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Tumour-stroma crosstalk in the development of squamous cell carcinoma.

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Abstract

Squamous cell carcinoma (SCC) represents one of the most frequently diagnosed tumours and contributes to significant mortality worldwide. Recent deep sequencing of cancer genomes has identified common mutations in SCC arising across different tissues highlighting perturbation of squamous differentiation as a key event. At the same time significant data have been accumulating to show that common tumour-stroma interactions capable of driving disease progression are also evident when comparing SCC arising in different tissues. We and others have shown altered matrix composition surrounding SCC can promote tumour development. This review focusses on some of the emerging data with particular emphasis on SCC of head and neck and skin with discussion on the potential tumour suppressive properties of a normal microenvironment. Such data indicate that regardless of the extent and type of somatic mutation it is in fact the tumour context that defines metastatic progression.

1. Introduction

Malignant transformation of stratifying epithelium commonly results in SCC. Stratifying epithelia are present in organs and tissues that provide a barrier between the environment and the organism such as lung, cervix, oesophagus, mouth and skin. Although much rarer SCC can also arise in other epithelial tissues such as prostate, thyroid and bladder (Kleer, Giordano et al. 2000; Shokeir 2004; Malik, Dakwar et al. 2011). Collectively stratifying epithelium constitute some of the most common tissues diagnosed with cancer and although SCC in skin (cutaneous SCC, cSCC) is the only recorded SCC registration, extrapolation of UK figures with percentages given by the American Cancer Society shows that SCC comprises approximately 15% of all cancer registrations (ACS 2014; ISD 2014). It is more difficult to relate these percentages to mortality figures but potentially up to 12% of all cancer related deaths are a result of SCC. This review will introduce SCC, discuss the numerous signalling pathways shown to be perturbed in tumour-stroma interactions during SCC development, before focussing on aspects of the tumour microenvironment such as inflammation, adhesion and extracellular matrix composition. We will also touch on the idea that normal tissue is itself tumour suppressive. We will concentrate on data derived from studying cSCC and to a lesser extent head and neck SCC (HNSCC), but many aspects herein will likely have relevance to other SCC for the reasons discussed. As with any review we are limited by space and acknowledge ahead of time that we are by no means comprehensive in our assessment of the literature and apologise to all individuals whose important studies we have overlooked in this article.

2. SCC

SCC manifests grossly as atypical epithelial growth with highly variable clinical appearance ranging from lesions that may be asymptomatic to ulcerations with intermittent bleeding. On the microscopic level, cellular atypia is usually coupled with varying degrees of squamous differentiation, such as the very prominent keratinous pearls seen in well differentiated cSCC (Figure 1). Tumour grade, as with most solid tumours, is determined by the degree of differentiation and cellular atypia, and in SCC a poorly differentiated tumour will exhibit very little or no keratinous pearls.

2.1 cSCC

Together with basal cell carcinomas, cSCC make up the vast majority of non-melanoma skin cancers (NMSCs), the most common group of malignancies in Caucasian populations, accounting for approximately 20% of all new cancer registrations and 90% of all skin cancers (NCIN 2013). cSCC

represents the most common skin cancer with high metastatic potential (Czarnecki, Staples et al. 1994; Brantsch, Meisner et al. 2008; Seo, Shim et al. 2011; Samarasinghe and Madan 2012) and cSCC incidence figures tend to be under-estimated because overall recording of NMSC is often incomplete. This is partly due to underreporting of minor cases treated in primary care but also due to a high frequency of recurrence when only the primary tumour is recorded – an estimated 30% of cSCC cases go unrecorded (CRUK 2014). In the US approximately 700,000 new cases of cSCC are diagnosed annually but as cSCC has been excluded from national cancer registries there is no precise data concerning incidence, metastases and related mortality (Karia, Han et al. 2013).

2.2 Common pathway perturbation in SCC

Much of the research to assess cross-talk between tumours and their surrounding environment, or stroma, has used cSCC as a model and although skin is distinct in many ways from other tissues such as lung or oesophagus recent deep sequencing of cancer genomes are now suggesting that common initiating mutation events occur in SCC arising across these different tissues implicating disruption of similar pathways (Durinck, Ho et al. 2011; Stransky, Egloff et al. 2011; Agrawal, Jiao et al. 2012; TCGA 2012). It has long been known that TP53 and CDKN2A are frequently mutated in SCC but recent findings of prevalent loss of function mutations in Notch receptors coupled with the described role of Notch signalling in terminal differentiation (Lefort and Dotto 2004) suggests that perturbation of this pathway is a common, early event in SCC development.

