Cancer Stem Cells in the Head and Neck Cancers

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Core Messages

- Cancer stem cells (CSCs), the proposed origin of cancer, are present in many cancer types including primary and metastatic cutaneous squamous cell carcinoma and malignant melanoma.
- CSCs are highly tumourigenic, resist conventional therapies and are responsible for loco-regional recurrence and distant metastasis.
- CSCs are regulated by the microenvironment in which the renin-angiotensin system (RAS) plays a vital role.
- The RAS consists of multiple components, its bypass loops that provide redundancies, and convergent signalling pathways that provide crosstalk.
- A novel treatment approach for cancer is by targeting CSCs by regulating the RAS and its related pathways.

1 Models of Cancer

There are two concepts guiding cancer research: (1) the prevailing *clonal evolution model*, also known as the *stochastic model of cancer*, which proposes that normal cells acquire tumourigenicity to become cancer cells by accumulating genetic mutations (Fig. 1a), and (2) the emerging *cancer stem cell (CSC)* concept of cancer, also known as the *hierarchical model of cancer*. The latter proposes CSCs—a small subset of highly tumourigenic cancer cells with embryonic stem cell-like (ESC) properties—as the origin of cancer (Fig. 1b) [1].

The clonal evolution model proposes that all tumour cells are clonally identical and have the same tumour forming ability and propensity for self-renewal (Fig. 1a) [2]. The CSC model proposes that a tumour consists of a heterogenous population of cells with CSCs sitting atop the cellular hierarchy, sustaining tumour cell diversity, tumourigenicity, and metastatic potential [3]. These CSCs divide asymmetrically giving rise to non-tumourigenic cancer cells that form the bulk of the tumour and identical CSCs that are highly tumourigenic, resist conventional therapies and are responsible for metastasis and recurrence (Fig. 1b) [4].

Processes involved in embryonic development are often reactivated under pathological conditions, such as carcinogenesis [5]. Cancer and embryogenesis share multiple common processes such as epithelial-to-mesenchymal transition (EMT) [5]. Another similarity between carcinogenesis and embryogenesis is the shared ability of ESCs and CSCs to undergo indefinite self-renewal and bypass the replicative barrier of 50–60 population doublings before senescence [6]. Both CSCs and ESCs can undergo differentiation giving rise to cells of all lineages and utilise signalling pathways such as the MAPK/ERK, PI3K/AKT, JAK/STAT and Notch pathways [7]. As somatic cells have a low rate of mutations and a relatively short lifespan, it raises the question of how cancer cells acquire so many essential genetic changes seen in ESCs. It is more plausible that cancer arises from CSCs that originate from resident adult stem cells or progenitor cells, which possess higher proliferative capacity and are more prone to mutations. ESCs undergo periods of high rates of clonal proliferation in a highly controlled manner, whereas the proliferation of cancer cells is not controlled. Furthermore, like ESCs, cancer cells can also establish themselves in various tissues in the body [7]. Using embryonic development as a framework for investigation of carcinogenesis could provide novel insights into the understanding and treatment of cancer.

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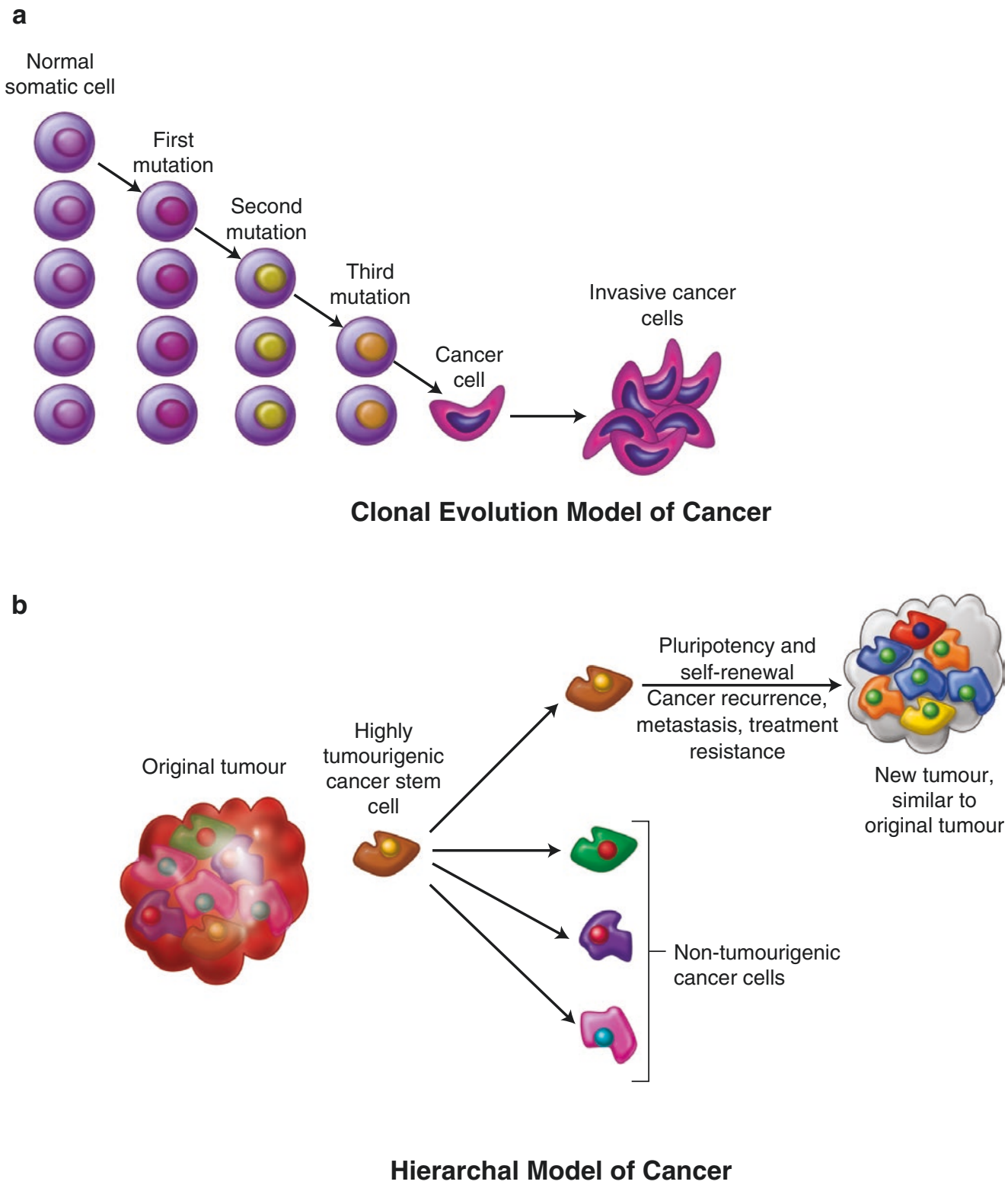

