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The Role of the “Cancer Stem Cell Niche” in Cancer Initiation and Progression

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Cancer stem cells: An introduction

Adult stem cells, also known as progenitor cells, have two major ascribed functions: (1) to replenish tissues throughout normal growth and development and (2) to repair tissues following damage by disease or injury. Classically, a stem cell is defined as possessing the capacity for self-renewal and potency. Self-renewal requires a stem cell to be able to divide in such a way as to maintain the pool of stem cells in an undifferentiated state, while potency (commonly referred to as pluri- or multi-potency) requires a stem cell to retain the capacity to differentiate into an array of specialized cell types. Cancer stem cells (CSC) represent a subset of tumour cells that exhibit the same properties as normal adult stem cells; namely the ability to self-renew, undergo asymmetric cell division and differentiate into a diverse range of cell types. In addition, the CSC population has the capacity to initiate tumours and has also been implicated in metastatic spread and in resistance to conventional anti-cancer therapies.

The majority of cells within a tumour are unable to sustain tumour growth and cannot initiate tumour establishment at secondary locations. The small population of cells that are inherently tumorigenic and commonly have a metastatic phenotype are referred to as CSC. CSC were initially identified and characterised in acute myeloid leukaemia (AML) [1]. In this study, a minority of the total tumour cell population (0.01-1%) was identified as having the unique capacity to induce leukemia following transplantation into immunodeficient mice implying that these cells alone had a tumour-initiating capacity. Following from this study, the ability

of cells to cause tumour development in immune-compromised animals has been used both as proof-of-principle for the existence of CSC and a means of identifying CSC or tumour-initiating cells. Since the initial discovery of CSC, our knowledge and understanding of this area has increased exponentially, with CSC identified in an array of solid tumours beginning with breast cancer [2] and glioma [3, 4] and subsequently extending to prostate [5], pancreatic [6], melanoma [7], liver [8] and head and neck [9] cancers, among others.

As discussed throughout this chapter, the reliance of the CSC on their specific niche is a current area of research focus. Importantly, as our understanding of the signalling pathways and specific interactions that modulate and maintain CSC function (and subsequently tumour initiation and development) increases, we will become better placed to formulate novel treatment modalities that will not only target the tumour cells for destruction but also focus on abolishing integral factors within the supportive CSC niche. Novel treatments such as these will aim to eradicate residual CSC, resulting in a decrease in tumour recurrence following therapy and better overall prognosis for cancer patients.

2. CSC markers

The isolation of CSC from total tumour cell populations has been made possible due to the identification of CSC-specific markers. The use of these markers to classify and identify tumour-initiating cells and circulating tumour cells has allowed the investigation of the importance of CSC in the developing tumour, particularly in metastasis, drug resistance and patient prognosis. However, due to the similarities in cell surface phenotype and marker expression between CSC and normal adult stem cells, further research is needed to aid in our ability to distinguish between malignant stem cells and those required for normal tissue regeneration processes. Ideally, in the future, CSC-specific markers will be used to therapeutically target CSC for eradication. Notably, inroads have already been made in this area.

CD44 has been identified as a CSC marker and its expression has been associated with high levels of metastasis, tumour recurrence and poor outcome in breast cancer, all factors associated with CSC sub-populations within tumours. Although it has been well characterised, there is some controversy regarding the specificity of CD44 to CSC as the full-length CD44 protein is widely expressed. However, recent studies have identified CSC-specific expression of a particular splice variant of CD44 [10, 11]. Aldehyde dehydrogenase isoform 1 (ALDH1) is another commonly used marker of CSC from a range of cancer types [12, 13], however similarly to CD44, ALDH1 expression is also associated with normal haematopoietic stem cells and therefore can be used as a marker of both normal and malignant stem cells [14]. A range of other markers have also been identified, with some CSC markers, including CD133, CD44 and ALDH1, remaining consistent across a number of tumour types. These are summarised in Table 1.

Tumour Type	CSC Phenotypic Markers	Selected References
Breast	CD44 ⁺ CD21 ^{-/low} Lineage ⁻ ALDH1 ⁺ CD133 ⁺ α_6 -integrin	[2, 13, 15, 16]
AML	CD34 ⁺ CD38 ⁻	[1]
Liver	CD133 ⁺ CD49f ⁺ CD90 ⁺ CD44 ⁺	[8, 17, 18]
Lung	CD133 ⁺ CD90 ⁺ ALDH1 ⁺	[19-21]
Glioma	CD133 ⁺ Nestin ⁺ CD90 ⁺ α_6 -integrin	[3, 4, 22-24]
Colon	CD133 ⁺ CD44 ⁺ CD24 ⁺	[25, 26]
Prostate	CD133 ⁺ CD44 ⁺ $\alpha_2\beta_1^{\text{high}}$ ALDH1 ⁺	[5, 27]
Pancreatic	CD44 ⁺ CD24 ⁺ CD133 ⁺ ALDH1 ⁺ EpCAM ⁺ Nestin ⁺ +ABCG2 ^{high}	[6, 28-30]
Melanoma	CD20 ⁺ CD166 ⁺ CD133 ⁺ Nestin ⁺ CD271 ⁺	[7, 31, 32]
Head and Neck	CD44 ⁺ CD133 ⁺ ALDH ⁺	[9, 33]

Table 1. Representative cell surface phenotypic markers for human CSCs.

