

## Wound Healing and Cancer Stem Cells: Inflammation as a Driver of Treatment Resistance in Breast Cancer

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**ABSTRACT:** The relationship between wound healing and cancer has long been recognized. The mechanisms that regulate wound healing have been shown to promote transformation and growth of malignant cells. In addition, chronic inflammation has been associated with malignant transformation in many tissues. Recently, pathways involved in inflammation and wound healing have been reported to enhance cancer stem cell (CSC) populations. These cells, which are highly resistant to current treatments, are capable of repopulating the tumor after treatment, causing local and systemic recurrences. In this review, we highlight proinflammatory cytokines and developmental pathways involved in tissue repair, whose deregulation in the tumor microenvironment may promote growth and survival of CSCs. We propose that the addition of anti-inflammatory agents to current treatment regimens may slow the growth of CSCs and improve therapeutic outcomes.

**KEYWORDS:** cancer stem cells, inflammation, tumor microenvironment, developmental pathways

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### Introduction

Tumors have been described as wounds that do not heal.<sup>1</sup> Recently, inflammatory processes that occur during normal wound healing have been linked to the pathological state of many tumors. Normal epithelial tissue exists in a state of homeostasis where tissue regeneration is tightly regulated by epithelial stem cells located within highly specialized niches. During tissue injury, replenishment of epithelial cell loss is ensured by the proliferation of these stem cells and their progeny in response to proinflammatory cytokines.<sup>2,3</sup> Additionally, numerous potent growth factors released by macrophages and lymphocytes during wound healing promote stem cell proliferation and plasticity. Among these are morphogens that are commonly associated with embryonic development.<sup>4</sup>

Many of the factors involved in the inflammatory microenvironment have also been identified as key contributors to the cancer stem cell (CSC) niche. CSCs have been identified in a number of solid tumors and are thought to be a subpopulation of tumor cells that resemble normal stem cells and continuously undergo differentiation to populate the heterogeneous tumor. In this review, we focus on breast CSCs and highlight proinflammatory factors, which are involved in regulation of normal adult stem cells during tissue repair and have also been shown to promote survival and proliferation of CSCs in breast cancer. We propose that the presence of inflammation in the tumor microenvironment may contribute to tumor aggressiveness and treatment resistance by shifting the equilibrium between differentiated and undifferentiated tumor cells toward a CSC phenotype.



## Wound Healing, Chronic Inflammation, and the Tumor Microenvironment

Wound healing is a dynamic process that consists of an inflammatory phase followed by epithelial cell proliferation and tissue remodeling. In normal tissue, the inflammatory phase is limited, lasting only 3–14 days. Tissue injury induces immediate recruitment of neutrophils, which are later replaced by macrophages and lymphocytes. Infiltrating leukocytes play a major role in secretion of inflammatory cytokines, growth factors, and chemokines, which stimulate proliferation of progenitor cells and recruitment of keratinocytes and endothelial cells during the proliferative phase of wound healing.<sup>2</sup> At this stage, granulation tissue forms, angiogenesis is induced, and new extracellular matrix (ECM) is secreted. Epithelial cells undergo epithelial-mesenchymal transition (EMT) and migrate to the edges of the wound to impart re-epithelialization of the damaged tissue. In the final phase of wound healing, the maturation phase, wound contraction, and differentiation of fibroblasts to myofibroblasts result in the formation of scar tissue. Failure to exit the inflammatory stage results in improper tissue remodeling and is associated with impaired wound healing in many disorders including diabetes mellitus, pressure necrosis, and vasculitis.<sup>5</sup>

Likewise, chronic inflammation has been linked to tumorigenesis, tumor progression, and metastasis in many different cancers. The tumor microenvironment shares many features of a chronic wound. Infiltrating leukocytes present within the tumor and associated stroma stimulate tumor growth, invasion, and angiogenesis. Tumor-associated macrophages (TAM), tumor-associated dendritic cells (TADC), and tumor infiltrating lymphocytes are a source of proinflammatory mediators in the tumor microenvironment. Environmental stimuli, such as hypoxia and DNA damaging agents, elicit secretion of chemokines from tumor cells that recruit pro-tumor inflammatory cells and help to shape the pro-tumor immune response.<sup>6</sup> Wound healing responses associated with tissue injury have been shown to promote the growth of breast cancer (Table 1). It has recently been shown that injection of wound fluid close to the tumor site in mice bearing syngeneic breast cancer xenografts resulted in increased tumor growth.<sup>7</sup> Additionally, acute tissue injury associated with biopsy in rodent models of breast cancer is reported to promote lung metastasis.<sup>8</sup> It has recently been recognized that the abundance of inflammatory cytokines and growth factors that occur during post-therapy wound healing can also contribute to survival and expansion of resistant CSCs.<sup>9–11</sup>

## CSCs and Treatment Resistance

CSCs are a subpopulation of cells within a tumor that share features with normal stem cells in which they have the ability to self-renew and differentiate into multiple lineages.<sup>12,13</sup> Initially described in leukemia,<sup>14,15</sup> CSCs have been identified in a variety of solid cancers including brain,<sup>16</sup> breast,<sup>17</sup> prostate,<sup>18</sup> pancreas,<sup>19</sup> melanoma,<sup>20</sup> and colon.<sup>21–23</sup> Injections of small numbers of CSCs into immunocompromised mice resulted in formation of heterogeneous tumors, suggesting that these cells

are the tumor-initiating population.<sup>17</sup> According to the CSC theory, tumors, like normal tissue, are organized in a hierarchy with a small number of CSCs at the top. Only the CSC population has the ability to initiate tumors and create differentiated progeny, which lack the ability to do so, and thus contribute to the heterogeneity of tumors. This is believed to be a dynamic process that is regulated by epigenetic events<sup>24</sup> and may be induced by inflammatory factors<sup>25</sup> or cellular stress.<sup>26</sup> Thus, the number of CSCs present within the tumor is highly variable and influenced by the tumor microenvironment.

