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Cancer Stem Cells in Squamous Cell Carcinoma

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Cancer stem cells (CSCs) are found in many cancer types, including squamous cell carcinoma (SCC). CSCs initiate cancer formation and are linked to metastasis and resistance to therapies. Studies have revealed that several distinct CSC populations coexist in SCC and that tumor initiation and metastatic potential of these populations can be uncoupled. Therefore, it is critical to understand CSC biology to develop novel CSC-targeted therapies for patients with SCC with poor prognoses. This review compares the properties of CSCs in SCC with normal stem cells in the skin, summarizes current advances and characteristics of CSCs, and considers the challenges for CSC-targeted treatment of SCC.

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INTRODUCTION

Nonmelanoma skin cancers, including basal cell carcinoma and squamous cell carcinoma (SCC), are the most common skin cancer types and have increased dramatically worldwide in recent years (Moore et al., 2015; Narayanan et al., 2010). SCC can metastasize to ectopic sites (Klein, 2013), and advanced SCCs have high mortality rates and are often refractory to conventional therapy (Geissler, 2015). SCCs contain subpopulations of cells with cancer stem cell (CSC) properties that are linked to SCC initiation, metastasis, and resistance to chemo- and radiotherapy (Biddle et al., 2011; da Silva-Diz et al., 2016; Oshimori et al., 2015; Schober and Fuchs, 2011; White et al., 2013; Zhang et al., 2010). Therefore, characterizing SCC CSCs will provide new insights into SCC treatment. This review covers similarities and differences between SCC CSCs and normal stem cells (SCs) in the skin and discusses therapeutic strategies to target CSCs.

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Abbreviations: ABC, ATP binding cassette; ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; K15, keratin 15; SC, stem cell; SCC, squamous cell carcinoma; SP, side population

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SCs VERSUS SCC CSCs IN THE SKIN

SCs are responsible for regenerating and maintaining tissues and have unique defining characteristics (Figure 1). First, normal SCs are capable of self-renewal. Each SC typically undergoes asymmetrical cell division to produce two daughter cells: one SC and one differentiating cell. Second, normal SCs are usually slow cycling with low proliferation rates, retaining tritium thymidine or BrdU labeling for long periods of time (also known as label retaining cells), yet maintain the capacity for clonogenic growth (Bickenbach, 1981; Morris and Potten, 1994). Third, they are rare in most tissues. Fourth, they are undifferentiated but can give rise to one or more cell lineages (multipotency or pluripotency). Fifth, normal SCs have a much longer lifespan than their progeny. Finally, normal SCs often have specific locations determined by their microenvironment (niche).

Epidermal SCs are located in the bulge of hair follicles, the basal layer of the interfollicular epidermis, and the base of the sebaceous gland (Levy et al., 2005). Hair germ cells, thought to arise from bulge cells, also contain BrdU label retaining cells (Ito et al., 2004). Although distinctive, the pattern of gene expression in hair germ cells is more similar to bulge cells than to transiently amplifying follicular matrix cells (Greco et al., 2009). Studies suggest that bulge cells and possibly hair germ cells contain multipotent follicular SCs that normally generate hair follicles, but can also regenerate the epidermis and sebaceous glands in response to skin injury (Ito et al., 2005; Jaks et al., 2008; Levy et al., 2005, 2007; Morris et al., 2004). Under normal conditions, SCs in the interfollicular epidermis and sebaceous glands are lineage specific, and generate their respective tissues without recruitment of SCs from the bulge (Claudinot et al., 2005; Clayton et al., 2007; Horsley et al., 2006; Ito et al., 2005; Levy et al., 2005; Morris et al., 2004).