3. CSC niche components and function

The function and maintenance of stem cells is highly reliant on the specific anatomical and physiological location in which these cells reside. This highly specialised microenvironment in which stem cells are located is commonly referred to as the stem cell niche. The niche is made up of stromal support cells, soluble factors, blood vessels and extracellular matrix proteins that have both direct and indirect effects on stem cell number, proliferation, self-renewal and fate determination. The niche is essential for the maintaining the balance between pro- and anti-proliferative signals to ensure a controlled stem cell environment. In fact, stem cells outside of their defined niche are reported to have limited function as they are highly reliant on cell-cell interactions and signals within their local microenvironment for their basic proliferative and tissue renewal properties [34]. Importantly, in the context of CSC, the niche plays an important role in maintenance of stem cell function, tumour initiation and protection against chemotherapeutic agents. The reliance of CSC on the niche is becoming increasingly evident as studies have shown that while CSC-like cells can be isolated from cancer cell lines *in vitro*, these cell populations are difficult to maintain in an *in vitro* setting [35, 36]. In contrast, xenograft models have proven to be effective in faithfully recapitulating the characteristics of the CSC as found in the original tumour [37]. This is likely due to the ability of the CSC to grow within a supportive tumour microenvironment, or niche, and as such respond to cellular interactions and local signalling pathways.

The CSC niche may be derived through one of two mechanisms; either the CSC specifically manufacture the niche through the production of numerous factors that signal between stromal cells of the local microenvironment and the CSC themselves or, conversely, the CSC utilise the pre-existing, tissue-specific stem cell niche. The latter option results in the CSC

“hijacking” the niche that would normally modulate the normal growth and development of local stem cells. It is clear that in both normal stem cell and CSC development and maintenance, there is a mutual dependence between the stem cells and their niche.

As discussed in detail throughout this chapter, both the CSC themselves and the stromal cells of the CSC niche secrete a variety of factors and signalling molecules to modulate pathways that are normally involved in growth and development. Together, these factors function to maintain CSC, support the maintenance of the CSC niche and promote tumour development. In addition, physiological consequences of tumour formation, such as the establishment of a hypoxic microenvironment and the subsequent induction of angiogenesis and neovascularisation, can result in a positive feed-forward effect on CSC. Furthermore, not only do CSC rely on factors expressed within the niche to ensure the maintenance of the CSC population, but the CSC themselves can regulate the composition and function of the niche [38, 39]. In this manner, the CSC and the niche function together to create a positive signalling loop to maintain a state conducive to the growth and further development of tumours.

4. CSC and metastasis

The metastatic potential of tumour cells relies not only on genetic alterations and expression of factors from the tumour cell itself, but also on interactions with structural, soluble and cellular components of the extracellular matrix (ECM) and stromal tissue compartment. These signals from the stromal microenvironment are required for the formation of what is termed the “pre-metastatic niche” and similarly to the CSC niche at the site of primary tumour establishment, this niche is a highly specialised microenvironment that aids in the migration, homing and colonisation of tumour cells, as well as subsequently enhancing tumour cell proliferation and disease development.

4.1. Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) was originally described as an important process in early embryonic development, and has also been described as a significant feature in a number of cancers. Molecular markers of EMT include an increase in the expression of N-cadherin and vimentin coupled with decreased expression of E-cadherin, increased accumulation of β -catenin within the nucleus, increased secretion of matrix metalloproteinases (MMPs) and increased expression and activity of a number of transcription factors including SLUG, SNAIL and TWIST. Cells that have undergone an EMT exhibit a loss of epithelial cell polarity and intracellular adhesion accompanied by reorganisation of the cytoskeleton, which together results in an increased capacity for migration, invasion and cell scattering (reviewed in [40]). EMT in tumorigenesis has been described in detail in recent years with a large number of studies supporting a role for EMT in tumour progression, particularly the process of metastasis. In addition, the EMT process has been closely associated with the formation, and subsequent behaviour, of CSC populations. A number of different stimuli regu-

late and drive EMT both in development and disease. These include: signalling through specific pathways (e.g. TGF β , Wnt, Notch and Hedgehog), direct cellular interactions and exposure to hypoxic conditions – all factors that also play key roles in the maintenance of CSC (as discussed throughout this chapter).

The process of EMT and the maintenance of CSC are closely linked, with the induction of EMT enriching for a CSC-like population. This has been observed by multiple means, including the observation that metastatic cells exhibit both an EMT- and CSC-like phenotype. Experimental evidence has demonstrated that circulating tumour cells in both prostate and breast cancers express both CSC and mesenchymal markers [41, 42]. In addition, induction of EMT in human mammary epithelial cells results not only in the acquisition of mesenchymal markers, but also the expression of a CSC phenotype, for example CD44⁺/CD24^{-/low} in breast cancer stem cells. The opposite is also seen to be true, with CSC-like cells expressing markers similar to those found on cells that have undergone an EMT, including decreased expression of E-cadherin and increased expression of N-cadherin, vimentin and Twist [43]. Notably, within a non-CSC population, the activation of EMT can in fact revert cells back to a CSC-like state, suggesting that this transition is a key player in modulating CSC function. It is likely, therefore, that the CSC population does, in fact, make up a large proportion of the tumour cells that have undergone EMT and as such can be found in the peripheral circulation and therefore play an integral role in the establishment of secondary/metastatic tumours. This has been demonstrated specifically in prostate cancer, with the vast majority of circulating tumour cells (those likely to be responsible for colonisation of distant metastatic sites) expressing both the stem cell marker CD133, as well as a range of mesenchymal-associated proteins [42].