The presence of CSCs in breast cancer specimens has been associated with poor prognosis. Higher levels of CSCs, identified by the phenotype CD44<sup>high</sup>/CD24<sup>-low</sup>/ESA<sup>+</sup>,<sup>27,28</sup> and high expression of the enzyme aldehyde dehydrogenase (ALDH)<sup>29</sup> predict poor clinical outcome for breast cancer patients.<sup>30–32</sup> Investigation of patients with surgically resected invasive ductal carcinoma after chemotherapy showed that patients with CD44<sup>high</sup>/CD24<sup>-low</sup> tumor cells had shorter cumulative disease-free and overall survival, suggesting that this population may be an important factor of malignant relapse in these patients.<sup>33</sup>

Expression of CSC markers in breast cancer is predictive of metastasis.<sup>34,35</sup> Breast CSCs have been linked to metastasis through enhanced expression of EMT proteins. Investigation of gene expression patterns of CD44<sup>high</sup>/CD24<sup>-low</sup> cells revealed upregulated expression of genes associated with EMT compared to non-stem like cells.<sup>36</sup> Overexpression of the EMT-associated protein Twist, or treatment with TGF- $\beta$ , a known inducer of EMT, leads to an increased number of cells bearing CSC phenotypes in non-tumorigenic human mammary epithelial cells.<sup>37</sup> Likewise, inhibition of EMT has been shown to reduce the number of CD44<sup>high</sup>/CD24<sup>-low</sup> cells as well as limit self-renewal properties in mammosphere forming assays.<sup>38</sup> Therefore, a higher amount of EMT-related proteins may promote increases in the breast CSC population and pose a greater risk of breast cancer metastasis.

Growing evidence suggests that breast CSCs are chemo- and radioresistant. CD44<sup>high</sup>/CD24<sup>-low</sup>/ESA<sup>+</sup> breast cancer cells were found to be more resistant to chemotherapeutic agents, such as paclitaxel and 5-fluorouracil, compared to the bulk of tumor cells.<sup>39</sup> Chemotherapy has been found to be less effective on breast CSCs because of multiple mechanisms that include overexpression of ATP-binding cassette transporters (eg, ABCG2), upregulation of multidrug resistance transporters (eg, MDR1), and enhanced ability to survive and repair damaged DNA.<sup>32,40–42</sup> The size of the breast CSC population may therefore determine chemotherapeutic sensitivity. Expression of CSC markers has been shown to be predictive of response to neoadjuvant chemotherapy in breast cancer patients, further confirming the resistance of this population to standard chemotherapy.

The breast CSC population has also been shown to be resistant to radiation therapy.<sup>40,43</sup> CD44<sup>high</sup>/CD24<sup>-low</sup> breast CSCs have an increased ability to scavenge free-radicals and

**Table 1.** Comparison of signaling pathways involved in wound healing and cancer.

PATHWAY	FUNCTION IN WOUND HEALING	FUNCTION IN CANCER
Hedgehog	*Regulation of stem cell proliferation and plasticity	*Regulation of cancer stem cell proliferation and plasticity
	*Induction of EMT to promote epithelial cell migration	*Induction of EMT in cancer cells
	*Angiogenesis	*Angiogenesis
	*Re-epithelialization and wound closure	*Chemoresistance
Notch	*Cell proliferation and migration of endothelial cells, keratinocytes, and fibroblasts at wound site	*Survival and proliferation of cancer stem cells
	*Recruitment of inflammatory cells to wound site	
Wnt	*Migration of epithelial cells	*Self-renewal of the cancer stem cells
	*Self-renewal of stem cells	*Cancer stem cell plasticity
	*Progenitor cell plasticity	*Contributes to radioresistance of cancer stem cells
TGF- $\beta$	*Angiogenesis	*Angiogenesis
	*Inflammatory cell infiltration	*Expansion of cancer stem cell population
	*Fibroblast proliferation	*Fibroblast proliferation
	*Re-epithelialization and wound contraction	*Regulator of EMT in cancer cells
	*Induction of EMT	
IL-1 $\beta$ /IL-6/STAT3	*Recruitment of inflammatory cells to wound site	*Self-renewal of cancer stem cells
	*Proliferation of progenitor cells	*Cancer stem cell plasticity
	*Initiates re-epithelialization and wound closure through activation of developmental pathways	*Tumor growth and metastasis
IL-8	*Chemoattractant to recruit inflammatory cells to wound site	*Regulation of genes in cancer progression
		*Cancer stem cell proliferation
		*Chemoattractant to recruit inflammatory cells to tumor microenvironment

overcome oxidative stress.<sup>44,45</sup> Although radiation preferentially kills non-tumorigenic cells resulting in the enrichment of CSCs,<sup>40</sup> the increase in the number of breast CSCs following ionizing radiation is not fully explained by preferential killing of non-tumorigenic cells.<sup>46,47</sup> It has been proposed that differentiated cancer cells may acquire stem cell traits under certain microenvironmental stressors.<sup>48,49</sup> Radiation-induced re-programming of CSCs has been observed *in vitro*,<sup>47,50</sup> however, *in vivo* conversion of non-stem cells into CSCs may require a complex network of signals from the microenvironment and signaling changes within the cancer cells.

The initiation and progression of breast cancer rely on changes within the malignant epithelial cells as well as the tumor microenvironment. Normal and tumorigenic non-stem cells have the ability to spontaneously convert to a stem-like state.<sup>51</sup> In addition, it has been shown that *in vivo*, differentiated cells can de-differentiate/re-program spontaneously under the right microenvironment conditions.<sup>52</sup> Conversion of non-stem cells to CSCs either occurring spontaneously or therapy induced is tightly regulated by the tumor microenvironment. Thus, changes in the cellular populations or signals within the tumor microenvironment may drive proliferation and survival

of the CSC population, promoting tumorigenicity, tumor recurrence following treatment, and metastasis.

### Inflammatory Cytokines in Wound Healing and CSCs

Inflammatory cytokines play an important role in wound healing and tissue repair. Cytokines such as interleukin (IL)-1 $\beta$ , IL-6, and IL-8 are strongly upregulated during the inflammatory phase of wound healing. They help to initiate re-epithelialization and wound closure through activation of developmental pathways.<sup>3</sup> These cytokines have also been reported to be present in the tumor microenvironment and contribute to tumor growth and increased stemness of tumor cells.