CSCs are certain tumor cells exhibiting stem cell-like properties. Whereas normal SCs have several distinct characteristics as described above, CSCs are primarily defined by one criterion: the ability to initiate tumors, and the term CSC is often used interchangeably with "tumor-initiating cell." CSCs can be derived from SCs (Morris et al., 1986) or from nonstem cells that acquire the capacity to self-renew (Jamieson et al., 2004). Unlike normal SCs, CSCs may not be multipotent, leading to single lineage tumors, such as SCC (epidermal lineage), various follicular tumor types (hair follicle lineage), or sebaceous gland tumors (sebaceous lineage). Furthermore, CSCs may not be quiescent. For example, normal slow cycling bulge SCs can acquire genetic mutations, such as Kras mutations or Smad4 deletions, that drive them into hyperproliferation (White et al., 2013). Finally, the number of CSCs varies widely, ranging from $\leq$1% to approximately 20% in SCCs, depending on tumor types and

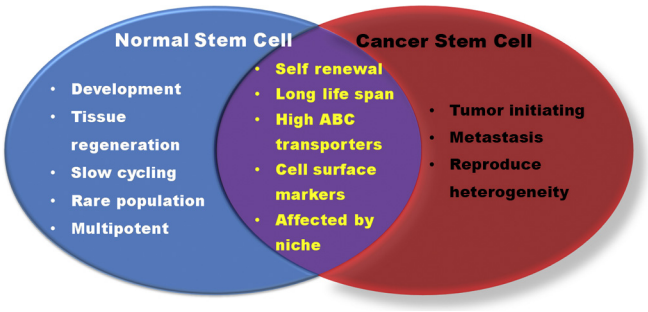

Figure 1. Venn diagram showing stem cell and cancer stem cell characteristics. ABC, ATP binding cassette.

experimental models used to assess tumor initiation, such as the severity of immune suppression of recipient mice in xenografts (Quintana et al., 2008; Song et al., 2010; White et al., 2013). For example, in our SCC mouse model, CSCs were rare in primary SCCs, but their numbers dramatically increased in metastatic SCCs and SCCs with epithelial to mesenchymal transition (White et al., 2013).

SCs and CSCs also share characteristics, such as the capacity for self-renewal, high levels of ATP binding cassette (ABC) transporters, certain cell surface markers, and being influenced by their niche. Also, CSCs share certain regulators with normal SCs. For example, SCC CSCs express factors that regulate self-renewal in embryonic SCs, such as SOX2, MYC, and OCT-4 (Bose and Shenoy, 2014; Boumahdi et al., 2014; Lim et al., 2014). Similarly, some common "stemness" pathways are activated in follicular SCs and CSCs, such as Wnt signaling (Malanchi et al., 2008; White et al., 2013).

SORTING CSCs

CSCs can be sorted by putative cell surface markers that can be either shared with or distinct from normal SCs depending on tumor type. CD34, a cell surface marker for mouse bulge SCs (Trempus et al., 2003), also serves as a CSC marker in mouse SCCs (Trempus et al., 2007) but is not expressed in human bulge SCs (Ohyama et al., 2006). CD200, a cell surface marker expressed in both mouse and human bulge SCs (Ohyama et al., 2006), is also enriched in metastatic SCC (Stumpfova et al., 2010). CD49f is a surface marker of quiescent label retaining cells, including bulge and interfollicular SCs (Blanpain et al., 2004; Jiang et al., 2010; Terunuma et al., 2003), and also serves as an SCC CSC marker (Schober and Fuchs, 2011; White et al., 2013). CD44 is high in SCC CSCs (Lapouge et al., 2012; Malanchi et al., 2008; Prince et al., 2007). CD133, a cell surface marker specific for hematopoietic SCs (Yin et al., 1997), was the first cell surface marker used to define tumor-initiating cells in human cutaneous SCC (Patel et al., 2012). In addition to cell surface markers, CSCs in SCCs can be sorted based on their aldehyde dehydrogenase (ALDH) and ABC transporter activity (Clay et al., 2010; Yang et al., 2014). The side population (SP) assay identifies stem-like cells based on their ability to pump out Hoechst dye and chemotherapeutic drugs via ABC transporters (Goodell et al., 1996; Zhang et al., 2009), and has been used as a marker for both normal skin SCs and SCC CSCs (Larderet et al., 2006; Song et al., 2010; Tabor et al., 2011; Wan et al., 2010; Zhang et al., 2009). SP cells are distinct from CD4f+ keratinocyte stem cells (Terunuma et al., 2003) and SCC CSCs (White et al., 2013). As discussed below, CSC markers can be altered by CSC plasticity and interactions with their niche. For instance, $CD44^+$ SCC CSCs express other CSC markers with a high degree of variability (Krishnamurthy et al., 2010).