At the same time as the new genetic data are emerging, numerous studies also demonstrate that changes in the stromal environment can drive cancer predisposition and overlaps also exist here when comparing SCC arising in different tissues.

2.3 Field Change in SCC

Environmental factors have a large influence on the risk of developing all SCC. In skin the primary cause is UV exposure while in cervix and head and neck viral infections are significant contributors. Tobacco, principally for lung SCC but coupled with alcohol use as well as air pollution and other factors, are all linked to increased risk of developing SCC (Bandera, Freudenheim et al. 2001; Liaw, Ting et al. 2008). As these environmental factors have the potential to affect large regions of tissue it has been proposed that precancerous fields can arise leading to areas at high risk of malignancy. This concept, termed field cancerisation was introduced by Slaughter and colleagues in 1953 to describe the observation that multiple primary HNSCC arise within close proximity, suggestive of a

predisposing area of tissue, or field (Slaughter, Southwick et al. 1953). Molecular evidence to support this notion followed in the late 1990s primarily concentrating on TP53 mutation (Franklin, Gazdar et al. 1997) but also describing unique epigenetic phenomena (Suzuki, Watkins et al. 2004). In the skin, field cancerisation characterised by TP53 mutation has been well described (Jonason, Kunala et al. 1996; Stahl, Stranneheim et al. 2011) and recent data now show that field change can be induced in mice through inhibition of Notch signaling within the stroma rapidly promoting spontaneous cSCC (Hu, Castillo et al. 2012). We have shown *NOTCH1* mutation arising in normal and tumor tissue in close proximity suggesting that such a mechanism may be active in human skin (South, Purdie et al. 2014). In addition and prior to this work, mosaic loss of *NOTCH1* in the epidermal compartment of mice can induce spontaneous tumours competent in Notch1 signaling with data again pointing to a direct influence from the stroma (Demehri, Turkoz et al. 2009).

Independent of Notch signalling, data from our laboratory has shown that altering the collagen composition of the dermal compartment can accelerate cancer progression directly implicating tumour-stroma interactions as the cause for rapidly developing cSCC in the rare inherited cancer prone disease, recessive dystrophic epidermolysis bullosa (RDEB, discussed below)(Ng, Pourreyaon et al. 2012).

2.4 Signalling pathways perturbed in tumour-stroma interactions in SCC

As shown in Table 1, nearly every major signalling pathway in normal homeostasis can be implicated in the development of SCC. Though by no means exhaustive, this list of recent discoveries demonstrating that involvement of more and more signalling mechanisms underscores the pivotal role of complex crosstalk in tumour development and metastasis. Given that many of these pathways have been long standing targets for cancer therapy and that molecular entities which efficiently do so are available in the clinic the possibility for targeting pathways involved in these tumor-stroma interactions now exists. However, as with all interventions a better understanding of the mechanisms involved is vital for effective utility. For example, we have identified numerous components of an altered extracellular matrix which is permissive to tumor development in patients with RDEB (discussed latterly) that are all targets of TGF- β suggesting that this signalling pathway is upregulated and important for cancer development in this context (Ng, Pourreyaon et al. 2012). Upregulation of the pathway in patient material has been independently verified (Kuttner, Mack et al. 2013) and leads to the tempting prospect of using TGF- β inhibitors for the treatment of this cancer. However, it is known that at later stages of cancer progression TGF- β can have pro-tumorigenic effects and further research is essential to avoid accelerating cancer progression in this patient group (Inman 2011).

<u>Signalling Pathways</u>	<u>Hypothesis of pathway</u>	<u>Interacting molecules</u>	<u>Attribution</u>
p63–FGFR2 Signaling	p63-induced FGFR2, coupled with its activation by overexpressed stromal ligand	p63, FGF/FGFR signalling	(Ramsey, Wilson et al. 2013)
ROS-induced PI3K/AKT-mTOR signalling	CCL2 from CAFs trigger ROS production, leading to activation of the PI3K/AKT-mTOR signaling pathway in both CAFs and oral SCC and an autoregulation loop	CCL2, ROS, NF-κB, STAT3	(Li, Xu et al. 2014)
TGF-β related signalling	Complex and often apparently contradictory effects depending on dose and context	-	Numerous works
Platelet-derived growth factor C (PDGF-C) pathway	CAFs may overcome inhibition of VEGF-mediated angiogenesis through upregulation of PDGF-C	PDGF-C, VEGF	(Crawford, Kasman et al. 2009)
Wnt/β-catenin signalling	Wnt2 from CAFs can promote oesophageal SCC by activating Wnt/β-catenin signalling and its downstream targets cyclin D1 and c-myc.	Wnt2, β-catenin, c-myc	(Fu, Zhang et al. 2011)
Shh/Gli1-3 signaling	Shh ligand from SCC could induce fibroblast proliferation and survival	Shh	(Bermudez, Hennen et al. 2013)
Notch signalling	Notch ligand Jagged1 induced by MAPK in HNSCC triggers Notch activation in neighboring endothelial cells (ECs) and promoting capillary-like sprout formation. Ablation of Notch signalling in dermal fibroblasts promotes SCC development via c-jun and c-fos upregulation	Jagged1 AP1 transcription factors	(Zeng, Li et al. 2005) (Hu, Castillo et al. 2012)