4.2. Migration and invasion

Recent studies suggest that the CSC population within tumours, although comprising a very small percentage of the total tumour, are critical for metastatic colonisation and have been shown to initiate tumour growth at secondary sites [44, 45]. The ability of cells to migrate and invade is closely linked with their intrinsic ability to metastasise and colonise secondary tumour locations. The mechanisms utilised by CSC to modulate their invasive capacity are similar to those employed by normal stem cells for homing and/or mobilisation. Within the haematopoietic stem cell (HSC) niche, MMPs play a role in HSC mobilisation through the proteolysis of ECM components [46]. In addition, stem cell migration within both the haematopoietic and neural systems displays a reliance on integrins [47, 48]. Specific roles for MMPs and integrins in cancer cell metastasis have also been described [49, 50]. The CXCL12/CXCR4 axis (described in detail below) is also a key player in both normal and cancer stem cell migration and invasion [51, 52].

There have been a number of recent discoveries that support a direct link between CSC and the development of metastasis in cancer, as common pathways (including EMT) have been identified as integral for both regulation of CSC and driving metastasis [53]. Furthermore, CSCs have been demonstrated to be directly involved in metastasis in xenograft models of cancer [54, 55], due largely to the enhanced invasive capacity of CSC. This can be attributed,

at least in part, to increased MMP secretion by CSC [56]. Furthermore, comparison of the genetic profiles of breast CSCs and normal breast epithelium has identified an “invasive” gene signature associated with the CSC population, which has been demonstrated to correlate with a metastatic phenotype in breast cancer patients [53, 57].

5. Extracellular matrix components of the CSC niche

The ECM has defined roles in cellular proliferation, differentiation and migration as well as tumour angiogenesis and protection from chemotherapy [58, 59] and represents an important component of the CSC niche. Receptors expressed within the ECM allow stem cells to anchor to specific locations within the niche and therefore maintain signalling and contact with other cells residing within the niche [58]. Loss of ECM contact with resident stem cells, through reducing and/or inhibiting the ECM glycoprotein components, results in a decrease in stem cell number [60, 61]. In addition, the ECM is also able to influence the behaviour of stromal cells within the niche, including endothelial cells, immune cells and fibroblasts [58]. Increased expression of specific ECM glycoproteins has been associated with various tumour types, including breast and pancreatic cancers [62-66], which in turn have been associated with poor prognosis [67]. The composition of the ECM is therefore critical in maintaining tissue and cellular homeostasis and in modulating the growth and proliferation of stem cells. Importantly, abnormal ECM dynamics compromise the role of the ECM as a physical barrier to tumour cell invasion, allowing for cellular migration and invasion. Modulation of the ECM, most commonly through enzymes such as MMPs that degrade the proteins making up the ECM, enhances cellular migration and subsequent colonisation of the pre-metastatic niche by CSC. High expression of Tenascin-C, a glycoprotein expressed within the ECM, has been linked to metastasis in a range of different cancer types [68-70]. Another component of the ECM, periostin (POSTN), is expressed by the stroma of primary tumours. Infiltrating tumour cells (i.e. cells undergoing metastasis) induce POSTN expression within the secondary target organ to initiate colonisation, a function that can be successfully inhibited by blocking POSTN [71]. The processes of cellular migration and invasion, and hence metastasis, therefore require significant modification of the ECM.

6. Stromal cells within the CSC niche

The CSC niche is composed of a range of specific cell types including fibroblasts, endothelial cells, mesenchymal stem cells (MSC) and immune cells. The “stemness” of CSC is in fact a dynamic quality that can be mediated by extrinsic cues derived from the local microenvironment. Direct cell-cell interactions between the stromal cell compartment and CSC, as well as signalling pathways mediated through the expression and secretion of a range of growth factors and cytokines play a role in the maintenance of the CSC population within the niche and in overall tumour growth.

6.1. Fibroblasts

The major cellular component of the tumour microenvironment is the stromal fibroblasts, commonly referred to as carcinoma-associated fibroblasts (CAFs). Factors that are secreted by fibroblasts within the tumour environment are able to revert differentiated tumour cells to a CSC-like phenotype, thereby maintaining the CSC population. CAFs have specifically been shown to alter the function of CSC through the expression of elevated levels of chemokine C-X-C motif ligand 12 (CXCL12) and MMP-1. The expression of CXCL12 by stromal fibroblasts within the tumour microenvironment adds to the supportive role of the niche, playing a role in the promotion of EMT in primary tumours ([72]; discussed in detail below). Coupled with the expression of MMPs, CAFs may directly stimulate the migration of CSC and therefore play a role in driving metastasis.

Tumour cells located in close proximity to stromal fibroblasts also exhibit increased expression of Wnt pathway regulated genes (as discussed below). The association between the tumour cells and their stromal environment is critical in modulating pathways and gene expression, both from the stromal cells (to create a permissive environment for CSC) and by the tumour cells (to further tumour development and CSC maintenance).

6.2. Immune cells

Tumour-associated macrophages (TAMs) represent the major immune component of stromal cells in tumour microenvironments. TAMs are identified as M2-type macrophages and as such express a range of factors that regulate matrix components resulting in remodelling of the ECM, activation of angiogenic pathways, suppression of adaptive immunity and enhancement of tumour cell proliferation and survival. Together, these functions cooperate to promote tumour development (Reviewed in [73]). TAMs are recruited to sites of tumour formation due to the expression of chemokines by the tumour cells. It has recently been suggested that the CSC population plays a significant role in the recruitment and modulation of TAM within the CSC niche. TAM recruitment factors, including soluble colony-stimulating factor 1 (sCSF-1), transforming growth factor beta (TGF β) and macrophage inhibitory cytokine 1 (MIC-1), are specifically expressed by tumour-derived CSC [74]. In glioma, the presence of TGF β -expressing TAM at the invasive tumour front has been correlated with the presence of glioma CSC and these CSC are shown to have a greater invasive potential in the presence of the TAMs [75]. In addition, CSC are able to promote the expression of IL-6 by TAMs [76], hence further enhancing the pro-growth signalling for CSC within the local environment. The specific depletion of TAMs in pancreatic cancers results in a concomitant decrease in the number of CSC [77]. It is apparent that the immune cell component of the CSC niche is important in the regulation of CSC number and activity in tumours.