Fig. 1 (**a**) A diagram illustrating the clonal evolution model of cancer. A normal somatic cell acquires oncogenic mutations in a stepwise manner and becomes a cancer cell that clonally expands to form a tumour. (**b**) A diagram illustrating the cancer stem cell (CSC) model of cancer. A highly tumourigenic CSC sitting atop the tumour cellular hierarchy which divides asymmetrically to form non-tumourigenic cancer cells that form the bulk of the tumour, and identical CSCs that form new tumours that are similar to the original tumour

2 Cancer Stem Cells

In 1937, Furth et al. [8] first showed that a single tumour cell from mouse leukaemia could establish a tumour following transplantation into another mouse. Identification of proliferating cells by radio-labelling and autography [9] in the ensuing decades enabled measurements of cell lifespan and the assessment of cellular hierarchy in normal tissues [10]. These methods led to a rapid advancement in stem cell research. In 1960, Pierce [11] demonstrated that teratocarcinomas contained tumourigenic cells that could individually differentiate into multiple differentiated non-tumourigenic cell types, resembling normal development. In 1963, haematopoietic stem cells were discovered, and stem-like cells were reported in multiple haematological malignancies in the ensuing decade [12]. Based on investigations using many other techniques over the subsequent decades, Pierce [13] advanced an early CSC concept—"A concept of neoplasms, based upon development and oncological principles, states that carcinomas are caricatures of tissue renewal, which have a marked capacity for proliferation and a limited capacity for differentiation under normal homeostatic conditions, and of the differentiated, possible benign, progeny of these malignant cells". Evidence supporting the notion that cancer originates from CSCs has been accumulating rapidly over the past two decades. CSCs have now been identified in numerous types of solid cancers affecting all major organ systems [4, 14].

3 Identification of Cancer Stem Cells

CSCs express stemness-associated markers that are present on ESCs and display ESC characteristics such as self-renewal and pluripotency—the ability to differentiate into cells of all lineages [15]. CSCs have been identified in many cancer types [4] including cutaneous SCC (cSCC) and malignant melanoma (MM) by specific markers [16–20]. Their presence is confirmed by functional studies, such as tumoursphere formation assays, organoid systems, and xenotransplantation of sorted tumour cells into immunodeficient mice [21]. Xenograft and teratoma experiments in animals are the gold standard for functional investigations that provide evidence of CSCs, and they remain valuable and perhaps essential for applications such as safety testing of therapies. However, teratoma assay protocols are often vague and inconsistent and are not highly standardised and reproducible [22]. To determine whether a cell population includes pluripotent cells, it is considered sufficient to employ directed or spontaneous differentiation and tumoursphere formation which can be sustained over multiple passages and an analysis of pluripotency marker expression [22]. It has become more acceptable to use stemness-associated markers such as OCT4, SOX2, NANOG, SSEA4 and TRA-1-60, to identify pluripotent cells [23–26].

Many markers that are expressed on ESCs have been used to identify CSCs [27, 28]. CD44 is a cell surface marker with many functions, including the transduction of microenvironmental signals to membrane-associated cytoskeletal proteins and the nucleus, which influences the expression of genes that alter cell functions [29]. As an important regulator of CSC properties including stemness, self-renewal and metastasis, CD44 has been used as a CSC marker [29]. As it is not essential for tumour formation [30], CD44 is now considered a marker of progenitor cells, further down the stem cell hierarchy, rather than an ESC marker [27].