Table 1. Major signalling pathways linked with SCC tumor-stroma interaction. Listed here are some of the studied pathways that have shown interdependence of SCC and stromal development, providing a glimpse of how various signalling processes can become hijacked in the tumorigenesis of SCC.

3 The tumor microenvironment

Cancers are complex organs which encompass not only the tumour cells but also cells within the surrounding stroma. Such cells facilitate many aspects of cancer development, like providing a structural and supportive framework or secretion of growth factors from both immune infiltrating cells and cancer associated fibroblasts (CAF), as well as neovascularisation through the proliferation of endothelial cells. Collectively, this tissue is referred to as the tumour microenvironment (TME) (Figure 2) (Hanahan and Coussens 2012). Arguably the most abundant cell type in the TME, CAFs can be distinguished from other, normal fibroblasts thru their increased rate of proliferation and differential expression of extracellular matrix (ECM) components (Bhowmick, Neilson et al. 2004; Kalluri and Zeisberg 2006; Erez, Truitt et al. 2010). It has been suggested at least in HNSCC, that resident and bone marrow (BM)-derived mesenchymal stem cells (MSCs) are actually precursors of the stroma and contribute to blood- and lymph angiogenesis, as well as produce tumor-associated

myofibroblasts (De Boeck, Narine et al. 2010). Markers traditionally used to identify CAFs have been smooth muscle actin, vimentin and PDGFR but significant heterogeneity can be observed even in murine models of cancer suggesting that one marker alone will not identify all CAF in the TME (Sugimoto, Mundel et al. 2006).

Parallels exist between the TME and that of healing tissues: both are characterised by inflammation and are rich in growth, chemotactic and angiogenic factors (Schafer and Werner 2008). Both contain similar cell types in the form of activated fibroblasts, infiltrating immune cells as well as proliferating epithelial and endothelial cells (Hanahan and Coussens 2012). In the case of wound healing however, cell proliferation enhanced by inflammation eventually subsides after the wound is healed. In the case of a tumour, malignant cells continue to thrive and sustain their activated microenvironments, coining the phrase “tumours: wounds that do not heal” (Dvorak 1986).

Thus the development of cancer is driven by and therefore inseparable from its microenvironment where interaction with the stroma is key to progression. Below we aim to touch upon a fraction of the data attesting to this fact.

3.1 Inflammatory crosstalk in the microenvironment of tumour stroma

As a site of chronic inflammation, tumours create a stroma populated by infiltrating inflammatory cells rich in growth factors that sustain cell proliferation and promote neoplastic risk (Figure 2B) (Coussens and Werb 2002). Rudolf Virchow made the first observations that provided clues linking inflammation to tumour development back in the nineteenth century (Virchow 1881). In this study, Virchow describes the observation that tumours are infiltrated by leukocytes. Such a trait is now considered to be one of the hallmarks of cancer (Hanahan and Weinberg 2011) but the interplay between tumours and the host immune system is finely balanced and still not fully understood. As well as promoting aspects of cancer progression immune cells can, and do, target tumour cells to eradicate the risk of neoplastic progression. Conversely tumours themselves can develop mechanisms to inhibit this immunological activity and thereby escape the host defence system. For example in HNSCC, cancer cells have been known to directly inhibit immune reactivity thru the production of soluble mediators, such as TGF- β and interleukin (IL)-10 (Qin, Valentino et al. 2001). In addition to producing mediators to evade host immunity, tumours can also influence host defences by skewing immune activity towards less effective or even immune suppressive functions (Rohrer and Coggin 1995; Schaefer, Kim et al. 2005).