DETERMINANTS OF SKIN SC AND CSC BEHAVIOR

Genetic and epigenetic modification

Within the same SC compartment, different genetic mutations have distinct effects on CSC behavior. For example, a $Kras^{G12D}$ mutation in keratin 15 $(K15)^+$ bulge SCs initiates benign papillomas in genetically engineered mouse models, but requires the loss of an additional tumor suppressor to induce SCC (Lapouge et al., 2011; Nassar et al., 2015; White et al., 2013). When combined with radiation, a heterozygous *Ptch* deletion is sufficient to induce basal cell carcinoma in $K15^+$ cells, and is exacerbated by the additional loss of *p53* (Wang et al., 2011). We found that a $Kras^{G12D}$ mutation in combination with *Smad4* deletion not only caused metastatic SCCs from $K15^+$ cells, but also produced tumors of other lineages, such as basal cell carcinomas, trichoepitheliomas, and sebaceous adenomas (White et al., 2013). However, because the K15 promoter could create leaky Cre expression, targeted mutations may not be limited to bulge stem cells. Nevertheless, not all genetic mutations caused multilineage tumor types despite being driven by the same K15 promoter, suggesting that specific stem cell mutations play an important role in determining tumor lineages. To validate if bulge stem cells are the source of tumor-initiating cells, additional bulge stem cell markers described in the Sorting CSCs section are used in studies summarized in Table 1.

Epigenetic regulation, including DNA methylation, histone acetylation, and miRNA expression, also plays an important role in skin SC and CSC behaviors. For example, enhancer of zeste homolog 2 is a major epigenetic component of polycomb repressive complex 2 and is required for epidermal CSC survival, migration, invasion, and tumor formation (Adhikary et al., 2015; Banerjee et al., 2011). miRNAs can also maintain SC populations. For example, miR-205 enhances phosphoinositide 3-kinase (PI3K) signaling and is required for the expansion of neonatal skin SCs (Wang et al., 2013). miR-203, the most abundant miRNA in normal skin, is downregulated by the Hras oncogene, and silencing of miR-203 is an early event in mouse and human SCC (Riemondy et al., 2015). miR-203 limits cell division in both early embryonic skin development and SCC CSCs, and its loss caused an expansion of CSCs, resulting in increased tumorigenesis in an experimental skin carcinogenesis model (Riemondy et al., 2015). Furthermore, we found that miR-9 overexpression contributes to the expansion of metastasis-associated CSCs by inhibiting α-catenin and subsequent Wnt activation (White et al., 2013).

Location and microenvironment

As discussed above, mutations in hair follicle bulge SCs potentially cause tumor formation representing lineages of the epidermis, hair follicles, and sebaceous glands, whereas lineage-committed mutant SCs only generate tumor types from that lineage. For instance, mutant interfollicular SCs

Table 1. Different tumor types develop in mouse models with genetic alteration in K15$^+$ cells

Genetic alteration in K15$^+$ cells	Tumor type
Kras^{G12D}	Papilloma (Lapouge et al., 2011)
Kras^{G12D}/p53$^{-/-}$	SCC (Lapouge et al., 2011; White et al., 2011)
Kras^{G12D}/p53$^{-/-}$/Pten$^{-/-}$	SCC (Nassar et al., 2015)
Ptch$^{+/-}$ + irradiation	BCC (Wang et al., 2011)
Kras^{G12D}/Smad4$^{-/-}$	SCC and metastases, BCC, sebaceous adenoma (White et al., 2013)

Abbreviations: BCC, basal cell carcinoma; K15, keratin 15; SCC, squamous cell carcinoma.

