Mammary epithelial and breast cancer stem cells

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Introduction

The perpetual renewal of tissues and organs is driven by resident stem cells and progenitors. These cells guarantee tissue maintenance and regeneration after injury or involution and most tissues and organs contain small populations of primitive stem cells and progenitors. These cells play a major role in the developing foetus and contribute to the generation of tissues and organs. Stem cells are positioned on top of the cellular hierarchy and give rise to progenitors with more restricted lineage potential. The stem cells can divide and self-renew to generate daughter stem cells or they can differentiate into a variety of mature cell types [1].

The stem cell hypothesis has been extrapolated to tumour tissues and the postulation of stem cells has many attractive conceptual and practical implications. Cancer cells are distinguished by multiple mutations which alter their cell cycle regulation, their sensitivity to apoptotic signals and their lifespan. Since stem cells persist within the organism for long periods of time, they have the potential to accumulate genetic damage and propagate them to their daughter stem cells and their downstream progenitors. Many tumours comprise minor populations of tumour-initiating cells, cells able to reconstitute a tumour upon transplantation; these cells have been called cancer stem cells (CSCs) [2]. CSCs share characteristics of normal stem cells, i.e. they can self-renew and they can give rise to heterogeneous cell populations within a tumour and maintain the tumour mass [3]. They are also more resistant to chemotherapeutic drugs and radiotherapy than their progenitors and thus represent a cellular reservoir for tumour recurrence. CSCs can be derived directly from mutational events in stem cells which confer proliferative properties or they can result from mutations in progenitor cells which provide them with self renewal capacity. Both pathways might be used and result in tumours with different pathological properties [4,5]. The characterisation of the signalling pathways which provide self renewal properties to CSCs and the targeted interference with limiting signal transduction components offer a promising new therapeutic approach for the treatment of cancer.

Evidence for the presence of stem cells in mammary tissue

The application of the stem cell concept to tumour tissues is persuasive and explains important observations, particularly the heterogeneous cell types and morphologies of cells present in tumours, the different potential of tumour cell subfractions to re-establish tumours upon transplantation, but also the differential sensitivity of cells within tumours to respond to chemotherapy and radiation therapy. Also, the course of disease, the progression and metastatic potential of tumours could possibly be explained by the particular genetic alterations which drive stem cell self-renewal and differentiation. Despite these important implications, the isolation and characterisation of normal stem cells or CSCs in breast tissue is still preliminary and in its infancy. Most of the evidence is based on functional properties of a small fraction of cells, rather than on histological or biochemical studies.

The cyclical nature of mammary gland growth and involution initially suggested the presence of stem cells in this organ [6]. The ducts of the epithelium are formed by a layer of luminal epithelial cells surrounded by basal myoepithelial cells and the basement membrane. The primitive branched ductal system present in virgin animals expands during pregnancy, stimulated by the systemic release of steroid and peptide hormones. Side-branches and alveoli are formed and secretory luminal epithelial cells fill the whole fat pad at parturition. Milk is produced during the suckling period, and the termination of suckling is followed by involution of the gland through massive apoptotic cell death. A ductal structure resembling the one found in the mature virgin gland is restored. Each cycle of pregnancy is thus accompanied by proliferation, differentiation and involution, a process driven by resident stem cells [7,8].

The functional description of the mammary gland has been complemented by a partial molecular characterisation of the main cell types involved in its structural organisation (Fig. 1A). The mammary epithelium consists of ducts and alveoli which are composed of basal and luminal cell layers. The basal cell layer comprises myoepithelial cells and harbours the mammary epithelial stem cell compartment. In mice the luminal cell layer is composed of two functional lineages which are distinguished by the expression of the cell surface proteins CD24 and Sca-1. $CD24^{+/high}Sca\text{-}1^{+}$ luminal cells express oestrogen receptor alpha (ER) and the prolactin (PrlR) and progesterone receptors (PR). The $CD24^{+/high}Sca\text{-}1^{-}$ luminal cells lack expression of the ER [12]. These cell types emerge from stem cells. Some of the signals which cause the generation of myoepithelial, luminal ER^{-} and luminal ER^{+} daughter cells and which control cellular homeostasis, fate determination and lineage commitment in the mammary gland have been identified. Paracrine cellular interactions, transcriptional regulators and epigenomic modifications have been implied [9].