Tumour cells can also express cell surface ligands that lead to immune evasion. Programmed cell death-1 ligand 1 (PD-L1) is a cell surface protein of the B7 family that is found to be up-regulated in

some solid tumours whilst absent in normal tissue (Nakanishi, Wada et al. 2007; Fife, Pauken et al. 2009). Cancer cells that express these ligands can interact with programmed cell death-1 (PD-1) present on infiltrating immune cells which can then suppress activation, proliferation and subsequently induce apoptosis in the immune cells (Blank, Gajewski et al. 2005; Karwacz, Arce et al. 2012). HNSCC cells (Tca8113) treated with inflammatory cytokines are capable of inducing significant apoptosis in tumour antigen-specific CD8+ T cells *in vitro* thru a mechanism that involves PD-L1 (Lu, Lu et al. 2013).

During tumour development the inflammatory environment encourages monocytes to migrate into surrounding stroma (thru chemotactic factors such as the tumour-derived chemokine CCL2) where they then differentiate into macrophages (Coussens and Werb 2002). Other factors such as fibroblast and macrophage derived cytokines IL-10, platelet-derived growth factor (PDGF), CCL3, CCL4, CCL5, CCL7, CCL8, CXCL12, and vascular endothelial growth factor (VEGF) also help to promote macrophage recruitment (Murdoch, Giannoudis et al. 2004; Allavena, Sica et al. 2008). Tumour associated macrophages (TAMs) in turn express growth factors and matrix-proteases, promote angiogenesis and suppress adaptive immunity even while helping the host to inhibit tumour growth and destroy neoplastic cells (Mantovani, Bottazzi et al. 1992; Sica, Allavena et al. 2008). For instance, VEGF-C produced by TAMs in cSCC leads to lymphangiogenesis which then results in increased lymphatic vessel density (Belkin, Mitsui et al. 2011), which in turn is linked to metastatic progression (Belkin, Mitsui et al. 2011; Moussai, Mitsui et al. 2011).

Using a murine model of cSCC Hanahan and colleagues demonstrated that CAF express a specific gene signature which can promote an inflammatory response, recruit macrophages and stimulate angiogenesis. Interestingly the work shows that tumour cells can “educate” normal fibroblasts to establish this pro-inflammatory CAF genetic signature and that this signature can be detected at the very early stages of neoplastic progression which in part is mediated through NF-kB signalling (Erez, Truitt et al. 2010).

It is also shown that IL-4 and IL-10 produced by macrophages and tumour cells respectively, polarize macrophages into the M2-subtype which are crucial for the development of VEGF-A-induced skin tumours through enhancing angiogenesis and also establishing an anti-inflammatory TME (Linde, Lederle et al. 2012).

Many examples of inflammation driven TME alterations which lead to tumour progression exist and offer one route for TME therapeutic targeting; tumour specific inflammatory drivers may be key to this process.

3.2 Adhesion

By governing mechanisms that mediate a cancer cells ability to navigate different environments, adhesion molecules are critical to the process of metastasis. As the range of environments are significant; from the primary tumour, through the vasculature or lymphatics and on to secondary sites; a cancer cell likely experiences a vast array of different adhesion molecules and will therefore likely need to react to such molecules in a manner governed by overall context. If this holds true then it should be of no surprise that studies of adhesion often yield conflicting results depending on the system used since many key molecules can act as both positive and negative modulators of metastasis.

For instance, CD44 isoforms have been shown to be down-regulated in HNSCC in several studies but findings were inconsistent concerning the relationship between CD44 expression and tumour grade, metastasis and prognosis (Herold-Mende, Seiter et al. 1996; Oliveira, Sherriff et al. 1998; Masuda, Kuratomi et al. 2000; Fonseca, Pereira et al. 2001). Examples exist for other cell-cell adhesion molecules such as E-cadherin which promotes homotypic tumour cell adhesion and helps to confine cells within the primary tumour site. Consequently the down-regulation of E-cadherin is well studied and known to be frequently associated with more aggressive tumours and poorer prognosis (Bankfalvi, Krassort et al. 2002; Berx and van Roy 2009). However, there is also evidence that the correlation with aggressiveness is not universal and indeed up-regulation of E-cadherin may be detrimental, presumably through promoting cell growth and maintenance of tumour architecture at sites of metastasis (Auersperg, Pan et al. 1999; Rodriguez, Lewis-Tuffin et al. 2012).