IL-1 $\beta$  is one of the first cytokines to be released during response to tissue injury. It is induced by platelet aggregation and leads to the recruitment of inflammatory cells to the site of tissue injury.<sup>3,53</sup> IL-1 $\beta$  signaling stimulates the production of IL-6 and IL-8 in inflammatory cells via an NF- $\kappa$ B-dependent pathway.<sup>54</sup> Activation of IL-6 and IL-8 signaling pathways results in phosphorylation and nuclear activation of signal transducer and activator of transcription 3 (STAT3), which is a key regulator of genes involved in tissue repair and



cancer progression.<sup>55</sup> Levels of IL-6 and IL-8 at the injury site increase exponentially during the initial phases of wound healing and decrease following re-epithelialization.<sup>53</sup> Elevated levels of IL-1 $\beta$ , IL-6, and IL-8 have been associated with chronic inflammation in many disorders including rheumatoid arthritis,<sup>56</sup> sepsis,<sup>57</sup> cardiovascular disease,<sup>58</sup> and diabetes.<sup>59,60</sup> IL-8 serves as a potent chemoattractant and is required for the recruitment of inflammatory cells to the site of injury.<sup>61</sup> IL-6 also plays a role in macrophage recruitment and is important for wound closure and re-epithelialization.<sup>62</sup> In addition, IL-6 knock-out mice demonstrate impaired wound healing. Punch biopsies in these mice took up to three times longer period to heal than those of wild-type mice and exhibited delayed reepithelialization.<sup>63,64</sup> Likewise, targeted knock-down of STAT3 resulted in impaired proliferation of epidermal progenitor cells and delayed wound healing, resembling that of the IL-6 knockouts.<sup>65</sup>

In breast cancer, high levels of IL-6 and IL-8 are associated with poor prognosis.<sup>66,67</sup> Activation of these signaling pathways within the tumor cells and the tumor microenvironment has been linked to tumor growth and metastasis.<sup>68,69</sup> High levels of IL-6 are critical for NF- $\kappa$ B-mediated transformation, mammosphere formation, and tumorigenesis.<sup>70</sup> Treatment of normal mammary tissues with recombinant IL-6 or IL-8 results in increased self-renewal and expression of stem cell markers.<sup>71-73</sup> Inhibition of IL-8 receptor binding<sup>68</sup> and STAT3 activation<sup>74</sup> has been shown to decrease tumorigenicity of breast cancer cell lines. Within the tumor microenvironment, IL-6 and IL-8 are secreted by TAM, TADC, mesenchymal stem cells, and CSCs.<sup>75</sup> Activation of IL-6 in tumor cells has been shown to activate a positive feedback loop, increasing both the secretion of IL-6 and the binding of IL-6 to its receptor.<sup>72</sup> In addition, STAT3 signaling in inflammatory cells induces transcriptional activation of NF- $\kappa$ B, leading to increased release of IL-6 and IL-8 within the tumor microenvironment.<sup>76</sup> Interestingly, activation of IL-6-STAT3 signaling induces a stem-like phenotype in non-stem cells.<sup>25,77</sup> IL-6 was shown to activate Oct-4 signaling and increase xenograft formation in non-CSCs, suggesting an effect on stem cell plasticity. Thus, inflammatory signaling within the tumor microenvironment may promote changes in differentiated cancer cells by increasing expression of stem cell markers and self-renewal properties. This may represent a change in function of existing tumor cells or the emergence of CSCs within inflammatory microenvironments.

### Developmental Pathways in Wound Healing and CSCs

In addition to the initiation of inflammatory cascades, wound healing results in reactivation of developmental pathways that, in some ways, recapitulate the tissue generation that occurs during embryogenesis. Developmental pathways are activated by inflammatory factors at the beginning of the proliferative phase of wound healing and are crucial to wound closure and tissue regeneration, which occur in the later phases. Similar to wound

healing, developmental pathways can be activated in the tumor microenvironment by immune cells,<sup>78</sup> inflammatory signaling cascades,<sup>79</sup> and in response to cellular stress.<sup>80</sup> These pathways are important for the proliferation and survival of breast CSCs, as well as stimulation of angiogenesis and initiation of metastasis. Key developmental pathways that are activated during wound healing are described in the following subsections.

**Hedgehog.** The hedgehog (HH) pathway is a key morphogenic pathway involved in many aspects of embryonic development, including regulation of embryonic stem cells, tissue patterning, and angiogenesis.<sup>81,82</sup> HH pathway activation occurs when a secreted ligand (Sonic, Desert, or Indian) binds to the surface receptor Patched (PTCH). This binding releases the inhibition of the transmembrane receptor SMOOTHENED (SMO), setting off a signaling cascade that results in the nuclear translocation of the Gli family of transcription factors. Transcriptional targets for Gli include genes controlling proliferation, angiogenesis, differentiation, EMT, and survival. Recently, a role for HH in wound healing and tissue repair has become evident. Activation of HH signaling has been reported to promote proliferation of epithelial progenitor cells during airway injury<sup>83</sup> and contributes to epithelial cell migration by inducing EMT.<sup>84</sup> Diabetic mice treated with topical Sonic hedgehog (SHH) experienced improvements in re-epithelialization and wound closure.<sup>85</sup> Inhibition of HH signaling has been shown to impair cutaneous wound healing both by inhibiting angiogenesis and by decreasing proliferation of progenitor populations.<sup>86</sup> HH signaling is activated in the area surrounding the wound and promotes plasticity of epidermal cells during re-epithelialization, leading to increases in stem cell populations.<sup>86</sup>

Prolonged activation of HH during cutaneous wound healing results in suppression of differentiation and reprogramming of cells to resemble interfollicular epidermal progenitor cells. These de-differentiated cells behave as tumor initiating CSCs, resulting in the formation of basal cell carcinoma (BCC).<sup>87</sup> In addition to BCC, HH signaling has been shown to be important for maintenance of both normal and malignant stem cells in the skin,<sup>88,89</sup> prostate,<sup>89</sup> pancreas,<sup>90</sup> brain,<sup>91</sup> and breast.<sup>92,93</sup> Similar to its role in wound healing, HH signaling in malignant tissues induces proliferation of progenitor populations and induces EMT within the stem cell compartment.<sup>94,95</sup> Although some tumors have constitutive activation of HH signaling, activation of the pathway can also arise from the tumor microenvironment. Release of SHH ligand by TAM has been reported in breast cancer models and promotes expansion of CSC populations and drug resistance through HH-mediated regulation of drug transporters.<sup>78</sup>