EpCAM, a cell adhesion molecule and a CSC marker, is expressed by nearly all carcinomas [31] including cSCC [31].

The surface marker CD133 has been used to identify CSCs in several solid cancers including glioblastoma [27] and pancreatic cancer and is associated with high tumourigenicity and metastasis [32]. Capan-1, a $CD133^{+}$ pancreatic cancer cell line derived from human pancreatic cancer, recapitulates tumours in a xenograft model [32]. As CD133 is also expressed on more differentiated cancer cells, further down the stem cell hierarchy, and given tumours can also be grown from $CD133^{-}$ cells in xenograft models, it is now considered a progenitor cell marker rather than an ESC marker [27].

Phosphorylated signal transducer and activation of transcription 3 (pSTAT3) proteins have a broad range of functions, including cell cycle signalling, cell survival, pluripotency and self-renewal capability [33, 34]. STAT proteins are activated by cytokines, and they regulate growth factor and cytokine responses [35]. Aberrant STAT3 signalling has been demonstrated in multiple types of head and neck cancers [36]. The role of pSTAT3 in pluripotency is regulated by leukaemia inhibitory factor pathway, resulting in STAT3 translocating into the nucleus and triggering the expression of the ESC markers KLF4, SOX2, SALL4 and c-MYC [37–39]. pSTAT3 is also expressed by more differentiated cells [27].

Yamanaka et al. showed that human [24] and mouse [25] fibroblasts can be induced into an ESC state [24] by introducing the transcription factors OCT4, NANOG, SOX2, KLF4 and c-MYC. Thomson et al. [26] showed that generation of such induced pluripotent stem cells (iPSCs) was also possible with NANOG and LIN28 in place of c-MYC and KLF4. These studies underscore the sufficiency of these stemness-associated markers in generating iPSCs. Expression

of these stemness-associated markers provides preliminary evidence of the presence of CSCs. Some or all of these markers have been used to identify and characterise CSCs in many cancer types [40–44] including primary head and neck cSCC (HNcSCC) [18], metastatic HNcSCC (mHNcSCC) [45], head and neck metastatic MM to the regional lymph nodes (HNmMM) [17] and metastatic MM to the brain (mMMB) [46].

The observation that stemness-associated markers SOX2, pSTAT3, CD44 and CD133 are expressed by ESCs and cells downstream of ESCs highlights the challenges of using these available markers for the identification and characterisation of CSCs [27].

4 Origin of Cancer Stem Cells

The origin of CSCs remains unclear. CSCs have been proposed to originate from normal progenitor cells that have unlimited potential to replicate and/or from normal resident adult stem cells that have acquired oncogenic mutations [47]. Differentiated non-tumourigenic cancer cells have also been shown to de-differentiate into CSCs by acquiring stemness through cellular adaptation under the influence of the surrounding microenvironmental *niche* [48]. This can occur via EMT by which the genes expressed by epithelial cells change, and the cells develop mesenchymal traits [1]. CSCs share properties of mesenchymal stem cells (MSCs), such as the ability to migrate, resist programmed cell death and degrade extracellular matrix (ECM), to facilitate cancer invasion [1]. These characteristics make the tumour cell more CSC-like. The ability for CSCs to acquire mesenchymal traits endows CSCs the ability to disseminate and form metastases [1]. The tumourigenicity of cancer cells and CSCs changes in response to environmental cues and other influencing factors, such as cancer therapy, a change in the microenvironmental *niche*, gene mutations and epigenetic factors [4].

5 Cancer Metastasis

Metastasis, one of the hallmarks of cancer, causes over 90% of cancer-related deaths [49]. There is increasing evidence showing metastasis is driven and sustained by CSCs [50], via haematogenous and/or lymphatic spread [51]. Less than 0.02% of disseminated tumour cells are capable of developing distant metastases [50], and it is theoretically possible that just one disseminated tumour cell is sufficient to initiate a metastatic lesion [52].

Metastasis is a complicated process involving multiple steps: invasion, intravasation, transport, extravasation and colonisation [53] (Fig. 2). First, tumour cells spread into adjacent tissues, invade the basement membrane and enter the blood and/or lymphatic system (known as intravasation) and then travel as circulating tumour cells (CTCs). By this stage, tumour cells have acquired the traits that enable evasion of the immune system, shear stress and survival mechanisms to escape programmed cell death such as *anoikis*, which occurs following detachment from the ECM. Disseminated tumour cells carry similar driver mutations present in the primary tumour and undergo further selection pressures at the metastatic site in the new microenvironment, known as the metastatic *niche* [49, 54]. In this complicated process, two crucial phenotypic transitions occur: EMT and mesenchymal-epithelial transition (MET) (Fig. 2). During the initial phase of metastasis, tumour cells undergo EMT, whereby epithelial cells acquire a mesenchymal phenotype that conveys increased invasiveness and migratory capacity. EMT is influenced by transcription factors such as Twist, Snail and FoxC2, as well as the cytokine transforming growth factor-β (TGF-β) [55], acting as core regulators of EMT [53]. Upon arrival at the metastatic site(s), CTCs undergo MET, a crucial process that enables cells to re-differentiate into an epithelial phenotype and form metastatic tumours [56] (Fig. 2)—a process that occurs naturally during embryogenesis when the mesoderm becomes epithelial tissue during organogenesis [57].