6.3. Endothelial cells

The CSC population is commonly located in what has been termed a "perivascular" niche [78-80], which can best be defined by its anatomical/physical location directly adjacent to the endothelial cells comprising the blood vessels. Indeed a number of studies have shown that

the removal of endothelial cells from the perivascular niche results in a reduction in CSC number, suggesting an absolute reliance on the cellular composition of the niche for maintenance and survival of CSC. Vascular endothelial cells interact directly with CSC and secrete a range of factors that influence both tumour growth and CSC maintenance. A number of studies have identified a range of secretory molecules that are produced by endothelial cells and subsequently support the growth, self-renewal and migratory capacity of CSC, including Bmi-1, Jagged-1, vascular endothelial growth factor (VEGF), interleukin 6 (IL-6), interleukin 8 (IL-8, also known as CXCL8) and epidermal growth factor (EGF) [78, 80-84].

6.4. Mesenchymal stem cells

Mesenchymal stem cells (MSC) are multi-potent stromal cells with the capacity to differentiate into osteoblasts, adipocytes and chondrocytes to make up bone, fat and cartilage tissues respectively. MSC are most commonly found within the bone marrow compartment; although, they have also been shown to reside in other tissue types. MSC have been implicated with a role in tumour progression. This has been described in haematological cancers, with an increase in MSC within the bone marrow being associated with myeloma disease [85]. In addition, a role for MSC in tumour development has been described in a number of solid cancers, including breast [86, 87], colon [88], lung [89] and prostate [90]. MSC injected into tumour-bearing mice exhibit preferential homing to the site of cancer formation and the presence of MSC within the local tumour environment was shown to accelerate tumour growth [86, 87]. The presence of MSC within the local tumour microenvironment was specifically shown to promote an increase in the CSC population, which is likely due to the expression of pro-proliferative factors by MSC, including CXCL12, IL-8 and IL-6 [87, 91-93]. These studies suggest that signalling between resident CSC and the MSC located within the niche results in enhanced proliferative and self-renewal signals.

7. Angiogenesis and hypoxia

Angiogenesis is a key player in the development of tumours and is defined by the proliferation of blood vessels within and around areas of established tumour, and aids in the supply of nutrients and oxygen to the malignant cells. In addition, as discussed above, vascular endothelial cells play an important role in maintaining the CSC niche. Exposure to hypoxic conditions, as are present in many solid tumours, results in the stabilisation of hypoxia inducible transcription factors (HIFs), which in turn mediate angiogenic pathways and stimulate neovascularisation within the tumour and at distant, metastatic sites. Angiogenesis is further regulated by a number of growth factors including VEGF and EGF.

The CSC population within the tumour, which (as previously discussed) has the potential to initiate metastatic growth at a distant site, will undergo an EMT in response to the hypoxic conditions present in the tumour microenvironment [94]. HIF-1 α has multiple roles within the tumour microenvironment, functioning as a master regulator of angiogenesis in hypoxic conditions, maintaining the CSC niche and directly modulating the CSC population. HIF-1 α

promotes EMT in a number of human cancers through the increased expression of EMT drivers (e.g. SNAIL) and the concurrent reduction of epithelial markers (e.g. E-Cadherin) [95]. As has already been discussed, the CSC population has distinct similarities to cells that have undergone an EMT, and as such it is plausible that this hypoxia-mediated EMT may also directly affect the CSC population. Indeed, the CSC population isolated from tumours following continuous cycles of hypoxia and re-oxygenation exhibit both stem-like and EMT-like phenotypes [94]. In addition, hypoxia is a critical physiological component of the CSC niche which functions to increase CSC number and maintain their stem-like state allowing for subsequent growth and metastasis [94, 96]. These effects are likely due to the aforementioned stabilisation of HIF-1 α , which has a direct effect on CSC, enhancing their self-renewal capacity, promoting cellular proliferation and increasing the tumorigenic properties of these cells [94, 96, 97]. Together, these studies outline an important role for the hypoxic microenvironment in establishing and maintaining the CSC niche.

In addition to the hypoxic environment and subsequent angiogenic induction having a pro-growth effect on CSC, CSC are also able to promote angiogenesis. This can occur via both direct and indirect mechanisms. Firstly, CSC differentiate into cells of the vascular endothelium, thereby functioning to create their own niche [39, 98]. Secondly, CSC secrete factors that promote angiogenic induction. The CSC component of tumours exhibit a much greater expression of the pro-angiogenic factor VEGF compared to the non-CSC population and transplantation of these cells results in the formation of highly vascularised tumours *in vivo* [38, 39]. Furthermore, expression of VEGF by vascular endothelial cells acts directly on CSC to promote proliferation and stemness [78, 99]. VEGF expression is also regulated by EMT and as such provides a possible link between the VEGF-mediated angiogenesis and EMT-induced cancer stemness that is commonly observed in relation to CSC [100].

8. WNT/Notch/Hedgehog pathways

The Hedgehog (Hh), Wnt and Notch signalling pathways are involved in normal development and have also been implicated in tumour biology. These pathways have specific roles in CSC maintenance and differentiation, as discussed in detail below. The CSC niche modulates expression of these pathways, highlighting the importance of extrinsic effects of the microenvironment on signalling pathways within the CSCs.