In acantholytic SCC, a variant of cSCC considered to be more aggressive than non-acantholytic, well-differentiated cSCC, it is thought that the decreased expression of intercellular adhesion proteins contribute toward this aggressive nature (Griffin, Wriston et al. 2013). The range of differences between how adhesion molecules affect tumour cells thus reflects how metastatic cells likely express a different repertoire of adhesion molecules or proteases (discussed below) depending on their context; what other cells, adhesion molecules and matrix are being encountered at a particular step along the neoplastic process. It is often postulated that selective pressures of the primary tumour differs from those that enable metastasis, and subsequently the establishment of a secondary site (Zetter 1993; Mendoza and Khanna 2009).

Aberrant function of adhesion molecules in SCC may even affect the ability of the SCC to survive intervention. It has been reported that in some cases of HNSCC, intercellular adhesion molecule 2 (ICAM2) appears to mediate survival signals that are sufficient to block apoptosis thru activation of the PI3K/AKT pathway in response to radiotherapy (Perez, Kinoshita et al. 2002). Absence of ICAM2

expression has also been associated with impaired angiogenesis *in vitro* and *in vivo*, as well as defective migration *in vitro* (Huang, Mason et al. 2005).

3.3 CAF and stromal composition

During the development of epithelial neoplasia, CAF provide not only structural and biochemical support and can recruit macrophages as described earlier but, as we will go on to discuss in this section, they can also provide direct interactions to drive invasion of tumour cells into surrounding tissue.

It has been shown that certain cancer cells cannot maintain growth and survival cues alone and require the presence of CAF and other supporting stromal cells (Tarin 2011). There are also well described differences between the structures of cancer associated stroma and normal stroma, and CAF themselves can show enhanced proliferation and migration *in vitro* when compared with normal fibroblasts (Schor, Schor et al. 1985; Schor, Schor et al. 1988; Carmeliet and Jain 2000). CAF often express α -smooth muscle actin (SMA) and are commonly found surrounded by dense accumulations of fibrillar collagens *in vivo* (Figure 2B) (Sappino, Schurch et al. 1990). Oesophageal SCC exhibit changes in stromal cell density shown to be increased in the peripheral tumour stroma when compared with stroma between tumour nests. The same study also showed that significantly higher SMA positive myofibroblasts which exhibited higher proliferative capacity correlated with tumour size (Liu, Li et al. 2012). Studies on initiated, pre-malignant epithelial cells indicate that neoplastic growth is not necessarily stimulated when cultured with normal fibroblasts even under ideal conditions. Vis versa, normal epithelial cells were similarly not stimulated for growth when cultured with CAF under identical conditions. However when these initiated epithelial cells are cultured with CAF, epithelial proliferation and malignant progression followed (Olumi, Grossfeld et al. 1999).

Physiological changes to normal fibroblasts can however directly affect malignant epithelial cells as well; it has been shown that senescent fibroblasts, like CAF, can promote proliferation of pre-malignant and malignant epithelial cells and can drive tumour formation in mice. Likely this indicates that senescent fibroblasts are able to produce similar oncogenic factors to those of CAF and may be partly responsible for the increasing risk of cancer with age (Krtolica, Parrinello et al. 2001). There is also some evidence of species-specificity in fibroblast stimulations of tumours. In one study cell proliferation and invasiveness of partially transformed oral keratinocytes were triggered by human, mouse and rat fibroblasts. However local invasion of the tumour cells was only observed in the presence of human fibroblasts or conditioned media from human fibroblasts, suggesting a difference in cytokine and collagenase production (Costea, Kulasekara et al. 2006).

CAF have also been shown to be capable of remodelling the physical matrix of the microenvironment and driving the collective invasion of HNSCC cells (SCC12 and A431) within a 3D organotypic culture model (Gaggioli, Hooper et al. 2007). HNSCC cancer cell invasion into the underlying matrix was only possible in the presence of CAF which generated tracks to lead collective invasion of SCC cells shown to be dependent on Cdc42 and MRCK mediated regulation of myosin light chain (Gaggioli, Hooper et al. 2007).

In a unique example of how fibroblasts can effect efficient tumour suppression, it was recently found that skin fibroblasts of the naked mole rat (*Heterocephalus glaber*) secrete high-molecular-mass hyaluronan (HMM-HA) that allow these rats to be almost immune to cancer. The cancer resistance results from an enhanced form of contact inhibition due to HMM-HA via the HA-CD44-NF2 pathway which enhances contact inhibition of cells at unusually low density. This enhancement is possible through several mechanisms that are uniquely evolved in these rats. The authors found that naked mole-rats accumulate HA abundantly due to the decreased activity of HA-degrading enzymes and a unique sequence of hyaluronan synthase 2 (HAS2). Naked mole rat cells are also more sensitive to HA signalling, possessing a higher affinity to HA compared with mouse or human cells in flow cytometric assays. Experiments demonstrated that RAS mediated carcinogenesis only occurred after perturbation of the HA-CD44-NH2 signalling pathway (Tian, Azpurua et al. 2013).