The transcription factor Gata3, for example, specifies commitment in the general luminal lineage [13] and Elf5 has the same effect on alveolar cell fate [14]. Wnt-4 is a signal which acts in a paracrine fashion downstream of the PR and is involved in ductal side-branching [15]. Amphiregulin, produced by ER^{+} cells in response to oestrogen, stimulates mammary stem cell activity [16] and Notch signalling regulates luminal cell fate [17].

More direct evidence for the existence of adult stem cells in the mammary gland is based on transplantation experiments [18]. The emerging mammary tree can be efficiently removed in very young mice and these cleared fat pads can be inoculated with exogenous mammary cells. Small fragments of mouse mammary tissue, very small numbers of marker enriched cells or unfractionated total mammary epithelial cells (our own unpublished observations) were able to reconstitute the epithelial component of the mammary gland and support its outgrowth and differentiation [9]. Although these stem cells have not been unequivocally identified, they have been found to be associated with particular cell surface markers. Cells sorted for CD24, CD29 (β1 integrin) and CD49f (α6 integrin) were found to be enriched in cells with a repopulation capacity [13], but they are not necessarily stem cell specific. Mouse CD24 is a marker expressed on epithelial cells and merely allows the distinction between epithelial and stromal components.

Since the stem cells are mainly functionally defined through their ability to repopulate cleared fat pads, different cell populations can be investigated for stem cell presence. The highest proportion of stem cells has been found in the terminal end buds of the developing gland in virgin mice, while only a few stem cells have been detected in the alveoli of lactating mice [6] and markers can be used to further enrich stem cells by flow cytometry. Flow cytometry allows the enrichment of stem cells and the removal of haematopoietic and endothelial cells. The combination of $CD24^{med}Sca\text{-}1^{low}CD29^{high}CD49f^{high}$ expression has led to the partial purification of cells with repopulating potential in mice; this subpopulation being about 1 to 2% of all cells [19,20].

These observations were made with mouse tissues, but attempts have also been made to investigate human mammary stem cells. The markers which have been employed for sorting experiments include the epithelial cell adhesion molecule, EpCAM, CD49f and the luminal cell-specific glycoprotein MUC1. EpCAM and CD49f are epithelial cell specific, expressed to different extents by luminal and basal cells, and reconstituting cells have been found in the $EpCAM^{low}CD49f^{high}MUC1^{-}$ cell population. They were tested for their ability to engraft humanised mouse mammary fat pads. Aldehyde dehydrogenase 1 (ALDH1) has also been identified as a marker for these cells [21]. Additional fractionation experiments were carried out with dispersed cells from human breast tissue. Bipotent progenitor cells ($EpCAM^{+}$/$CD49f^{hi}$/CALLA $(CD10)^{+}$/$Thy1^{+}$/$CD133^{-}$), luminal committed progenitor cells ($EpCAM^{+}$/$CD49f^{+}$/$MUC1^{+}$/$CD133^{+}$/$CD10^{-}Thy1^{-}$) and differentiated luminal cells were identified by surface markers from normal human breast tissue [22]. ER^{low}/PR^{high} expression was found in the bipotent cell population and ER^{high}/PR^{low} expression in the luminal committed progenitor population.

Breast cancer stem cells

Cancer cells are thought to originate through the accumulation of multiple mutations, a process which requires many cell divisions. The self renewal capacity of stem cells is a prerequisite for the maintenance of progressive genetic changes and allows mutations to be propagated through cellular generations. The activities of tumour suppressor proteins might counterbalance detrimental mutations temporarily [23,24].