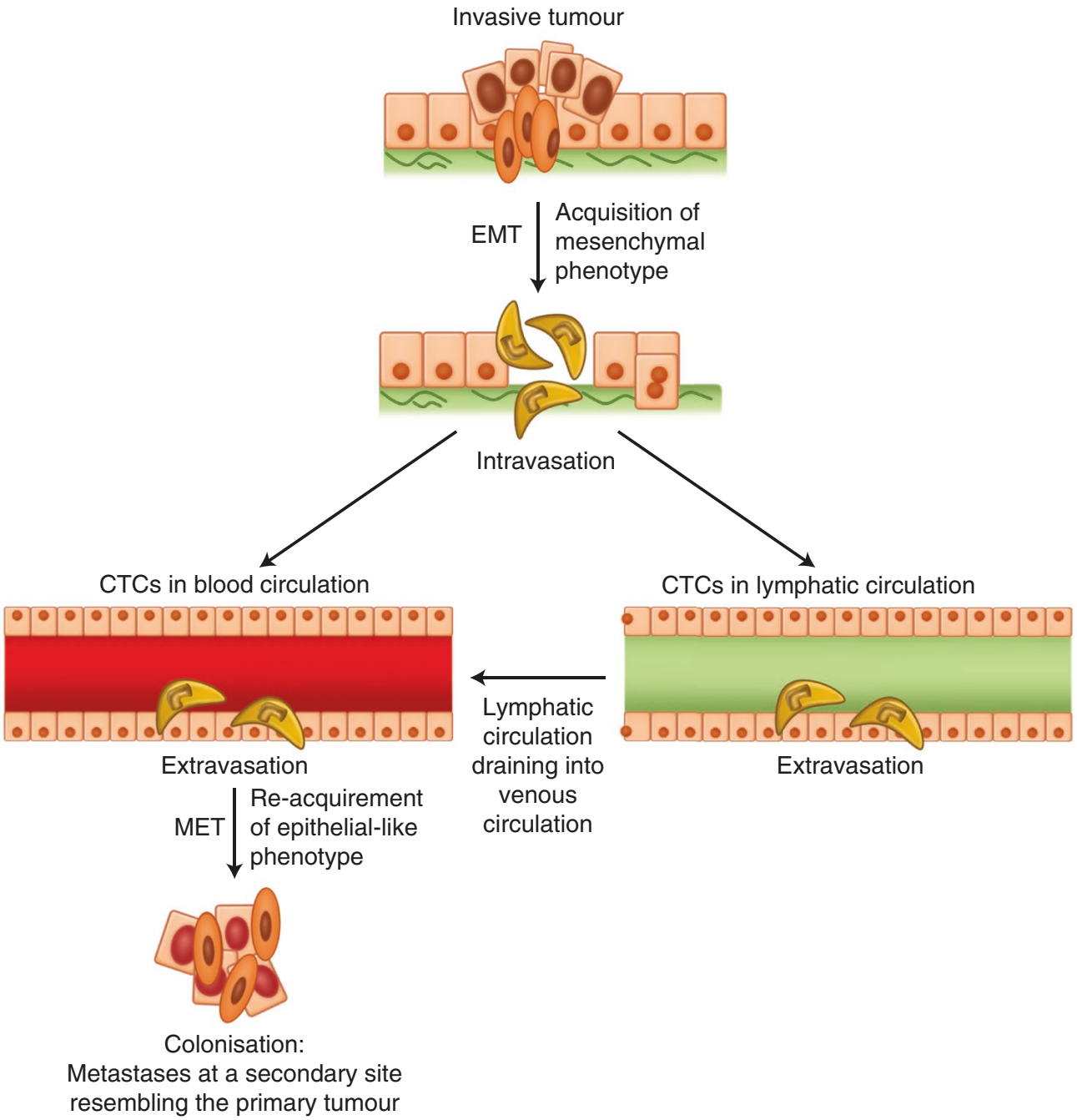

Fig. 2 A diagram showing the role of epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) in cancer metastasis. Tumour cells within a cancer undergo EMT to form mesenchymal-like cells, which undergo intravasation to enter the blood and/or lymphatic circulations as circulating tumour cells (CTCs). These CTCs then undergo MET and extravasate into distant tissue sites, where metastatic tumours may be established

6 Circulating Tumour Cells

The number of CTCs far exceeds the number of macrometastases that eventually develop [58]. Even a small tumour is capable of shedding millions of cancer cells [50]. However, many patients remain in remission or develop recurrence after a long latent period [50]. Metastases arise from complex processes involving CTCs, which bear CSC characteristics that enable them to colonise sites with a favourable microenvironmental *niche*. To be successful, CTCs need to be able to infiltrate tissue, evade immune mechanisms, adapt to a favourable microenvironmental *niche* and survive as slow cycle tumour initiating cells before eventually undergoing further genetic and epigenetic changes to form metastatic lesions [50].