Increased activity of the Hh pathway is associated with normal adult stem cell development, with down-regulation of the pathway components common following stem cell differentiation. Similarly, increased expression and activation of the Hh pathway is a common feature of CSC and has been described as an essential signalling component in CSC self-renewal and tumour-initiating properties [101-105]. These studies outline a role for the Hh signalling pathway in increasing CSC number and regulating the expression of CSC-related genes. Furthermore, a reliance on Hh signalling has been demonstrated for the recurrence and metastasis of xenograft tumours, most likely through the induction of EMT [105].

Activation of the Wnt signalling pathway is common throughout numerous developmental processes and is usually associated with the promotion of cell growth. This occurs through ligands binding to specific cell surface receptors, including Frizzled and Lrp5/6, which in turn mediates the inhibition of GSK3 β and the subsequent accumulation of β -catenin within the nucleus. Therein, β -catenin functions as a transcription factor, modulating the expression of a range of genes that promote cellular growth and proliferation, including MYC [106], IL-8 [107] and Cyclin D [108, 109]. It therefore follows that abnormal or constitutive activation of the Wnt pathway will result in continuous signalling which promotes cell proliferation. Consequently, the Wnt inhibitors DKK1 and sFRP play a key role in regulating this pathway to ensure a balance in proliferative signals is maintained.

Wnt signalling is known to play a role in promoting normal adult stem cell activation and expansion, a function which is kept in check by the presence of specific Wnt inhibitors [110, 111]. In addition, bone morphogenic proteins (BMPs), which in general function to inhibit cell growth, work in concert with the Wnt signalling pathway to provide a balance between anti- and pro-growth signals to regulate stem cell self-renewal. However, in the context of cancer, this balance can be disrupted, through both loss of BMP signalling or aberrant activation of Wnt signalling, leading to uncontrolled proliferation of CSC [112]. The increase in Wnt signalling activity in tumour cells has been associated with cells in close proximity to the stromal fibroblasts, suggesting that modulation of this pathway may be through extrinsic factors and as such a niche-dependent function [113].

The Notch signalling pathway has also been associated with increased growth and tumorigenicity of CSC. High levels of Notch are observed in CSC populations, which have been demonstrated to lead to EMT and increased capacity to form tumours upon transplantation into immune-compromised animals [114–116]. Silencing Notch pathway signalling in CSC results in reduced growth, migration and invasion as well as enhanced apoptotic induction [117]. Furthermore, inhibition of Notch signalling has been demonstrated to inhibit tumour growth and, more specifically, reduce the number of CSC in a number of cancer types including colon and brain [115, 118, 119]. In addition, expression of hairy and enhancer of split 1 (Hes1), a target of the Notch signalling pathway, correlates with the expression of stem cell markers in colon cancer and is associated with self-renewal and increased tumorigenicity [120]. This recent study suggests that activation of the Notch signalling pathway and subsequent expression of target genes is an important component of maintaining CSC function.

The CSC population exhibits reliance on expression and deregulation of these developmental pathways that are modulated by stromal cells of the CSC niche. Together, these findings substantiate a reliance of the CSC on specific components of the niche. Specifically, these pathways provide novel opportunities for targeting the niche and signals emanating from it in an attempt to reduce CSC number *in vivo* and hence reduce the incidence of tumour recurrence following treatment.

9. The role of the CXCL12-CXCR4 axis in CSC

CXCL12 (also known as SDF-1) is a strong chemo-attractant initially identified with a role in the attraction of lymphocytes during haematopoiesis. In addition, CXCL12 has been demonstrated to play a role in immune signalling and inflammation and hence is an important regulator of key physiological processes in both development and disease. However in recent years, knowledge of its role has greatly diversified, particularly in the area of stem cell regulation and homing. The cognate receptor for CXCL12, CXCR4, is highly expressed on cells of the haematopoietic lineage, thereby promoting the localisation of HSC within their specific niche [121, 122]. Importantly, CXCR4 is also expressed on a range of stem cells including endothelial, haematopoietic, neural and embryonic stem cells, enabling these cells to respond to gradients of CXCL12, which are generated following tissue damage and irregular physiological events such as hypoxia [123]. This results in the efficient migration and homing of CXCR4 positive stem cells to areas of high CXCL12 expression, making the CXCL12-CXCR4 axis critical for normal development and tissue regeneration and repair.

In the context of human cancers, CXCR4 is highly expressed in many tumour types [124] and has been shown to not only regulate invasion of cancer cells to metastatic sites, but to be a marker of the CSC population of multiple tumour types [125, 126]. The CXCL12-CXCR4 axis has been implicated in metastatic processes in a range of cancers, including breast, prostate, lung, colon, kidney, melanoma, brain, leukaemia and myeloma, with expression of CXCL12 highest in tissues that are common sites of metastasis, such as liver, bone marrow and lungs [127], suggesting that tumour cells hijack this mechanism of normal tissue regeneration and stem cell localisation to aid the metastatic process. In all, it is clear that the CXCL12-CXCR4 axis plays an integral role in the development of metastasis and the definition of a pre-metastatic niche. Importantly, it is the CSC population within the tumour that is likely to utilise this axis for the development of distant metastases.

10. Cytokines and CSC regulation

Cytokines are a group of small molecules that are secreted by a broad range of cells, including immune cells, endothelial cells and fibroblasts. In general, following release by cells, cytokines act through binding their specific receptors on target cells and modulating immune responses as well as regulating cell growth and behaviour. A number of cytokines have been implicated in CSC maintenance and therefore are important secreted factors present within the CSC niche.