The functional properties of stem cells, which comprise self-renewal and the generation of proliferating

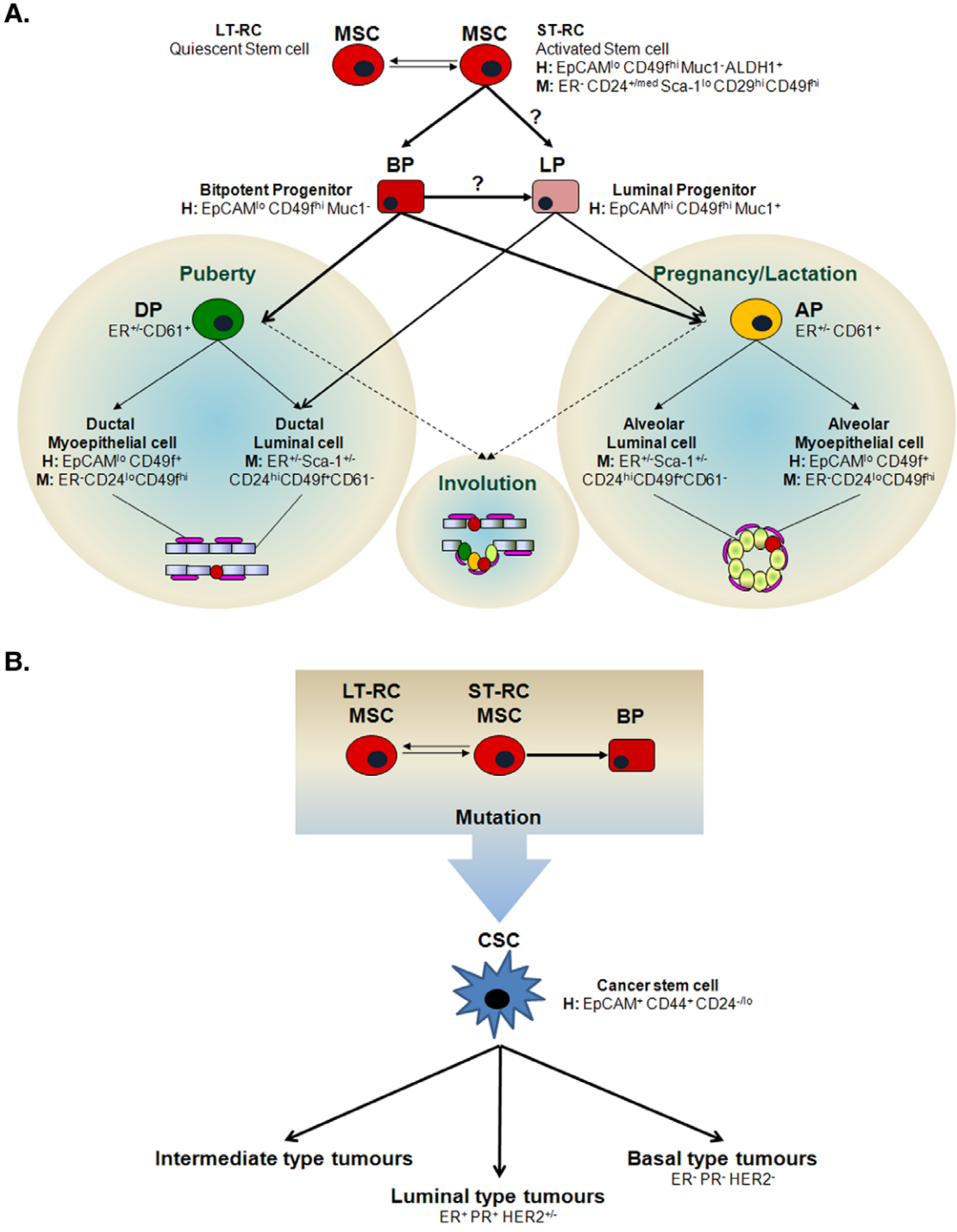

Fig. 1. Epithelial cell hierarchy in human and mouse mammary gland and tumours. (A) Mammary stem cells (MSC) give rise to bipotent (BP) and luminal restricted progenitors (LP) by asymmetric cell division. BPs in turn produce either ductal or alveolar cells. Myoepithelial and luminal cells are derived from ductal precursors (DP) during puberty or from alveolar precursors (AP) on initiation of pregnancy. Luminal cells are also derived from LPs. The alveolar luminal cells terminally differentiate into milk secreting cells. A ductal system containing multipotent (red), committed ductal (green) and alveolar (orange) precursor cells persists after involution. They develop into a fully functional epithelium in subsequent pregnancies. (B) CSCs potentially arise from MSCs through oncogenic transformation or from more developmentally advanced, replication competent progenitor cells (BP). Their origin and the peculiarities of the acquired mutations cause the different breast tumour subtypes and breast cancer heterogeneity. LT-RC, long-term-repopulating cell; ST-RC, short-term-repopulating cell. Adapted from [9–11].

progenitor cells and differentiated tissue cells, can be extended to the investigation of tumours [25]. Human breast tumours have been dissociated, cells were fractionated into subpopulations and the potential of individual fractions to cause tumour growth upon transplantation into immuno-deficient mice was tested. The assumption in these experiments was that not all tumour cells are functionally equivalent, but that a subpopulation of tumour cells can be functionally defined as CSCs (Fig. 1B). For this purpose human breast tumours were investigated [10]. The separation of dispersed tumour cells according to particular cell surface markers was performed and the resulting subpopulations were investigated for their potential to form tumours upon transplantation into immuno-deficient recipient mice. Only a minor population of cells obtained from human breast cancers is able to cause tumour growth when introduced into NOD/SCID mice. These cells are distinguished by the expression of $CD44^{+}$, $CD24^{-/low}$ and represent about 20% of the total cancer cells [7]. The tumours which grew in the transplanted mice showed a phenotypic heterogeneity similar to the one found in the parental tumours. Cells expressing $EpCAM^{+}CD44^{+}CD24^{-/low}$ were most effective in causing tumour growth upon transplantation into mice [26]. Breast cancer stem cells were also found in established cell lines [27]. However, the correlation with the markers described above was not necessarily found [28].