As metastasis is a feature of CSCs, it is proposed that some CTCs are CSCs that form new metastatic lesions at distant sites [59]. The observation that most injected cancer cells do not form macrometastases, e.g., only 0.02% of injected melanoma cells into the portal circulation develop metastases at distant sites [60, 61], suggests a very small proportion of CTCs are circulating CSCs [62].

7 Cancer Stem Cells and Tumour Microenvironment

The observation that certain cancers preferentially metastasise to certain organs suggests the presence of an environmental *niche* within these organs that favours the formation of metastases for that particular cancer [50]. Both the CSCs—the "seed" and the surrounding microenvironmental *niche*, the "soil"—are critical ingredients for the development of metastasis [63]. The microenvironmental *niche* is a specialised set of environmental conditions that includes different elements such as cytokines, prostaglandins, growth factors, ECM components, immune cells, endothelial cells and cancer cells [49, 63]. Understanding CSCs and their environmental *niche* may lead to novel therapeutic targeting of CSCs directly, or their microenvironmental *niche*, in the treatment of cancer [47, 64].

Like adult stem cells, CSCs require input from their microenvironment to maintain a balance between self-renewal and differentiation [63]. Changes to factors influencing this *niche* affect CSC characteristics such as plasticity, tumour initiation ability, tumour progression and patient outcome [63, 65, 66]. EMT is a crucial step that enables cancer cells to acquire a CSC-like state, underscoring tumour development and progression, local invasion and distant metastasis [63]. Interaction between cancer cells and the microenvironmental *niche* can induce EMT. For example, EMT can be induced by tumour-associated fibroblasts residing in the *niche* by releasing TGF-β, which has been shown to cause proliferation of CSCs in breast cancer [67] and oral cavity SCC [68].

CSCs use signalling pathways, such as STAT3, Wnt/β--catenin, Notch, Sonic hedgehog, NF-$_{K}$B and epidermal growth factor signalling pathways [63], that regulate stem cell maintenance, self-renewal and pluripotency. STAT3 mediates signalling by the cytokines interleukin-6 (IL-6) and IL-10 released by immune cells and growth factors in the surrounding microenvironmental *niche*. When activated, STAT3 influences the expression of genes involved in tumour initiation, invasion and metastasis and angiogenesis [69]. STAT3 has also been shown to maintain CSCs and promote EMT [69]. NF-$_{K}$B is an inflammatory regulator that contributes to tumourigenesis and chemotherapy resistance [63, 70]. Activation of NF-$_{K}$B in ovarian CSCs has been correlated with drug resistance [71] and may play a role in other cancer types.

Tumour activating macrophages (TAMs), which are abundant in solid cancers [72], play an important role in the tumour microenvironmental *niche* that influences CSCs and the microenvironmental *niche* before and after tumour initiation [63]. Before tumour formation, TAMs cause DNA damage and contribute to oncogenic mutations and cancer-related inflammation by releasing inflammatory cytokines such as IL-6 and tumour necrosis factor (TNF) [63]. In advanced tumours, TAMs influence angiogenesis and immunosuppression and promote invasiveness, proliferation and survival of cancer cells [63]. Tissue macrophages have also been shown to regulate homeostasis in the haematopoietic stem cell *niche*, and it is interesting to speculate whether TAMs also influence CSCs [73]. Like CSCs, TAMs are plastic and vary in phenotype depending on their location and how they interact with the tumour microenvironment [74]. Furthermore, tumour cells release cytokines that attract cells which create an immunosuppressive microenvironment [75, 76].

MSCs residing in the microenvironmental *niche* promote tumour formation and metastasis by releasing various cytokines such as IL-6 and IL-8 [49]. Cancer cells can also release cytokines such as IL-1 to stimulate MSCs to produce

prostaglandin E2 [77], and other cytokines, to activate β-catenin signalling and facilitate CSC formation [78].

If CSCs play a crucial role in metastatic seeding, then measures that target them at diagnosis of the primary tumour, or factors that contribute to the pre-metastatic *niche* that support seeded CSCs, may improve treatment outcome [79].

The endocrine RAS (Fig. 3), classically known for regulating blood pressure and body fluid homeostasis, is an important constituent of the microenvironmental *niche* that influences stem cell maintenance and differentiation [80]. Different components of the RAS drive MSC differentiation into different cell types [81, 82]. For example, angiotensin-converting enzyme (ACE) enables expansion of haemangioblasts—multipotent haematopoietic precursor cells. Angiotensin II (ATII) receptor 1 (AT_1R) or ATII receptor 2 (AT_2R) signalling can determine the cell lineage haemangioblasts differentiate into [83], underscoring the important role of the RAS in determining stem cell fate. The RAS also influences developmental processes such as vasculogenesis, erythropoiesis and haematopoiesis [80].

Cathepsin B and cathepsin D contribute to renin activation. Cathepsin D and chymase mediate conversion of angiotensinogen into angiotensin I (ATI). Cathepsin G promotes generation of ATII from ATI or directly from angiotensinogen [64] (Fig. 3).

The RAS interacts with stem cell signalling pathways [85]. Critically, pro-renin receptor (PRR) induces Wnt/β--catenin [85], by activating multiple genes for the RAS [86]. Wnt/β-catenin signalling is also important for normal stem cell development and cancer development [87]. For example, two of its downstream targets are the CSC markers CD44 and c-MYC [88], which regulate CSCs [89].