typically generate SCCs, mutant sebaceous gland SCs cause sebaceous tumors, and mutant transit-amplifying cells of the hair follicles cause hair follicle tumors (Owens and Watt, 2003). The microenvironment controls CSC fate via cell-cell interactions between CSCs, tumor cells, and the neighboring stroma, including immune cells, cancer-associated fibroblasts, and endothelial cells. A mouse model of SCC showed that CSCs exist in a vascular niche, and vascular endothelial growth factor secreted by endothelial cells is associated with their expansion (Beck et al., 2011). SCC CSCs in close proximity to endothelial tumor cells often express high levels of SOX2, which promotes the expansion of SCC CSCs along the tumor-stroma interface (Siegle et al., 2014). Neighboring stromal cells provide cues to regulate the cell cycle of SCC CSCs (Schober and Fuchs, 2011), demonstrated by a study showing that transforming growth factor-β secreted by neighboring endothelial and stromal cells bestowed slower cycling properties to SCC CSCs in mice (Oshimori et al., 2015).

Quiescence paradox

Adult SCs and certain CSC populations are typically quiescent (Fuchs, 2009). Quiescence in normal SCs limits proliferation and protects genomic integrity (Coller et al., 2006; Sang et al., 2008; Viatour et al., 2008; White et al., 2014). The tumor suppressor phosphatase and tensin homolog (PTEN) plays a role in maintaining quiescence in hair follicle SCs, even in the presence of tumorigenic stimuli (White et al., 2014). However, quiescent CSCs may contribute to cancer progression by increasing epithelial to mesenchymal transition, enhancing colony formation, invasion, and tumor initiation (Moore and Lyle, 2011). In a chemical carcinogenesis SCC model, tumors grow from the skin after rapidly proliferating epidermal cells were killed by the chemotherapeutic agent 5-fluorouracil, suggesting that tumors rise from quiescent CSCs (Morris et al., 1997). Quiescent CSCs are delayed in entering late S phase and have high DNA repair activity, making them more resistant to therapeutics that inhibit cell cycle progression or promote DNA damage-induced cell death, encompassing the mechanisms of many chemotherapeutic drugs and radiation therapy (Ahsan et al., 2009; Masunaga et al., 1991; Oshimori et al., 2015). Indeed, rescuing hematopoietic SCs from a quiescent state increased their sensitivity to 5-fluorouracil (Essers et al., 2009).

CSC PLASTICITY AND HETEROGENEITY

CSCs display functional heterogeneity that is less location dependent than normal SCs. In the dynamic CSC model, CSCs and non-CSCs can interconvert in response to environmental cues, such as secreted factors within the niche that activate downstream signaling. For instance, hepatocyte growth factor secreted from myofibroblasts activates Wnt signaling that converted non-CSCs to CSCs in a human colon cancer transplant model (Vermeulen et al., 2010). In human breast cancer CSC transplant models, IL-6 secretion converts non-CSCs to CSCs (Iliopoulos et al., 2011), and transforming growth factor-β activates Zeb1 transcription, causing conversion of CD44^{low} cells to CD44^{high} CSCs (Chaffer et al., 2013). Because of this "dynamic stemness," SCC tumors likely contain multiple CSC populations with distinct characteristics. One study found that CSCs from human SCCs fall into two phenotypes: one was similar to normal epithelial SCs and associated with growth and proliferation, whereas the other became migratory (Biddle et al., 2011). Subpopulations of metastatic CSCs have also been identified, suggesting that CSCs might be the "lethal seeds" responsible for metastasis (Hermann et al., 2007; Pang et al., 2010; Patel et al., 2011; Sun and Wang, 2010). We found two distinct CSC populations (SP and CD34$^+$CD49f$^+$) in a mouse SCC model developed by *Smad4* deletion and *Kras^{G12D}* activation in K15$^+$ SCs (*K15.Kras^{G12D}.Smad4$^{-/-}$*). Although both SP and CD34$^+$/CD49f$^+$ CSC populations were tumorigenic, only tumors initiated by SP cells underwent epithelial to mesenchymal transition and metastasized to the lung (White et al., 2013).