HH signaling plays a role in stem cell expansion, both in wound healing and within the tumor microenvironment. Components of the pathway including Gli-1, Gli-2, and PTCH are highly expressed in normal and malignant mammary stem cells. Activation of the HH signaling is necessary for mammosphere formation and for treatment of mammary stem cells with SHH increasing the size of mammosphere, indicating that the HH



signaling is involved in regulation of self-renewal and proliferation of mammary stem cells.<sup>92</sup> We have recently shown that release of SHH from tumor cells is induced by chemotherapy treatment in breast cancer cells and serves to expand stem-like populations.<sup>9</sup> Breast cancer cell lines treated with docetaxel showed an expansion of cells bearing the CD44<sup>high</sup>CD24<sup>-low</sup> phenotype, as well as increased mammosphere formation and expression of the stemness gene *OCT4*. This expansion is dependent on the HH signaling as it can be blocked with SHH blocking antibodies or SMO inhibitors. It is unknown whether the expansion of CSCs driven by chemotherapy-induced HH is due to the exit of CSC from G0 and increased proliferation, or enhanced resistance of the CSC populations through SHH-induced upregulation of drug transporters and DNA repair enzymes. Alternatively, it is possible that HH stimulation, induced by inflammation associated with chemotherapeutic stress, may alter the plasticity of non-stem like populations, causing them to acquire a CSC phenotype. Several studies have shown that lineage committed cells may have the capacity to acquire stemness features during alterations in homeostasis such as those that occur during wound healing and the tumor microenvironment.<sup>96-100</sup> For example, release of SHH from peri-follicular sensory nerve endings has been shown to result in permanent conversion of hair follicle progenitor cells into intrafollicular epidermal stem cells during wound healing. The progeny of these converted cells is capable of generating long-lasting epidermal clones suggesting the conversion of committed follicular cells into self-renewing epidermal stem cells.<sup>98,99</sup> Further research is needed to determine if SHH contributes to plasticity of breast cancer cells in the context of an inflammatory microenvironment.

**WNT pathway.** Canonical WNT signaling occurs when secreted WNT glycoproteins bind to the membrane protein Frizzled and low-density lipoprotein receptor-related protein 5/6. This binding stimulates a downstream signaling mediator, Dishevelled, which stabilizes the transcriptional co-activator  $\beta$ -catenin, leading to increased nuclear levels of  $\beta$ -catenin. In the absence of stimulation by WNT ligands,  $\beta$ -catenin is targeted for proteasomal degradation by glycogen synthase kinase 3 $\beta$ . WNT signaling is an important mediator of embryonic development and regulates differentiation and EMT. During tissue injury, WNT ligands are highly expressed during the early phases of wound healing and promote migration of epithelial cells.<sup>101</sup> WNT signaling is also necessary for formation of new hair follicles during cutaneous wound healing by driving self-renewal of interfollicular epidermal stem cells<sup>102</sup> and initiating de-differentiation of epidermal cells to follicular progenitor cells.<sup>103</sup>

Activation of WNT signaling is predictive of poor prognosis in breast cancer.<sup>104</sup> Immunohistochemical expression of nuclear  $\beta$ -catenin has been associated with a basal-like gene signature in breast cancer, and higher levels of cytoplasmic and nuclear  $\beta$ -catenin have been found in cells bearing the CD44<sup>high</sup>CD24<sup>low/-</sup> phenotype.<sup>105</sup> Evidence of the role of the WNT pathway in the maintenance of the CSC population comes from

transgenic mouse models in which overexpression of WNT1 under control of the Mouse Mammary Tumor Virus promoter leads to an increase in Thy1<sup>+</sup>CD24<sup>+</sup> tumor progenitor cells.<sup>106</sup> Additionally, WNT signaling has been shown to promote de-differentiation of normal mammary cells, suggesting that it may also play a role in stem cell plasticity.<sup>107</sup> Targeted knockdown of WNT1 in breast cancer cell lines decreases the expression of stem cell markers such as ALDH, inhibits sphere formation, and decreases *in vivo* tumor forming ability.<sup>108</sup> In addition, WNT signaling has been implicated in the radioresistance of CSCs. Overexpression of WNT/ $\beta$ -catenin signaling promotes survival of mammary epithelial progenitor cells after exposure to clinically relevant doses of radiation through upregulation of survivin.<sup>43</sup> These findings suggest that the presence of WNT ligands in the tumor microenvironment may promote survival and resistance of CSCs.

**TGF- $\beta$ .** The transforming growth factor-beta (TGF- $\beta$ ) superfamily consists of a large number of structurally related proteins that include TGF- $\beta$  cytokines (TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3), bone morphogenic proteins (BMP), anti-mullerian hormones, and activins among other growth factors. Although originally discovered in malignant tissues, TGF- $\beta$ s have many physiological functions in normal tissue processes such as embryonic development, immune responses, and wound healing. TGF- $\beta$ s are secreted from many cell types during tissue injury including platelets, macrophages, endothelial cells, keratinocytes, and fibroblasts.<sup>70</sup> They can also be released upon disruption of the ECM.<sup>109,110</sup> During the wound healing response, TGF- $\beta$ s stimulate pleiotropic effects that are dependent on cell type, spatial concentration, and temporal distribution.<sup>111</sup> TGF- $\beta$ s are involved in angiogenesis, inflammatory cell infiltration, fibroblast proliferation, and wound contraction. TGF- $\beta$ 1 knockout mice exhibit defects in re-epithelialization and formation of granulation tissue.<sup>112</sup> Activation of TGF- $\beta$ 1 is important for induction of EMT in keratinocytes during re-epithelialization of cutaneous wounds.<sup>113</sup>