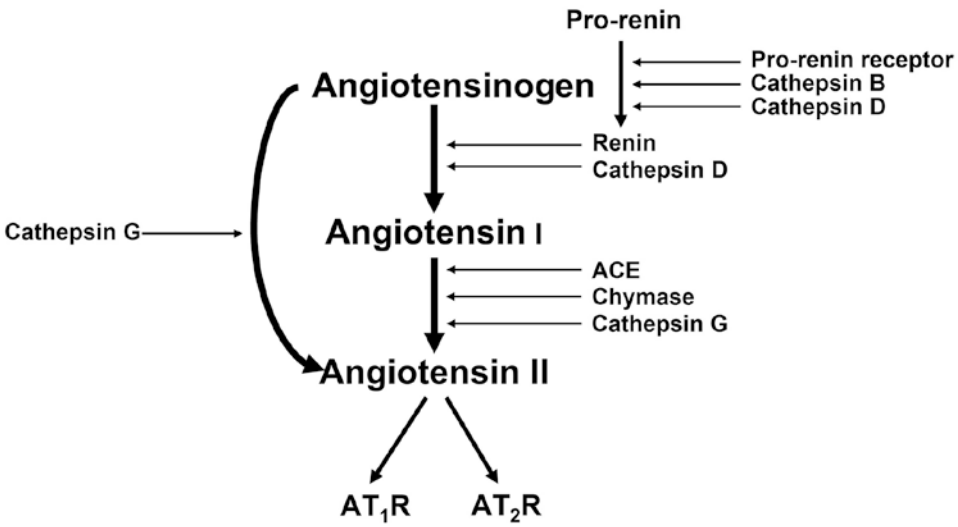

Fig. 3 A schema demonstrating the classical renin-angiotensin system, with cathepsins B, D and G and chymase, acting as bypass loops. Activation of pro-renin occurs upon binding with pro-renin receptor. Renin then converts angiotensinogen into angiotensin I (ATI), which is cleaved by angiotensin-converting enzyme (ACE) to produce the active peptide angiotensin II (ATII). The actions of ATII are mediated through interactions with ATII receptor 1 (AT_1R) and ATII receptor 2 (AT_2R). Cathepsin B and cathepsin D contribute to renin activation. Cathepsin D and chymase mediate conversion of angiotensinogen into ATI. Cathepsin G promotes generation of ATII from ATI or directly from angiotensinogen [84]

8 Cancer Stem Cells and Treatment Resistance and Cancer Recurrence and Metastasis

There remain significant shortcomings in current cancer therapies, especially for patients with cancer recurrence and metastasis [1]. CSCs sustain and drive carcinogenesis and cause loco-regional recurrence and distant metastasis [47, 90, 91]. CSCs resist chemotherapy, radiotherapy [91] and immunotherapy [92] which target rapidly dividing cancer cells. The persistence of CSCs may explain why these treatments may decrease tumour size but do not affect survival [1].

Resistance of CSCs to chemotherapy, radiotherapy and immunotherapy is multifactorial, with one factor being the relatively slow cell cycle of CSCs compared to cancer cells [93]. CSCs also sustain less DNA damage during treatment, as they accumulate less reactive oxygen species (ROS) than cancer cells [94], due to increased expression of genes that protect CSCs against ROS [1]. Another factor is the tumour microenvironment, which contains various cytokines and growth factors, such as TGF-β, that promote survival of CSCs [55]. Conventional cancer treatments increase CSC properties in cancer cells and can even convert these cells into CSCs [95]. Conventional cancer treatments exert selection pressures that increase the relative proportion of CSCs to cancer cells [55]. The enrichment of CSCs, the increase in their stemness, and the conversion of cancer cells to CSCs cause the recurrent tumour to be more resistant to treatment with a poorer prognosis than the original tumour [55].

9 Cancer Stem Cells in Cutaneous Malignant Melanoma

Australia and New Zealand have the highest incidence of MM [96, 97]. MM affected >350,000 people globally in 2015 [98] and causes 60–80% of all deaths from skin cancers [99]. MM has been assumed to arise from a mature melanocyte. However, there is increasing evidence suggesting a melanocyte stem cell [100] or CSC [101] origin.

The mainstay treatment for primary MM is wide local excision, and surgery and radiotherapy for metastatic MM [102]. Over the past decade, targeted therapies including BRAF and MEK inhibitors [103] and immune checkpoint blockers [104] have improved outcomes of patients with advanced (stages III and IV) MM, compared to dacarbazine chemotherapy [105]. Adjuvant immunotherapy with pembrolizumab has also shown efficacy for stage III MM, with a recurrence-free survival at 1 year of 75.4%, compared to 61% in the placebo group [106].