MODELING HUMAN SCC IN MICE

The most widely used SCC models are genetically engineered mouse models and human tumor xenograft models. Genetically engineered mouse models (examples in Table 1) phenotypically and histologically mimic human SCC and are powerful tools for dissecting driver mutations that contribute to tumorigenesis and metastasis. Some driver mutations found in human SCCs are required to initiate SCCs in mouse models (Table 1), but some oncogenic mutations, such as *p53*, are insufficient to cause SCC. This is also observed in human skin, which normally harbors many oncogenic driver mutations (Martincorena et al., 2015). Therefore, mouse models provide a valuable tool to identify which combinations of driver mutations initiate SCC (examples in Table 1). Compared with the mutation burden of 2–6 mutations/Mb/cell in chronically UV damaged and aged skin (Martincorena et al., 2015), the mutation burden in cutaneous SCC ranges from 1 to 380 mutations/Mb or averaged 61.2 mutations/Mb depending on sample sources (Martincorena et al., 2015; Pickering et al., 2014). Similarly, mouse SCCs initiated by few oncogenic driver mutations typically harbor numerous subsequent genetic alterations (Bornstein et al., 2009; Torchia et al., 2012). UVB signature *p53* mutations were found in approximately 58% of cutaneous SCCs (Brash et al., 1991), and *Ras* mutations (9% *Hras*, 7% *Nras*, and 5% *Kras*) were detected in 21% of cutaneous SCCs (Bamford et al., 2004). In mice, the combination of *Kras^{G12D}* and *p53* deletion in bulge SCs or their hair follicle progeny is required to induce skin SCC (Lapouge et al., 2011). Smad4 expression is lost in 70%

of patients with SCC (Hoot et al., 2008), and *K15.Kras*^{G12D}*.Smad4*$^{-/-}$ mice developed metastatic SCCs with stage-specific histotypes including epithelial hyperplasia, dysplasia, and primary and metastatic SCC, which are comparable to human lesions and tumor progression (White et al., 2013).

Although it is commonly believed that cutaneous SCC arises from the interfollecular epidermis, follicular SCC, which is derived from a pre-existing hair follicle structure (e.g., from the scalp), may arise from bulge SCs (Shendrik et al., 2013). Because it is difficult to confirm the origin of these tumors in patients, mouse models of SCC arising from mutations in bulge SCs provide unique tools for lineage tracing of these mutant bulge cells. Among them, *K15.Kras*^{G12D}*.Smad4*$^{-/-}$ mice bearing SCCs developed lung metastases (White et al., 2013). However, when *Smad4* deletions were targeted to the epidermis by promoters not restricted to bulge SCs, such as K5, K14, and mouse mammary tumor virus (MMTV), the resulting SCCs failed to metastasize to the lung, even in the presence of spontaneous *Ras* activating mutations (Bornstein et al., 2009; Owens et al., 2010; Qiao et al., 2006; Yang et al., 2012). In those models, SCCs mimic interfollicular SCCs or oral SCCs in human patients, which can arise from many different progenitor cell locations. Taken together, genetic alterations in bulge SCs appear to result in more aggressive SCCs than the same genetic alterations in broader keratinocyte populations.

Human SCC xenografts are commonly used to characterize CSCs. However, these models are limited by a lack of native tumor stroma and interactions with an intact immune system. To overcome these constraints, humanized xenograft models are being developed. One example is XactMice, in which the bone marrow of nonobese diabetic/severe combined immunodeficiency (NOD/SCID)/IL2rg$^{-/-}$ mice is replaced with human hematopoietic stem and progenitor cells (Morton et al., 2016). Tumors grown in XactMice contained infiltrated human CD45^{+} hematopoietic cells, including CD3^{+} T cells, CD4^{+} T-helper cells, CD19^{+} B cells, and α-smooth muscle actin-positive cells (activated fibroblasts) (Morton et al., 2016). Moreover, tumors can at least partially reverse the genetic drift observed in classical patient-derived xenograft models (Tentler et al., 2012), and RNA sequencing data suggested that stromal signatures in XactMice SCCs revert back to signatures similar to primary SCCs (Morton et al., 2016). Thus, XactMice provide an advanced model to study human SCC CSCs and experimental therapeutics within a native stromal environment.

THERAPEUTICALLY TARGETING CSCs

Strategies to target CSCs are being explored using experimental therapeutics and clinical trials in the following areas.

