a new strategy, revealing an unrecognized dependence of certain mutant BRAF, NF1 and KRAS proteins on upstream SHP2 activity (Nichols et al., 2018). SHP2 is a scaffold protein that promotes RAS/MAPK signaling by co-ordinating the early steps at the cell membrane leading to RTK activation (Nichols et al., 2018). In effect, the SHP2 phosphatase inhibitor decouples RTK activation from downstream RAS/MAPK pathway activation, serving to decrease cancer growth as demonstrated *in vitro* in cell lines of multiple cancer types and *in vivo* in xenograft mouse models (tumor inhibition and tumor regression) (Nichols et al., 2018). As technologies evolve, this brings hope for targeting previously pharmacologically intractable proteins that lead to cancer development and drug resistance.

10. Future prospects

Cancer cells with the ability to initiate tumors *in vivo* that can be maintained for multiple passages have been granted the title of 'cancer stem cells' and have been distinguished from other cancer cells by their differential expression of surface antigens (CSC markers). These so-called 'CSCs' also exhibit inherent resistance against chemotherapies as demonstrated by their enrichment following cessation of treatments. In addition, recent evidence of cancer cells toward the tumor periphery behaving as CSCs despite not necessarily expressing the typical CSC markers suggests that potentially two fundamentally different processes

are operating – one driving the initial stages of tumor development and the other driving tumor expansion. This raises the following questions: can we decouple these two processes and therapeutically target them individually or together? At what point does the microenvironment driven regulation of clonogenicity and tumor expansion at the tumor-stroma interface take precedence over the mechanisms governing initial tumor establishment by CSC marker-positive cells? Do CSC marker-positive cells also rely upon microenvironment interactions along with cell-autonomous processes for their function in tumor-initiation? After all, xenotransplantation assays involve subcutaneous injection or surgery to transplant the cells which conceivably damages ECM in the process. We have seen in the case of metastasis, evidence of CSCs requiring niche-modifications prior to arrival and during early stages of metastasis for successful colonization (Erler et al., 2009; Lambert et al., 2017). It could then be argued that CSC-microenvironment interactions are also as important for the perpetuation of primary tumors as they appear to be for the expansion of established tumors. As our understanding of the mechanisms controlling CSC fate, maintenance, metastasis and resistance continues to evolve, this will inevitably provide more opportunities for designing therapeutic agents to undermine the influence of key proteins responsible for cancer progression and survival. Together, this knowledge from interdisciplinary efforts, along with advancements in technologies, brings promise for finding a cure for the disease that has defied researchers for centuries.

Disclosure statement

The authors have nothing to disclose.

Author contributions

Y.B. drafted the manuscript and prepared the figure. P.S.T. and S.H. critically reviewed the manuscript for important intellectual content. P.S.T. supervised the study, provided financial support, editing and final approval of the manuscript.

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