3.4 Extracellular Matrix can profoundly influence tumour progression

Homeostasis in many cell types is tightly controlled by ECM. Examples of ECM proteins with significant impact on cellular dynamics are the laminins (Aumailley 2013). In normal tissues, the laminins form a major component of the basal lamina which functions to anchor the epithelium to the underlying stroma (Rousselle and Beck 2013). Laminins are secreted by the epithelial cells and directly link transmembrane components to other secreted components of the basal lamina. Laminin 332, previously known as laminin 5, is highly expressed in several types of SCC and other epithelial tumours (Berndt, Hyckel et al. 1997; Skyldberg, Salo et al. 1999). Laminin receptor expression, such as $\alpha 6\beta 4$ integrin, have also been shown to play an important role in SCC progression by regulating processes such as adhesion and migration (Zhang and Kramer 1996; Salo, Haakana et al. 1999; Janes and Watt 2006).

When mutated, certain molecules responsible for cellular adhesion via ECM interactions cause severe monogenic disease that can lead to neoplasia (Ng, Dayal et al. 2011). In patients with RDEB caused by mutations in the gene encoding type VII collagen, the epidermis fails to adhere to the underlying dermis as type VII collagen is the main component of anchoring fibrils which contribute to the

hemidesmosome-anchoring filament complex. The disruption of this complex leads to a weak dermal-epidermal junction and the epidermis can easily separate from the dermis leading to debilitating widespread blistering. This condition is complicated by the subsequent development of aggressive and often metastatic cSCC (Fine, Johnson et al. 2009).

Although not fully understood recent studies indicate that the stroma in RDEB patients is tumour permissive and drives accelerated cSCC progression; RDEB CAF confer increased adhesion and facilitate the invasion of cSCC (Ng, Pourreyron et al. 2012). The loss of functional *COL7A1* in RDEB CAF was found to be associated with an altered gene expression pattern in these cells. Re-expression of wild type *COL7A1* in RDEB fibroblasts reverted components of this gene signature leading to decreased type XII collagen, thrombospondin-1, and Wnt-5A expression, reduced tumour cell invasion in organotypic culture, and restricted tumour growth *in vivo*. Type V collagen, type XII collagen, and thrombospondin-1 were also found to be upregulated in the stroma of a panel of cSCC, including RDEB, and modulated by siRNA depletion of *COL7A1* in normal dermal fibroblasts (Ng, Pourreyron et al. 2012). Similar gene expression changes have been found in the stroma of other SCC correlating with prognosis (Roepman, de Koning et al. 2006; Saadi, Shannon et al. 2010). Type VII collagen has also recently been found to modulate the expression of an organic anion transporting polypeptide known as OATP1B8 in tumour cells. The lack of type VII collagen induced expression of OATP1B8 while recombinant type VII collagen reduced OATP1B8 and coincided with an acquisition of front to rear polarity. Importantly, type VII collagen delivered by the stromal compartment modulated OATP1B8 expression and increased the structural organisation of 3D spheroid co-cultures of RDEB cSCC and stromal cells (Dayal, Cole et al. 2013).

Collagens are major constituents of ECM representing as much as 30% of total mammalian protein mass (Shoulders and Raines 2009). Among them type I collagen is most abundantly expressed in the human body and is often associated with type III collagen fibres. Type IV collagen is known to be important as a core component of basement membrane and the distribution of type IV collagen correlates with the differentiation status of HNSCC, with a significant loss in cases of poorly differentiated SCC (Agarwal and Ballabh 2013). The loss of normal collagen orientation and the development of desmoplasia, characterised by an accumulation of fibrillar collagen types I and III and increased degradation of type IV collagen (Zhu, Risteli et al. 1995; Kauppila, Stenbäck et al. 1998; Huijbers, Iravani et al. 2010), is often referred to as ‘cancer-associated collagen’ and is well observed in the development of many cancers (Schedin, O’Brien et al. 2007). Type XII collagen has been found to be expressed not just by the CAF, but also by cancer cells lining the desmoplastic front of invading tumour subpopulations (Karagiannis, Petraki et al. 2012). It is interesting to note that cancer cells at this invasive or budding front (Figure 2A) are also known to be less differentiated. These findings

imply a link between the differentiation state, invasion of cancer cells and type XII collagen secretion (Xing, Saidou et al. 2010).