For years, it has been known that TGF- $\beta$  is a key regulator of EMT in cancer cells.<sup>114,115</sup> Because of its role in EMT, TGF- $\beta$  is involved in the acquisition of CSC-like properties, which is necessary for breast cancer cell metastasis.<sup>37,114,116,117</sup> Treatment of immortalized human mammary epithelial cells with TGF- $\beta$  increases the CD44<sup>high</sup>/CD24<sup>-low</sup> population and the ability of cells to form tumorspheres. Treatment of breast cancer cells with TGF- $\beta$  not only increases stem cell populations but also induces a mesenchymal phenotype, suggesting that they have entered into EMT.<sup>37</sup> This link between EMT and breast CSC properties may be a prerequisite for metastasis and TGF- $\beta$  is believed to be the driving force behind this.<sup>117</sup>

Important sources of TGF- $\beta$  are present in the tumor microenvironment. Stromal cells, cancer cells, and cancer cell-associated platelets are among the cell types in the niche responsible for secreting TGF- $\beta$ .<sup>118,119</sup> Once CSCs metastasize to different sites, they primarily produce TGF- $\beta$  and induce EMT in order to create their own niche.<sup>120</sup> In addition, TGF- $\beta$  signaling can be



induced by chemotherapy and leads to increases in IL-8 expression as well as increases in cells bearing CSC phenotypes. Inhibition of TGF- $\beta$  signaling is able to block IL-8 induced expansion of CSC and sensitizes breast cancer xenografts to chemotherapy.<sup>11</sup> Thus, TGF- $\beta$  within the tumor microenvironment may regulate the breast CSC population to aid in chemoresistance.

**NOTCH.** The Notch signaling pathway regulates cell fate decisions during development, including cell fate specification, differentiation, proliferation, and survival.<sup>121–124</sup> Studies have shown that Notch signaling is critical for normal embryonic development since the absence of Notch or Notch ligands in mice is embryonically lethal because of angiogenic vascular remodeling defects that affect the embryo, yolk sac, and the placenta.<sup>125,126</sup> Notch pathway activation occurs when any of the four transmembrane Notch receptors (Notch1–4) interact with one of the five membrane-bound ligands from the protein families of Delta or Jagged (Jagged-1 and -2, Delta-like (DLL)-1, -3, and -4), which are located on the surface of adjacent cells.<sup>127–129</sup> Following binding of the ligand to the Notch receptor, cleavage of the extracellular Notch domain by the metalloprotease TNF- $\alpha$ -converting enzyme (ADAM 17), a member of the ADAM (a disintegrin and metalloprotease domain) family of metalloproteases,<sup>130</sup> generates a short-lived intermediate that is then cleaved by the  $\gamma$ -secretase complex.<sup>131,132</sup> This final cleavage releases the active intracellular domain of Notch, NICD, which translocates to the nucleus and functions as a transcriptional activator to upregulate the expression of a number of genes that are associated with differentiation and survival including the family of transcription factors, HES and HEY,<sup>133,134</sup> cyclin D1,<sup>135</sup> and c-Myc.<sup>136</sup> Recent studies suggest that Notch signaling is also crucial for tissue homeostasis in adults including angiogenesis and vascular homeostasis,<sup>123</sup> lymphocyte expansion and immune function,<sup>137</sup> synaptic plasticity,<sup>138</sup> and neural cell responses to injury.<sup>139</sup>

There is also evidence of a role for Notch signaling in wound healing. Notch1 and Jagged-1 are highly expressed in the epidermis after tissue injury<sup>140</sup> and inhibition of Notch signaling has been shown to decrease cell proliferation and migration of cultured vascular endothelial cells, keratinocytes, and fibroblasts in a mechanical scratch wound assay. When Notch signaling is reduced or inhibited by a  $\gamma$ -secretase inhibitor in mice, the healing of dermal wounds is delayed, whereas treatment with the Notch ligand, Jagged peptide, enhances wound healing.<sup>141</sup> However, in healing corneal epithelium, Notch1 expression is reduced at the leading edge of the wound and inhibition of Notch signaling increases cell migration *in vitro* and accelerates corneal epithelial wound closure *in vivo*,<sup>142</sup> indicating that the role of Notch signaling in wound healing may be tissue dependent. Notch has also been found to be involved in the inflammatory response of wound healing. Myeloid-specific Notch1 deletion in mice results in decreased macrophage recruitment and TNF- $\alpha$  expression in a wound healing assay.<sup>143</sup> In addition, both activation and deletion of Notch1 disrupt the epidermal barrier and can trigger an inflammatory infiltrate.<sup>144,145</sup>

Although Notch plays a role in epidermal differentiation in development,<sup>133,146</sup> the effects of Notch signaling on differentiation of epidermal progenitors during wound healing have not fully been investigated. However, Notch has been shown to crosstalk with HH and WNT pathways,<sup>133</sup> both of which are involved in regulation of progenitor proliferation and plasticity during cutaneous wound healing.

In adult skin, loss of Notch1 expression can lead to tumor development through alterations of the stroma and creation of a wound-like environment in the skin,<sup>147</sup> indicating that the changes in Notch signaling consistent with increases in inflammation can promote tumor growth. Expression of Notch receptors and its ligands has been found to be upregulated in a number of hematopoietic and solid tumors including colon, lung, pancreas, and breast.<sup>148–153</sup> In breast cancer, high levels of Notch1 are associated with poor prognosis.<sup>154,155</sup> Likewise, expression of Jagged-1 protein or mRNA has been associated with lower disease-free survival, basal-like phenotype, and poor prognosis in breast cancer.<sup>156,157</sup>

Inhibiting Notch signaling by  $\gamma$ -secretase inhibitors, anti-Notch1, or anti-DLL-4 monoclonal antibodies has been shown to result in antitumor activity in a variety of cancers.<sup>158–161</sup> Promotion of tumor development through altered Notch signaling may be in part through survival and proliferation of CSCs. Specific inhibitors to Notch receptors or ligands have been reported to reduce breast tumor initiating cell frequency or depletion of stem-like breast cancer cells.<sup>162–164</sup> Inhibiting Notch1 in triple-negative breast tumors results in tumor growth inhibition, decreased tumor growth upon reimplantation, and delayed tumor recurrence possibly due to reduction in the CSC population.<sup>165</sup> In addition, knockdown of the Notch1 receptor inhibits breast cancer cell growth by induction of apoptosis and can enhance chemosensitivity of breast cancer tumors through reduction of CSC frequency.<sup>166</sup> Therefore, including Notch inhibitors in the therapeutic regimen for certain cancers may be useful to target the CSCs to enhance chemo- and/or radiosensitivity and prevent cancer recurrence.