IL-6 is a pro-inflammatory cytokine which normally functions to stimulate the immune response following injury or infection. Importantly, IL-6 is expressed by a range of cells within the CSC niche, including macrophages and stromal fibroblasts, as well as CSC themselves, and has been demonstrated to directly regulate CSC behaviour. IL-6 signalling specifically through the STAT3 pathway has been identified as a key mechanism for the modulation of CSC. STAT3 is highly expressed in CSC derived from liver, bone, cervical and brain cancers

[128]. Furthermore, inhibition of the STAT3 pathway, through use of a specific STAT3 inhibitor, has been demonstrated to reduce the formation of glioblastoma CSC [129]. IL-6 signalling is a direct regulator of breast cancer CSC self-renewal, leading to subsequent increased expression and secretion of IL-6 and a resultant feed-forward loop that functions to further enhance CSC function [130]. In addition, glioblastoma CSC are reliant on increased expression of IL-6 to mediate growth, invasion and anti-apoptotic functions [131, 132]. Conversely, the loss of IL-6 in mouse models results in reduction in gastric tumour formation [133]. These mechanistic studies provide an explanation as to why patients with advanced or metastatic cancers exhibit increased serum expression of IL-6, which in turn is correlated with poor prognosis [134, 135].

IL-6 function within the CSC niche is not strictly limited to the direct maintenance of CSC self-renewal and growth, rather IL-6 also has an indirect effect on CSC through modulation of the stromal cell composition of the CSC niche itself. In breast cancer, the production of IL-6 by CSC acts as an attractant for MSC, resulting in the migration of MSC directly to sites of breast tumour growth [87]. This is particularly important as MSC have been described to play a major supportive role for CSC within their local microenvironment, promoting both tumour growth and angiogenic induction (as described above).

Aside from IL-6, a number of other interleukins play important roles in the regulation of CSC. IL-17 is expressed by cells within the tumour microenvironment and promotes the self-renewal of CSC in ovarian cancer [136]. High levels of IL-1 have been associated with advanced metastatic disease [137, 138], suggesting that it may also play a role in modulating CSC and the metastatic niche. Notably, increased IL-1 production by TAMs has been shown to increase angiogenesis and tumour cell growth as well as enhance metastasis [139]. Similarly, increased levels of IL-8 in serum are also correlated with more aggressive cancer and subsequently poor prognosis [140, 141]. This is likely due to the ability of IL-8 to modulate CSC self-renewal, and hence tumour growth, as the IL-8 receptor (CXCR1) is found to be highly expressed on CSC [142]. IL-8 in the tumour microenvironment is secreted by endothelial cells located within the perivascular niche, providing another mechanism through which the vasculature can induce CSC growth. In addition, the expression of IL-8 can also increase the expression of the CXCR1 receptor on CSC, making the CSC even more responsive to further IL-8 signalling [81].

11. Prognosis

The identification of CSC within the bulk tumour, as made possible through the use of phenotypic markers (see Table 1) has been shown to correlate with patient prognosis and survival. The expression of CD44, a common CSC marker, has been associated with high levels of metastasis, increased incidence of tumour recurrence following treatment, and as a result, poor patient outcome. This is likely due to the key role the CSC sub-population plays in the growth and development of tumours, both at the primary site and for establishment within the metastatic niche. An increase in the CSC isolated from breast cancer patients using the

CD44^{high}/CD24^{-/low} phenotype was shown to correlate with more aggressive forms of disease and poor patient prognosis [143, 144]. A similar correlation was also noted in pancreatic cancer, with patients exhibiting increased expression of CD44 presenting with a median survival of only 10 months, compared to 43 months in patients with low levels of CD44 [145]. High ALDH1 expression was also demonstrated to correlate with poor prognosis in breast cancer [13], rectal adenocarcinoma [146] and colorectal cancer [147]. Furthermore, ALDH1 expression has also been shown to be indicative of tumour recurrence following treatment [148]. However, there remains some controversy in this area as high stromal cell expression of ALDH1 within the tumour microenvironment has been associated with improved patient outcome in breast cancer [149, 150], suggesting that use of stem cell markers as prognostic indicators must be approached with some caution. CD133 expression was correlated with metastasis in colorectal cancer patients, despite having no significant effect on overall survival in the same study [147]. However, high levels of CD133 have been demonstrated to correlate with poor clinical outcome in other studies [151, 152]. Other less commonly described markers of CSC, such as Nestin, have also been demonstrated to correlate with tumour size and lymph node metastasis in non-small cell lung carcinoma and poor patient prognosis in adenocarcinoma [153]. The analysis of CSC within a patient's tumour, through the use of defined CSC markers, may aid clinicians in defining more precise prognostic factors.

12. Resistance to therapy

Resistance to standard treatments, including radiation and chemotherapy regimens, frequently results in tumour relapse following a clinical response, due to the presence of residual tumour cells. One of the key attributes of the CSC, which highlights their importance in tumour development and maintenance, is the ability to resist chemotherapy- and radiation-mediated treatment strategies. This has been observed in breast cancer patients, with the remaining tumour cells following standard chemotherapy exhibiting both stem- and EMT-like gene expression profiles [154]. The CSC-enriched component from breast cancer cell lines also display a decreased sensitivity to radiation [155]. In addition, following radiation treatment in glioblastoma, the recurring tumours tend to exhibit a distinct nodular pattern, which is consistent with the theory of the recurring tumour arising from a clonal or sub-clonal source [156], likely due to the expansion of radio-resistant CSC. More recent studies have identified a specific increase in the percentage of CSC within the radio-resistant tumours in glioblastoma and breast cancer both in patients and in culture [38, 155, 157]. Furthermore, the CSC population is specifically involved in the generation of new tumours following treatment. This, coupled with the identification of both CSC and non-CSC cells within the recurring tumour, is supportive of a specific subpopulation of cells, namely the CSC, being responsible for the recurrent tumours.