Treatment with the BRAF inhibitor vemurafenib for stages IIIc and IV MM shows a complete or partial response rate of 50% and a median progression-free survival (PFS) of 5.3 months, compared to a 7% complete or partial response and 1.6 months of PFS for dacarbazine chemotherapy [107]. However, at 8 months, the PFS is similar between the two groups [107]. For resected BRAF V600-mutant stage III MM patients, an adjuvant BRAF inhibitor regime of dabrafenib plus trametinib results in a recurrence-free survival of 54% at 4 years, versus 38% among patients receiving placebo [108]. Despite improved outcomes with immunotherapy, the 5-year overall survival for patients with stages III and IV MM are 41–71% and 9–28%, respectively [109]. Treatment failure has been attributed to the presence of CSCs which underscore cancer invasion and metastasis and treatment resistance [110, 111].

CSC subpopulations expressing some or all of the five stemness-associated markers involved in generation of iPSCs [17] have been identified in HNmMM [17] and mMMB [46]. A Melan-A^+ and a Melan-A^- subpopulation expressing one or more of the ESC markers OCT4, SALL4, SOX2 and NANOG, and a $pSTAT3^+$ subpopulation localising to the endothelium of the tumour microvessels have been demonstrated in mMMB [46]. In HNmMM an $OCT4^+$/$SOX2^+$/$KLF4^+$/$c\text{-}MYC^+$ CSC subpopulation has been demonstrated within the tumour nests (TNs) and the peritumoural stroma (PTS) with some of these CSCs also expressing NANOG [17]. Cells derived from HNmMM express these stemness-associated markers and form tumourspheres in vitro [17]. Zimmerer et al. [112] demonstrated in vitro tumoursphere formation capacity, a core feature of CSCs, by the metastatic melanoma cell lines Na8, D10 and HBL. Other established cell lines from metastatic MM also demonstrated typical features of CSCs [112]. Cells derived from human MM can undergo melanogenic, adipogenic, chrondrogenic and osteogenic differentiation [113]. Furthermore, the self-renewal capability of MM CSCs is preserved both in vitro and following xenotransplantation into mice [101].

10 Cancer Stem Cells in Cutaneous Squamous Cell Carcinoma

cSCC makes up 15–25% of all skin cancers [114], with its incidence rising rapidly globally [115]. It is the second most common skin cancer, affecting 118/100,000 people in New Zealand [116]. Risk factors for cSCC include European descent, pale complexion, immunosuppression and advancing age [114, 115]. 60% of cSCC occurs in the head and neck region with a 2% risk of metastasis, mostly to the parotid and/or neck nodes [117]. The 5-year survival for metastatic head and neck cSCC (mHNcSCC) is 34–48% despite intensive treatment with surgery and adjuvant radiotherapy [115, 118, 119]. This poor outcome has been attributed to the presence of CSCs [120].

Several CSC markers have been used to identify CSCs in cSCC including the five aforementioned transcription factors involved in the generation of iPSCs [18, 45], CD133 [19], CD49f [121] and CD44 [122].

$CD133^+$, but not $CD133^-$ cancer cells from primary cSCC recapitulate cSCC histology and the heterogeneous tissue hierarchy, and demonstrate self-renewal capacity, upon xenotransplantation into mice [18].

The transcription factor SOX2 is the most abundant stemness-associated marker expressed by cSCC in mice [123]. Tumour formation from chemical-induced tumour carcinogenesis significantly decreases following the deletion of *SOX2*, and ablation of $SOX2^+$ cells leads to tumour regression [123].

CSCs have been identified in both primary HNcSCC [18] and mHNcSCC [16] and many other cancer types [124]. An $OCT4^+/NANOG^+/SOX2^+/KLF4^+/c\text{-}MYC^+$ CSC population within the TNs and the PTS and an $OCT4^+/NANOG^-/SOX2^+/KLF4^+/c\text{-}MYC^+$ in the PTS have been demonstrated in primary HNcSCC [18]. An $OCT4^+/NANOG^+/SOX2^+/KLF4^+/c\text{-}MYC^+$ in the TNs and the PTS and an $OCT4^+/NANOG^-/SOX2^+/KLF4^+/c\text{-}MYC^+$ CSC subpopulation in the TNs have been demonstrated in mHNcSCC [16]. Primary cell lines derived from mHNcSCC tissues that express these stemness-associated markers form tumourspheres in vitro [17].

CSC properties can be induced in cSCC by inhibition of PTEN—a protein encoded by the tumour suppressor gene *PTEN*, by the microRNA has-miR-142-5p [19]. Hsa-miR-142-5p can induce CSC characteristics, suggesting this microRNA may be a potential therapeutic target [19].

11 Cancer Stem Cells in Basal Cell Carcinoma

Basal cell carcinoma (BCC) is the most common skin cancer [125], comprising 65–75% of all skin cancers [126, 127]. BCC was thought to arise from the epidermal basal layer [125]; however, more recent studies suggest a follicular origin [125]. Stem cell markers including CD34, Bmi-1 and p63 have been demonstrated on BCC [125]. However, currently available stem cell markers are unable to confirm an adult somatic follicular stem cell or an intrafollicular CSC origin [125]. BCC may arise from resident follicular adult stem cells that acquire oncogenic mutations in a stepwise fashion or from a CSC residing in the follicle. However, the presence of CSCs within BCC remains to be proven.