### Pharmacological Inhibition of Inflammatory and Developmental Pathways

Recently, new therapies targeting developmental pathways such as WNT, Notch, and HH have entered clinical trials (Table 3). Additionally, many anti-inflammatory agents that are currently under development for treatment of chronic inflammatory conditions may also be beneficial to cancer patients (Table 2). Multiple strategies have been used to target the IL-1 $\beta$ -IL-6-STAT3 pathway for both autoimmune diseases and cancer. Inhibition of the IL-1 signaling cascades via IL-1 receptor antagonists (IL-1Ra; Kineret(R) or anakinra/Amgen, Inc) or blocking antibodies Riloncept (IL-1 Trap/Arcalyst Regeneron Pharmaceuticals) effectively block IL-6-STAT3 signaling in autoimmune disorders and have some success in the treatment of inherited autoimmune diseases.<sup>167</sup> These agents are currently in clinical trials for treatment of

**Table 2.** Current clinical trials of anti-inflammatory agents in chronic inflammatory diseases and cancer.

TARGET	INHIBITOR	OTHER NAME	CONDITION	PHASE	NCT NUMBER
IL-1	Kineret	Anakinra	Rheumatoid arthritis; Juvenile rheumatoid arthritis; Myocardial infarction and heart failure; Type I and II Diabetes; Advanced cancers including breast, pancreatic, colorectal	I/II/III/IV	NCT02018458; NCT01802970; NCT02021422; NCT01175018; NCT00789724; NCT00645840; NCT01950299; NCT00117091; NCT02236481; NCT02021422; NCT02090101; NCT01300650; NCT00213538; NCT01936844; NCT00037648
	Ilaris	Canakinumab; ACZ885	Autoinflammatory disease; Systemic juvenile idiopathic arthritis; Schnitzler syndrome; Type I and II diabetes; Atrial fibrillation; COPD; Gout	I/II/III	NCT01390350; NCT01676948; NCT00891046; NCT00947427; NCT01080131; NCT00663169; NCT01356602; NCT02204293; NCT00819585; NCT00927810; NCT01068860; NCT00605475; NCT01805960; NCT00581945; NCT00487825; NCT00424346; NCT00504595
	Arcalyst	Rilonacept; IL-1 Trap	Autoinflammatory disease; Gout; Systemic sclerosis; Juvenile Idiopathic arthritis; Cryopyrin-associated periodic syndromes; Type I Diabetes; Chronic kidney disease; Cardiovascular disease	I/II/III/IV	NCT00962026; NCT00288704; NCT00417417; NCT01663103; NCT00534495; NCT00855920; NCT01538719; NCT00094900; NCT00582907
IL-6	Tocilizumab	Actemra	Rheumatoid arthritis; Juvenile idiopathic arthritis; schizophrenia; Type I diabetes; HIV infection; systemic sclerosis; Non-ST elevation myocardial infarction; recurrent ovarian cancer	I/II/III/IV	NCT02087696; NCT01194414; NCT02010216; NCT01941095; NCT01347983; NCT01696929; NCT02034474; NCT00868751; NCT01603355; NCT00642460; NCT01904292; NCT02165345; NCT01637532; NCT01532869; NCT01491074; NCT02049437; NCT02293837
	Sirukumab	CNTO 136	Rheumatoid arthritis; Lupus nephritis; Lupus erythematosus	I/II/III	NCT01606761; NCT01273389; NCT01702740
IL-8	Reparixin	–	Rheumatoid arthritis; Pancreatic islet transplantation in type 1 Diabetes Mellitus; Delayed graft function after kidney or lung transplantation; HER2 negative breast cancer; stage II breast cancer	I/II/III	NCT01220856; NCT02001974; NCT00248040; NCT01861054; NCT01967888; NCT01817959; NCT00224406
	Danirixin	GSK1325756	COPD; Respiratory syncytial virus (RSV) infection	I	NCT02130193; NCT02201303
	AZD5069		Asthma; COPD; Bronchiectasis	I/II	NCT01890148; NCT01704495; NCT01962935; NCT01233232; NCT01255592
	Navarixin	SCH 527123; MK-7123	Asthma; COPD; Psoriasis	I/II	NCT00632502; NCT00688467; NCT00441701; NCT00684593
	SB-656933		COPD; Cystic fibrosis; Ulcerative colitis	I/II	NCT00551811; NCT00748410; NCT00903201; NCT00605761
STAT3	OPB-31121		Advanced and/or metastatic solid tumors	I/II	NCT00955812; NCT01406574
	AZD9150	ISIS-STAT3Rx; ISIS 481464	Advanced and/or metastatic solid tumors	I/II	NCT01563302; NCT01839604
	Daraprim	Pyrimethamine	Chronic lymphocytic/small lymphocytic lymphoma	I/II	NCT01066663
	OPB-51602		Advanced and/or metastatic solid tumors and hematologic malignancies	I	NCT02058017; NCT01423903; NCT01184807; NCT01867073; NCT01344876

multiple myeloma, as well as some solid tumors. Additionally, IL-6 blocking antibodies are in clinical trials for various diseases. Siltuximab, a chimeric antibody to IL-6 developed by Johnson and Johnson, recently gained Food and Drug Administration (FDA) approval for treatment of Castleman's disease and is currently in clinical trials for cancers such as multiple

myeloma and prostate cancer as well as other solid tumors. Recently, results from a double-blinded randomized clinical trial indicated improvement in response rates to standard therapy in patients with multiple myeloma, but no change in overall survival or progression-free survival.<sup>168</sup> Phase I/II dose escalation trials in solid tumors have shown that the drug is