The mechanism of therapy resistance in CSC is still largely unknown, however a number of signalling pathways and molecules have been implicated in this process. Notably, resistance is mediated by both extrinsic and intrinsic functions of the CSC, with extrinsically mediated resistance governed by the CSC niche. The tumour environment, or niche, is well-documented

ed to mediate drug-resistance. This can be mediated both through the release of soluble factors and via cell adhesion mechanisms, with the latter commonly referred to as cell adhesion-mediated drug resistance (CAM-DR) (Reviewed by [158]). Both forms of niche-mediated drug resistance are well established in haematological malignancies. The bone marrow represents the most common site of metastases in these tumours and is characterised as an environment rich in IL-6 and fibronectin, with both of these factors being demonstrated to contribute to the acquisition of drug resistance [159]. As described earlier, the CXCL12-CXCR4 axis is an integral component in CSC homing to the niche and the colonisation of the pre-metastatic niche. This axis has also been shown to be a key player in tumour drug-resistance, with CXCR4 positive cells responding to stromal cell-derived CXCL12, resulting in increased activation of Akt/PKB and ERK signalling pathways which, in turn, mediate resistance by inducing anti-apoptotic pathways [160-162]. Direct cellular interactions mediated by adhesion between integrins and their receptors (often components of the ECM, including collagen and fibronectin), have also been directly associated with a decrease in drug-induced apoptosis [163, 164]. These studies show that CAM-DR is a key feature of chemotherapy resistance in tumours that is modulated by the niche. Due to the expression of CXCR4 and a range of integrins by CSC, coupled with the key interactions observed between CSC, the ECM and the CSC niche, it is likely that these mechanisms may be involved in CSC-specific resistance pathways.

In addition, activation of signalling pathways and increased expression of key molecules within the CSC themselves, provide an intrinsic means of modulating CSC resistance. The Wnt and Notch pathways that are commonly involved in regulating stem cell growth and behaviour have shown increased activation in response to irradiation and may confer radio-resistance on the CSC population. Inhibiting the Notch pathway or specific knockdown of Notch1 or Notch2 in glioma CSC sensitises these cells to radiation [165]. Furthermore, radiation-resistant CSC exhibit increased levels of stabilised β -catenin [157, 166], which suggests abnormal activation of the Wnt signalling pathway. Further to these signalling pathways, a population of stem-like cells identified in AML patients have been shown to exhibit a higher degree of drug efflux than non-stem cells isolated from the tumour [167], suggesting a mechanism by which the CSC component of tumours may be able to escape the effects of chemotherapeutic agents. This phenomenon is likely due to the increased expression of drug transporter genes ABCG2 and ABCA3 on CSC [168]. A further mechanism through which CSCs have been demonstrated to exhibit radiation-resistance is through enhanced activation of DNA damage checkpoint responses. A study utilising glioblastoma-derived CSC, prospectively isolated by their CD133+ phenotype, showed that although DNA damage was initiated equally in both CD133⁻ and CD133⁺ cells following radiation, the CD133⁺ cells (representing the enriched CSC population) were better able to repair the damage and hence displayed a lower rate of apoptosis [38]. It was subsequently shown that the DNA damage checkpoint proteins CHK1 and CHK2 showed preferential activation in the CD133⁺ cells. In addition, these cells also showed a basal level of activation of another component of the DNA damage checkpoint, rad17. These data suggest that the CSC component of the tumour is specifically primed to respond to DNA damage caused by external stress stimuli, such as radiation, and as such exhibit increased survival following treatment. Furthermore, these

studies suggest that radiation and chemotherapy resistance may be mediated by enhanced Wnt and/or Notch signalling, enhanced DNA damage responses and differential expression of drug transporter enzymes.

The observations detailed above explain why current cancer therapies are generally ineffective – or at least why tumour recurrence is extremely common in aggressive tumour types. Coupled with differential expression of key genes and pathways that can mediate cell survival following radiation and chemotherapy, the CSC are generally slow cycling and are not targeted by conventional therapies. In addition, CSC homing to the niche and subsequent adhesion provides important support for the CSC and in turn mediates drug resistance. Therefore, surviving CSC following treatment regimens continue to grow and differentiate and are able to re-populate the tumour.

13. Targeting CSC and the CSC niche in cancer therapeutics

Tumour regression following treatment does not always correlate with patient survival, and this is due largely to the remaining treatment-resistant CSC (as described above). Therefore, directly targeting the CSC, in conjunction with existing therapeutics, may provide a novel treatment strategy to eradicate the residual CSC and hence prevent tumour recurrence. When investigating suitable target pathways, it is also important to factor in both the targeting of the CSC population directly, as well as the CSC niche that supports their growth and survival. As has been discussed throughout this chapter, the niche provides essential support for CSC and disruption of these supportive processes presents an attractive focus for the development of new therapies.

CSC rely on increased expression of the developmental signalling pathways – namely Wnt, Notch and Hedgehog – to regulate their increased growth and survival. These pathways therefore represent key targets for the development of novel treatment strategies that may significantly inhibit the growth of the CSC population. Targeting the Notch pathway as a means of specifically targeting the CSC component of tumours is reviewed in detail by Pan-nuti *et al.* [169]. Inhibition of the Notch signalling pathway has been demonstrated to be effective in reducing the frequency of CSC derived from colon, medulloblastoma, glioblastoma and breast cancer [115, 118, 119, 170]. Importantly, inhibition of Notch signalling prevented breast cancer metastases [170] and when used in combination with a common chemotherapeutic agent delayed tumour recurrence [119]. As the Hedgehog pathway represents an essential signalling component in CSC to modulate self-renewal and tumour-initiating properties, targeting the Hedgehog pathway is another feasible therapeutic option (reviewed by [171]). Proof-of-principle for Hedgehog as a therapeutic target is demonstrated as inhibition of the hedgehog pathway results in a reduction in the tumourigenic capacity of human gliomas in immune-compromised mice [101]. The resident CSC population is required for the inherent tumourigenicity of glioma cells; therefore this data supports a role for inhibition of hedgehog in directly affecting the CSC component of the tumour. Specific inhibition of the hedgehog signalling pathway using cyclopamine is effective in preventing