Vismodegib, a targeted therapy for advanced BCC, targets the Sonic hedgehog pathway, which plays an important role in embryonic development, and is active in stem cells, follicular cells and skin cells [128]. The transmembrane receptor Patched (PTCH) normally inhibits Smoothened (Smo) resulting in suppressor of fused (Sufu) inhibition of the transcription ability of Glioma-1/2 (Gli-1/2) [128]. PTCH suppression of Smo ceases, in the presence of a mutation affecting PTCH or Smo, or when hedgehog ligand is present, causing inhibition of Sufu and release of the transcriptional ability of Gli-1/2 [128]. Vismodegib blocks the Sonic hedgehog pathway by inhibiting Smo, which causes suppression of Gli-1/2 transcriptional activation [128]. Inhibition of this pathway utilised by stem cells results in an overall response rate (ORR) of 50% for patients with metastatic BCC. Of the 15 patients with locally advanced BCC treated with vismodegib, two had a complete response, seven had a partial response, four had stable disease, and two had progressive disease—an ORR of 60% [128]. Given the therapeutic benefit of vismodegib results from inhibition the Sonic hedgehog pathway that regulates stem cells, it is interesting to speculate the presence of CSCs in BCC. Further research into the presence of CSCs in BCC is warranted.

12 The Renin-Angiotensin System, Its Bypass Loops and Novel Cancer Treatment

The endocrine RAS (Fig. 3) regulates cardiovascular homeostasis. Pro-renin is converted to renin upon binding to PRR. Physiologically, renin, released by the kidneys in response to reduced blood volume or blood pressure, cleaves angiotensinogen to form angiotensin I (ATI), which is converted to ATII by ACE. ATII exerts its effects by binding to AT_1R and AT_2R. Binding of ATII to AT_1R causes vasoconstriction to increase blood pressure, whereas binding of ATII to AT_2R causes vasodilation [80]. A local RAS that acts in an autocrine and paracrine fashion is also present in multiple tissue types [129], including the kidney [129], infantile haemangioma (IH) [130], vascular malformations [131, 132], microvessels in fibrotic conditions [133, 134], and cancer [47, 135, 136].

The RAS plays a critical role in carcinogenesis, and numerous studies have demonstrated its involvement in many cancer types [80]. For example, propranolol, which blocks renin, inhibits the growth of breast cancer in vivo [137], and ACE inhibitors (ACEIs) prevent tumour growth and invasion in different cancer types [80]. Similarly, angiotensin receptor blockers (ARBs) inhibit development of various cancer types [80]. The effect of different RAS modulators on tumour growth, invasion and metastasis in many cancer types warrants further investigation into repurposing these medications for cancer treatment [47, 80].

As RAS inhibitors (RASIs) are commonly used in the treatment of hypertension, their effects on cancer have been observed [80]. An early study demonstrated the association between administration of ACEIs and reduced risk of devel-

oping certain cancers, especially those affecting women [138]. There has been extensive epidemiological data on reduced cancer risk associated with RASIs depending on the cancer types, cohort characteristics and the type of RASI [80]. However, several large meta-analyses have shown mixed results, which may be attributed to the methodologies of the studies included in the analyses [80]. A recent meta-analysis of 55 studies demonstrates significant improvements in overall survival (HR = 0.82; 95% CI: 0.77–0.88; $P < 0.001$), PFS (HR = 0.74; 95% CI: 0.66–0.84; $P < 0.001$) and also disease-free survival (HR = 0.80; 95% CI: 0.067–0.95; $P = 0.01$) in patients taking ACEI and ARB compared to those who did not [139]. In subgroup analyses, a better overall survival in patients with head and neck SCC (HR = 0.38; 95% CI: 0.12–1.20; $P < 0.10$) and MM (HR = 0.41; 95% CI: 0.10–1.68; $P = 0.22$) is associated with ACEI and ARB use, compared to non-users [139].

Components of the RAS are expressed by CSCs in many cancer types [140–143], including primary HNcSCC [144] and mHNcSCC [145], mHNMM [146] and mMMB [136]. Cathepsins B, D and G, which constitute bypass loops of the RAS, are expressed by CSCs in metastatic colon adenocarcinoma to the liver [135], oral tongue squamous cell carcinoma [147], glioblastoma [148] and HNcSCC [149]. It is interesting to speculate that the RAS acts in a paracrine fashion within the microenvironmental *niche* to influence CSCs, as with tumour stem cells in IH [130, 150].

Several clinical trials targeting the RAS, its bypass loops and other converging pathways are underway for multiple cancer types. A phase II clinical trial on patients with metastatic renal cell carcinoma treated with perindopril (an ACEI) or candesartan (an ARB), combined with other agents such as cyclooxygenase-2 (COX-2) inhibitor, demonstrates stabilisation of metastatic disease and reduced cancer recurrence [151]. A randomised clinical trial demonstrates reduced biomarkers of invasion and inflammation in patients with breast cancer treated with propranolol, combined with a COX2 inhibitor, which is well tolerated [152].

The RAS with its multiple components, the presence of bypass loops providing redundancy and the convergent signalling pathways onto the RAS with crosstalk require a system-wide approach using a combination of medications to provide optimal blockade of the RAS to target CSCs [47, 80]. This novel therapeutic approach has been shown to be safe and well tolerated with a trend towards improved survival in a phase I clinical trial on glioblastoma [153].

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