**Table 3.** Current clinical trials of pharmacological inhibitors of developmental pathways.

TARGET	INHIBITOR	OTHER NAME	CONDITION	PHASE	NCT NUMBER
Hedgehog	Vismodegib	GDC-0449	Advanced and/or metastatic solid tumors	I/II	NCT02073838; NCT01088815; NCT01713218; NCT00887159; NCT00957229; NCT00959647; NCT00636610; NCT00739661; NCT00968981; NCT00991718; NCT00833417; NCT01546519; NCT01209143; NCT01174264; NCT01064622; NCT00878163; NCT01195415
	Ipilimumab	IPI-926	Advanced and/or metastatic solid tumors	I/II	NCT01255800; NCT01371617; NCT01130142; NCT00761696; NCT01310816
	Erismodegib	LDE225	Advanced and/or metastatic solid tumors	I/II	NCT02151864; NCT01911416; NCT01579929; NCT01431794; NCT01757327; NCT01954355; NCT01694589; NCT01125800; NCT01787552; NCT01350115; NCT00880308; NCT01208831; NCT01769768; NCT01576666
	PF-04449913	–	Advanced and/or metastatic solid tumors	I/II	NCT01842646; NCT01841333; NCT01286467; NCT01546038; NCT04449913; NCT0203877; NCT02226172
	LEQ506	–	Advanced solid tumor, recurrent or refractory medulloblastoma or locally advanced or metastatic basal cell carcinoma	I	NCT01106508
	TAK-441	–	Advanced nonhematologic malignancies	I	NCT01204073
	Itraconazole	–	Relapsed prostate cancer	II	NCT01787331
	LY2940680	–	Small cell lung carcinoma	I/II	NCT01722292
	Sonidegib	–	Myeloid malignancies	I	NCT02129101
	BMS-833923	–	Extensive stage small cell lung cancer (ES-SCLC)	I	NCT00927875
Notch	Notch inhibitor	–	Advanced and/or metastatic solid tumors	I/II	NCT01158404; NCT02069730
	MK0752	–	Advanced and/or metastatic solid tumors	I/II	NCT00106145; NCT01098344; NCT00645333
	BMS-906024	–	Advanced and/or metastatic solid tumors	I	NCT01653470; NCT01363817; NCT01292655; NCT01986218
	PF-03084014	–	Desmoid tumors/aggressive fibromatosis; AIDS-related Kaposi sarcoma; Advanced solid tumor malignancy and t-cell acute lymphoblastic leukemia/lymphoblastic lymphoma	I/II	NCT01981551; NCT02137564; NCT00878189
	RO4929097	–	Advanced and/or metastatic solid tumors	I/II	NCT01238133; NCT01122901; NCT01196416; NCT01151449; NCT01198184; NCT01120275; NCT01232829; NCT01193881; NCT01218620; NCT01119599; NCT01145456; NCT01131234; NCT01096355; NCT01158274; NCT01141569; NCT01189240; NCT01200810; NCT01198535; NCT01149356; NCT01175343; NCT01217411; NCT01116687
Notch & Hedgehog	Vismodegib & RO4929097	GDC-0449 & RO4929097	Metastatic or unresectable breast cancer; Advanced or metastatic sarcoma	I/II	NCT01071564; NCT01154452
Wnt signaling pathway	Resveratrol	–	Colon cancer	I	NCT02137421; NCT00256334; NCT00578396
	Diclofenac & Calcitriol	Diclofenac & topical vitamin D3	Basal cell carcinoma	III	NCT01358045
	Genistein	–	Metastatic colon cancer	I/II	NCT01985763



well tolerated, but so far the advantage of including Siltuximab in standard therapy regimens has not been clinically proven.<sup>169</sup> Antibodies to the IL-6 receptor gp-130 are also undergoing clinical trials for treatment of autoimmune diseases. Tocilizumab, an IL-6R blocking antibody developed by Hoffmann-La Roche AG, has recently gained FDA approval for treatment of rheumatoid arthritis. Although it has been shown to have an effect on cachexia associated with non-small cell lung tumors, its role as a treatment for solid tumors has not yet been investigated clinically. Additionally, several inhibitors of STAT3 are currently under preclinical/clinical development for treatment of solid tumors.<sup>170-172</sup> To date, Phase I dose escalation studies of STAT3 inhibitors have shown poor pharmacokinetics and severe dose-limiting side effects.<sup>173</sup>

Several agents targeting developmental pathways are also in clinical development. The first HH inhibitor to gain clinical approval for the treatment of BCC was Vismodegib (GDC-0449) developed by Genentech. This agent was found to have a significant effect on metastatic and resistant BCC. Preliminary findings indicate that this agent may also have anti-tumoral activity in medulloblastoma.<sup>174</sup> Phase II trials of Vismodegib for treatment of other solid tumors are currently underway. Several other inhibitors to the HH pathway are also under clinical development, including Sonidegib (also known as Erismodegib or LDE225, Novartis) which has shown acceptable toxicity profiles in Phase I trials and is currently progressing into Phase II trials to examine efficacy in solid tumors.<sup>175</sup> Likewise, multiple agents are under preclinical/clinical development for inhibition of  $\gamma$ -secretase, a key enzyme involved in Notch signaling. Phase I trials of RO4929097, a  $\gamma$ -secretase inhibitor developed by Roche, have shown acceptable toxicity profiles and some evidence of anti-tumor activity.<sup>176</sup>

Significant crosstalk exists between developmental pathways in both wound healing and the tumor microenvironment. For this reason, combinatorial approaches with targeted inhibitors for multiple developmental pathways are currently being explored. A Phase II study is currently underway to determine the effects of concurrent inhibition of the HH and Notch pathways in breast cancer. If successful, the combination of Vismodegib and RO4929097 may offer new treatment options for advanced or metastatic tumors. Additionally, crosstalk between developmental and inflammatory pathways within the tumor microenvironment has been reported. Currently, a Phase I trial of combination therapy using Vismodegib and the immunosuppressive agent Sirolimus (rapamycin; Pfizer) are underway. Advancement of this approach will depend on an acceptable toxicity profile, but may yield promising results for the treatment of solid tumors including pancreatic and breast cancer. It is likely that combination therapies targeting both developmental and inflammatory pathways may lead to better responses. Additionally, studies have shown that treatment with radiation and standard chemotherapy induces a substantial wound healing response, which may further promote resistance and expansion of CSCs.<sup>9,10,177,178</sup> Activation of Notch signaling during