Changes in the expression of collagens such as those described above do not function alone and work in the context of proteins such as fibronectin and other proteoglycans which can bind the increased collagen present and affect overall fibril organisation and the underlying mechanical properties of ECM (Levental, Yu et al. 2009). An increase in collagen production, changes in collagen organisation and cross-linking can produce an overall stiffer matrix that imparts biochemical and mechanical influences that are being increasingly recognised as correlating with malignant progression (Levental, Yu et al. 2009; Evans, Armstrong et al. 2013). The fibronectins themselves are large extracellular matrix glycoproteins often found in the plasma and other body fluids. They participate in cell adhesion, migration, invasion, and survival by activating integrin and proteoglycan receptors through specific signalling pathways such as the extracellular signal-regulated kinase (ERK) (Kamarajan and Kapila 2007). Studies using an altered fibronectin protein showed that SCC cells escape suspension-induced death (anoikis) by forming multicellular aggregates which avail themselves of fibronectin survival signals mediated by integrin α V and focal adhesion kinase activation (Zhang, Lu et al. 2004).

3.5 Proteases

As discussed above, tumour cells interact with their environment through cell surface adhesion receptors and components of the ECM. As a tumour outgrows its primary site and begins to invade and penetrate through the basement membrane into the underlying stroma, malignant cells directly interact with ECM, CAF, immune cells, blood and lymph vessels. To do this cancer cells employ a number of proteolytic enzymes capable of degrading and remodelling components of the stroma. One such group of enzymes are the Matrix Metalloproteinases (MMPs); zymogens which are synthesized in a latent form and secreted as pro-enzymes that require extracellular activation. Activation itself is controlled through specific endogenous inhibitors of metalloproteinases (called TIMPs). Proteolytic activity within the stroma not only removes physical barriers and regulates tissue architecture but also activates or deactivates proteases, growth factors and cytokines through cleavage events, many of which yield active products from ECM (Whitelock, Murdoch et al. 1996; Sternlicht and Werb 2001). The non-collagenous domain of type IV collagen α 1 also contains a 26-kDa fragment known as arresten that, once cleaved becomes an endogenous angiogenesis inhibitor shown to also efficiently inhibit proliferation, migration and invasion of a highly metastatic human tongue SCC cell line (Aikio, Alahuhta et al. 2012). Arresten is also known to inhibit the proliferation, migration and tube formation of different types of endothelial cells through α 1 β 1 integrin receptors (Nyberg, Xie et al.

2008). The cleavage of laminin-332, or collagen IV results in the exposure of cryptic sites which are able to promote migration (Giannelli, Falk-Marzillier et al. 1997; Xu, Rodriguez et al. 2001).

Not surprisingly due to their ability to modify collagen morphology and content, proteases are considered to be key factors in the invasion and growth of epithelial tumours. It has been shown that peripheral blood concentrations of degradation products from type I and type III collagen reflects the invasive activity of HNSCC and predicts patient survival (Nurmenniemi, Koivula et al. 2012).

Tumour cells, either co-cultured with normal fibroblasts or with fibroblast-conditioned media, can initiate a proteolytic cascade that ultimately ends in the activation of pro-MMP-1 and tumour cell invasion (Ohuchi, Imai et al. 1997; Sabeh, Li et al. 2009). Cleavage of type I collagen by MMP-1 is thought to be necessary for keratinocyte migration on type I collagen and MMP-1 is invariably expressed by basal keratinocytes migrating across the dermal matrix. It has been shown that contact with native type I collagen, as well as epidermal growth factor (EGF) induces MMP-1 expression in primary keratinocytes, but not after contact with basement membrane proteins or other components of the dermal matrix (Pilcher, Dumin et al. 1997; Ziober, Turner et al. 2000). Work on an invasive oral SCC cell line (HSC-3) indicates that MMP-1 expression can be induced in the presence of 10% serum (Ziober, Turner et al. 2000). These findings are supported by others that show sustained MMP-1 production requires autocrine EGFR activation (Ito, Nakajima et al. 1995). Human SCC are known to often overexpress epidermal growth factor receptor (EGFR), and the up-regulation of EGFR correlates with increased cell motility and invasion *in vitro*, often associated with poor prognosis (Pilcher, Dumin et al. 1999; Ali, Gunduz et al. 2008; Uribe and Gonzalez 2011).