Targeting ABC transporter proteins

ABC transporter overexpression leads to higher drug efflux and therapeutic resistance in SCC. SCC SP cells display increased ABCG2 expression and activity, which may mediate resistance to diverse cancer drugs including platinum compounds, bortezomib, and 5-fluorouracil (Sun et al., 2010; Tabor et al., 2011; Yajima et al., 2009; Yanamoto et al., 2011). Indeed, SCC cells selected for high cisplatin resistance show enhanced ABCG2 expression and a CSC-like phenotype (Tsai et al., 2011), and SCC SP cells become sensitized to chemotherapy on general inhibition of ABC transporters by the calcium channel blocker verapamil (Loebinger et al., 2008). Our observation that SP cells were associated with SCC metastasis prompted us to investigate how to therapeutically target them. In our study, *K15.Kras*^{G12D}*.Smad4*$^{-/-}$ SCCs were treated with docetaxel, a first-line chemotherapeutic for SCC, alone or in combination with verapamil. Although docetaxel alone had no effect, the combination with verapamil significantly reduced lung metastasis (White et al., 2013). Although a number of other inhibitors specific for ABC transporters have been identified, including MS-209, PSC833, VX710, and the third-generation inhibitor tariquidar (Dean et al., 2005; Modok et al., 2006), their use in clinical trials has yielded largely negative results or been terminated early because of an increased incidence of adverse effects (Pusztai et al., 2005). Because CSCs rely on several mechanisms to escape drug sensitivity, ABC transporter inhibitors will need to be combined with other strategies to efficiently eliminate CSCs in vivo, and safer ABC transporter inhibitors need to be developed.

Inhibiting Wnt signaling

Wnt activation plays an important role in SCC CSC maintenance as depleting β-catenin, a key component of Wnt signaling that is activated in human SCCs, in mouse SCCs initiated by CD34^{+} CSCs resulted in tumor regression (Malanchi et al., 2008; White et al., 2013). Further, we have found that Wnt signaling is linked to metastasis-associated CSCs because knocking down miR-9, a miRNA responsible for activating Wnt signaling and expanding SP CSCs, reduced SCC lung metastases in mice (White et al., 2013). Currently, although no Wnt antagonists are in clinical trials for treating SCC, the Wnt inhibitor LGK974 is undergoing a phase I trial to treat a variety of other cancers, such as melanoma, breast cancer, and pancreatic adenocarcinoma (http://clinicaltrials.gov/show/NCT01351103), and could provide insight for future SCC treatment strategies.

Enhancing antitumor immunity

Immune escape is defined by the inability of the immune system to recognize and eliminate transformed cells during disease progression. Immune escape is essential for SCC development, and organ transplant recipients with immune suppression demonstrated a 50- to 100-fold increased risk of SCC (Harwood et al., 2003). Additionally, high expression of ABC transporters, such as ABCB5, have immune suppressive effects in addition to their role in chemoresistance as mentioned above (Schatton et al., 2015). Thus, targeting ABCB5 may reduce both chemoresistance and immune evasion. ALDH is a potential CD8^{+} T-cell antigen in SCC (Visus et al., 2007), and ALDH^{high} CD8^{+} T cells were effective against models of nonsmall cell lung cancer (Luo et al., 2014). Because ALDH is also a SCC CSC biomarker (Adhikary et al., 2013), stimulating a CD8^{+} T-cell response against ALDH antigens may preferentially eliminate CSCs. The development of cancer vaccines that target CSCs through dendritic and other antigen-presenting cells is also promising (Li et al., 2015; Ning et al., 2012; Xu et al., 2009), but it

remains to be determined if immunotherapy will be effective at eradicating CSCs in SCCs.

CONCLUSIONS

Our understanding of CSCs in cutaneous SCC can be summarized as follows: first, although SCs and CSCs share some features, CSC heterogeneity is less location dependent when compared with normal SCs. Second, genetic alterations in bulge SCs may cause more aggressive SCCs than the same genetic alterations in broader keratinocyte populations. Third, the capacity for tumor initiation by CSCs may not always be linked to their metastatic potential. Finally, although CSCs may be difficult to eradicate, therapeutic interventions can be designed to target specific functions of CSC populations responsible for metastasis. We foresee that these research discoveries will translate into CSC-targeted therapeutic interventions in the near future.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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