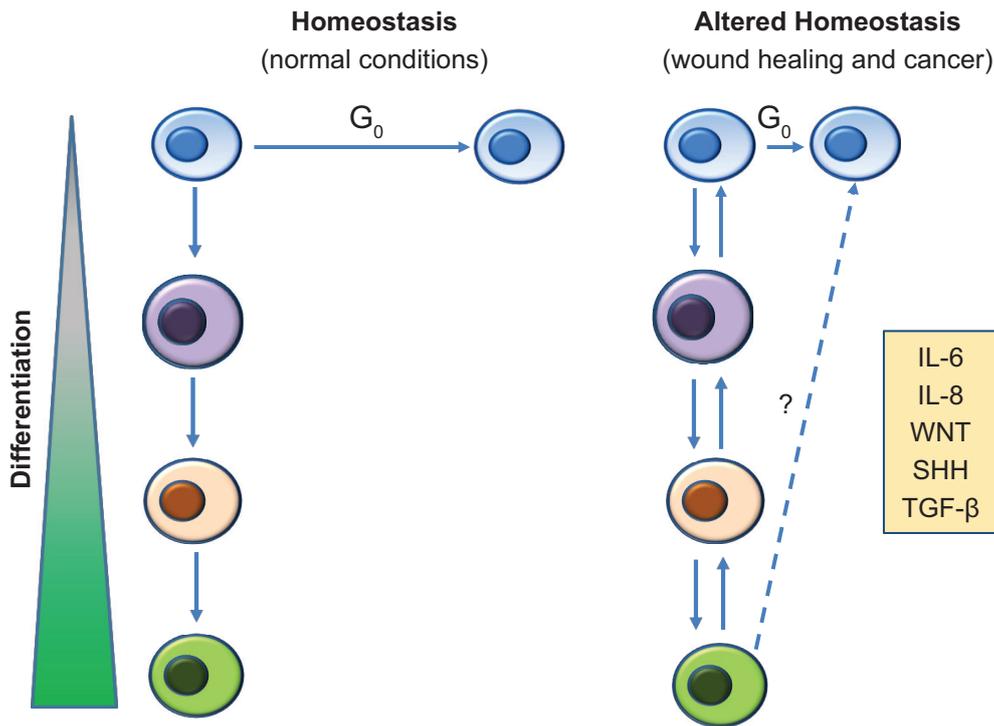
post-surgical wound healing has been linked to increased metastasis.<sup>10</sup> Thus, combinations of anti-inflammatory drugs with standard treatment approaches may prevent activation of developmental pathways responsible for cancer resistance and metastasis and lead to better clinical outcomes.

## Conclusion

During the course of malignancy, tumor cells invade neighboring tissues, stimulate angiogenesis, remodel the ECM, undergo EMT, and metastasize. In doing so, they activate a chronic inflammatory response involving numerous cytokines, developmental pathways, and growth factors involved in the normal wound healing process. The presence of these factors within the tumor microenvironment is linked to an increase in the proportion of cells bearing stem-like phenotypes, enhanced tumor-initiating properties, and increased resistance to standard therapies.

It is currently unclear whether the tumor microenvironment confers a survival advantage through selection of resistant CSC or through upregulation of stem-like properties in non-stem cells. Although evidence for cells bearing stem cell signatures in resistant breast cancer is compelling, the origin of these cells is controversial. The question remains as to whether signals within the tumor microenvironment lead to proliferation of a small population of cells arising from genetically altered stem cells, or do they induce a stem-like phenotype in differentiated cells through upregulation of developmental signaling pathways and stem cell markers. Further, does this change in phenotype confer a functional change in differentiated cells so that they now behave as CSC, exhibiting self-renewal and tumor-initiating properties? As shown in Figure 1, we propose that when normal homeostasis is disrupted, either by tissue injury or by the tumor microenvironment, activation of inflammatory and developmental pathways alters the ratio of stem cells to non-stem cells in two possible ways. The first is by driving existing slow-cycling, quiescent stem cell populations into the proliferative phase of the cell cycle. The second is by altering plasticity of differentiated cells so that they obtain stemness properties. The latter of these processes is supported by recent evidence showing significant cellular plasticity in normal tissue during wound healing processes. Lineage tracing experiments have shown that committed progeny in the lung and intestine can revert to a stem-like phenotype and contribute to tissue regeneration.<sup>4,96,179</sup> Likewise, interconversions between stem-like and differentiated states have been reported in glioblastoma<sup>26</sup> and breast cancer<sup>180</sup> cells after chemotherapy. Although both wound healing and the tumor microenvironment represent states of disrupted homeostasis in which stem cell numbers may be increased, they differ significantly in that the level of stemness during wound healing is tightly regulated, with a number of stem-like cells returning to normal during the final phases of the healing process. In the tumor microenvironment, stem cell plasticity may be constant with an overall increase in stem-like cells.

The effect of inflammatory signals within the microenvironment on the cellular plasticity of tumor cells has yet to



**Figure 1.** Cellular plasticity in normal vs altered homeostasis. (Left panel) Under normal conditions, stem cell/non-stem cell ratios are maintained at a consistent level via slow cycling stem cells (blue), which undergo a unidirectional differentiation to lineage restricted cells. (Right panel) In conditions of altered homeostasis, the ratio of stem cell/non-stem cell is increased by proliferation of stem cells and a bidirectional differentiation whereby lineage restricted cells are able to dedifferentiate and acquire stem-like features. In both wound healing and the tumor microenvironment, this cellular plasticity is driven by inflammatory and developmental factors. However, in wound healing, expression of these factors is transient, homeostasis returns, and the ratio of stem/non-stem cells returns to normal levels. In the tumor microenvironment, continuous expression of these factors may lead to a permanent expansion of stem-like cells.

be fully determined. Thus, it will be important to investigate whether blocking specific inflammatory signals, alone or in combination with other stem cell pathway inhibitors, can decrease CSC populations in a therapeutic setting and promote better responses to standard therapies. The answer to this question would help to uncover a link between inflammation and CSCs and determine whether, together or separately, they impact the response to therapy.

### Author Contributions

Contributed to the writing of the manuscript: KMA, LMO, JSM. Jointly developed the structure and arguments for the paper: JSM, DF. Made critical revisions and approved final version: JSM, DF. All authors reviewed and approved of the final manuscript.

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