Another crucial proteolytic pathway involved in remodelling basement membrane and associated ECM components is the serine protease of the plasminogen (Plg) activation system (Chapman 1997). Plasminogen activators uPA and tPA convert Plg into its active form, plasmin, which then degrades ECM proteins, either as a direct interaction or through activation of MMPs and inappropriate expression of the Plg protease system has been associated with SCC development (Romer, Pyke et al. 2001).

4 The Differentiation Potential of the normal stroma

Differentiation, the process by which progenitor cells acquire characteristics or specific function of a tissue or system, is often subverted in cancer. In the skin for instance, keratinocytes support function through continual terminal differentiation and renewal; progenitor cells divide and progeny migrate upwards and eventually outwards forming a barrier through a process of programmed cell death. In order for a tumour to develop, this program needs to be subverted and although SCC retains aspects of

differentiation, cellular proliferation predominates. An attractive approach to cancer therapy would be to switch on the intrinsic ability to undergo terminal differentiation in an SCC cell thereby skewing tumour dynamics away from proliferation. An example of such a therapy exists in the haemopoietic malignancy, acute promyelocytic leukemia (APML). APML is caused by a chromosomal translocation t(15;17) leading to a fusion of the promyelocytic leukemia (PML) and retinoic acid receptor α (RAR α) genes. This mechanism drives the pathogenesis of APML thru defective transcriptional regulation that leads to aberrant proliferation of undifferentiated cells. This understanding allowed the development of the drug Tretinoin that could dissociate PML-RAR α /HDAC complexes and result in the resumption of normal myeloid differentiation in APML cells demonstrating that it is possible to artificially induce cancer cells to differentiate into a benign phenotype (Pitha-Rowe, Petty et al. 2003).

Differentiation of normal cells to specialised cellular phenotypes can also be regulated by changes in the ECM. For instance, mammary gland alveoli cells plated and cultured in the presence of physiologically appropriate hormones do not produce milk, and only do so when suspended in an artificial ECM containing laminin which binds integrins on the epithelial cells ~~and~~ triggering the required signalling cascade for spherical cyst formation and milk production (Pullan, Wilson et al. 1996). Continuing with this theme, breast cancer cells can be induced to form differentiated structures called acini which are indistinguishable from normal breast cells after simple manipulation of ECM components. (Weaver, Petersen et al. 1997).

Finally, direct evidence of induction of differentiation and reversion of malignant SCC keratinocytes through co-culture with normal epithelial cells (Javaherian, Vaccariello et al. 1998) demonstrates that a normal stroma/ microenvironment is capable of reverting a malignant SCC phenotype. In this study Garlick and colleagues used the HaCat model of malignant conversion (through introduction of oncogenic RAS) (Boukamp, Stanbridge et al. 1990) to show that admixing malignant RAS transformed keratinocytes with normal primary keratinocytes induced differentiation rather than invasion.(Javaherian, Vaccariello et al. 1998). In experiments along a similar theme a normalised stromal scaffold was able to ionduce similar differentiation characteristics in malignant cells (Willhauck, Mirancea et al. 2006).

The reversion of a malignant cancer phenotype through triggering normal differentiation is an attractive therapeutic avenue. Crucial to delivering this potential will be an increase in our understanding of how differentiation pathways are perturbed in SCC; for example through loss of Notch signalling. Clues to such an understanding will come from demonstrating how these signaling pathways drive normal cells into the differentiation processes in relation to environment.

5 Conclusion

The limited investigations reviewed above clearly indicate that although successive mutation and genetic disruption are required for cells to undergo malignant progression, cross talk with the developing stroma plays an equally important role in providing a permissive environment for initiated pre-cancerous cells to proliferate, escape host defences and eventually metastasize.

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Figure Legends

Figure 1: Key features of cutaneous squamous cell carcinoma (cSCC): growth of atypical cells with squamous differentiation. H&E section of human cSCC. cSCC can vary in degree of differentiation from well differentiated tumours exhibiting clear boundaries with extensive keratinisation (keratinous pearls), to poorly differentiated tumours with severe cellular atypia and little or no observable squamous differentiation.

Figure 2: Different types of stromal reaction to cancer: Arrows indicate are invasive fronts of development cSCC extending into the surrounding stroma (A); Inflammatory stromal response with arrows pointing to the presence of infiltrating immune cells (B); Irregular nests of malignant cells surrounded by dense collagenous stroma (Arrow) (C, stromal fibrosis); Irregular nests of malignant cells surrounded by retracted stroma / edema that indicated by the arrow as empty spaces (D, stromal retraction).