cancer growth as well as invasion and metastasis (another feature attributed to the CSC population) [172, 173]. Cyclopamine has also been shown to specifically reduce the number of CSC in pancreatic cancer [174], providing evidence that inhibition of the Hedgehog pathway can directly affect the CSC population. In addition to targeting the Notch and Hedgehog signalling pathways, as described here, inhibitors of Wnt signalling have also been developed in recent years and have been shown to be effective in reducing tumour cell growth [175-177]. Further development of these inhibitors and specific investigation of the effect of treatment on the CSC population is warranted.

Further studies have demonstrated a possible role for targeting a range of small molecules and proteins that play an integral role in the survival and growth of CSC. For example, the CXCR1/CXCR2 inhibitor repertaxin functions to inhibit IL-8 responses, and as such depletes the CSC component of breast cancer xenografts, resulting in a reduction in tumour growth and metastases [142]. In addition, CXCR4 antagonists have been established and are under investigation for clinical efficacy in treating leukaemia [91, 178]. Due to the high expression of CXCR4 on CSC and the role of the CXCL12-CXCR4 axis in mediating metastasis and drug resistance, these antagonists may also have a therapeutic role in targeting CSC and modulating their ability to colonise the pre-metastatic niche. The use of CSC phenotypic markers as possible additions to anti-tumour therapies is also beginning to be developed. A proof-of-principle study has demonstrated that use of an anti-CD44 antibody was able to reduce the number of tumour-initiating cells, both *in vitro* and in a xenograft model. In addition, this reduction of CSC resulted in decreased growth, metastasis and post-radiation tumour recurrence in mice [145]. In addition, targeting CD44 in AML has shown promise in eradicating the CSC population and reducing the tumorigenic capacity of cells *in vivo* [179]. The resistance of CSC to conventional therapeutics is perhaps the most critical function of CSC that must be overcome in the identification of novel therapeutic strategies. To this end, targeting critical enzymes in the DNA damage response pathway, CHK1 and CHK2, has been shown to reverse the radio-resistance observed in CD133 positive glioma CSC [38]. In addition, pharmacological inhibition or targeted knockdown of ABC drug transporter enzymes may provide useful tools to reverse chemoresistance of CSC [180, 181].

Perhaps as important as targeting the CSC specifically, is targeting the CSC niche. Disruption of critical components of the CSC niche has the potential to inhibit CSC growth and subsequently reduce tumour development and metastases. As tumour angiogenesis is supportive of CSC survival, anti-angiogenic treatments, when used in combination with cytotoxic chemotherapies, can reduce the CSC population [182]. In particular, VEGF has been shown to be expressed directly by CSC and by the tumour microenvironment and is an important molecule in regulating vessel formation as well as CSC growth. Targeting VEGF specifically with bevacizumab has been demonstrated to disrupt the CSC niche, and as such result in a reduction in the number of CSC and subsequently tumour growth [79, 183]. Specific disruption of cellular interactions between the CSC and stromal cells that comprise the CSC-and pre-metastatic-niche may also contribute to the inhibition of CSC growth and metastasis. For example, the fibronectin receptor VLA-4 is required for interactions between tumour cells and stromal cells of the pre-metastatic niche and antibodies targeting this re-

ceptor are able to prevent this association and reduce tumour burden following treatment [163, 184]. In addition, targeting this and similar interactions involving integrins in breast cancer cells can restore cells to an epithelial-like state [185, 186] and therefore may be useful in reversing the EMT which enhances the ability of malignant cells, specifically CSC, to metastasise.

14. Concluding remarks

CSC display a dependence on their niche for growth and survival. Signalling through various developmental pathways and via a range of cytokines and growth factors not only modulates CSC growth and function, but can also alter the composition of the CSC niche. The ability of tumours to metastasise is a feature of CSC, due to their enhanced capacity for migration and invasion. This process is reliant on the formation and regulation of a pre-metastatic niche, which is conducive to the colonisation of CSC and subsequent tumour growth at a secondary site. Metastasis accounts for almost 90% of all cancer-associated deaths. A better understanding of what drives tumour cells to metastasise and the changes in the host tissue and local microenvironments that may accommodate the establishment and colonisation of circulating tumour cells will increase our capacity to develop novel therapeutic agents that may inhibit or delay metastasis and as such have a significant impact on patient outcome. In addition, understanding the radio- and chemo-resistant properties of CSC has allowed us to ascertain possible mechanisms for tumour recurrence and poor patient outcome following initial tumour regression.

Described in this chapter are but a few of the possible novel therapies that target CSC-specific factors and/or components of the CSC niche. Further investigation of these, and others, as well as the development of novel agents, is still required to better understand the feasibility of direct targeting of CSC and/or their niche in cancer therapy. Furthermore, reliable biomarkers for the CSC population are required in order to accurately determine the efficacy of these treatments. Importantly, the development of these novel therapeutics would not replace existing effective treatment such as radiotherapy and cytotoxic chemotherapy, but rather would be used in conjunction with these known and proven agents. Combination therapies, such as these, as has already been demonstrated in a small number of *in vivo* studies, would better enable us to effectively inhibit the CSC and the functionality of the CSC niche, and subsequently lead to improved overall survival and reduced incidence of tumour recurrence.

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