The subfractionation experiments combined with transplantation of the sorted cells into immuno-compromised mice indicate that $CD44^{+}/CD24^{-/low}$ cell population is enriched in tumour initiating cells, but these markers do not provide for the purification of CSCs. Additional markers, like aldehyde dehydrogenase (ALDH1), have to be included to identify the stem/progenitor population [21]. Just like normal tissue stem cells, tumours might harbour specific cell populations which undergo self-renewal as well as various degrees of differentiation. CSCs could arise from normal stem cells through oncogenic transformation or from more developmentally advanced, replication competent progenitor cells possibly through the deregulation of self-renewal pathways. A cell population results which is characterised by distinct genetic and epigenetic changes and which gives rise to the cellular heterogeneity of tumours. Instead of generating ductal and alveolar cells, the CSCs undergo aberrant and limited differentiation. Their origin and the peculiarities of the acquired mutations cause the different breast tumour subtypes and breast cancer heterogeneity [29]. Alterations in gene expression patterns can also be caused by epigenetic changes and cell-cell interactions which define the stem cell niche.

Mouse models have shown the close relatedness of tissue stem cells and CSCs. Tumors induced by targeted oncogene expression in the mammary gland, MMTV-Wnt [30] and MMTV-polyoma middle T transgenic mice, or p53 deletion [31] showed that CSCs and mammary stem cells share similarities. The mammary progenitor marker CD61 identifies CSCs in mouse models of mammary tumours [32]. Human mammary stem cells are sensitive to oncogenic mutations. HER-2 expression, activation of Notch and hedgehog signalling and loss of expression of BRCA1 enhance their engraftment in immune-deficient mice [33]. Human repopulating cells or progenitor cells can function as a source of CSCs [34].

Signaling pathways responsible for stem cell properties of CSC and their implications for therapeutic interventions

Cellular phenotypes are usually determined by extracellular cues which act through the mediation of a cell surface receptor system and the induction of an intracellular signal transduction pathway. It is therefore not unreasonable to assume that CSC properties are induced and maintained by pathways able to ascertain self-renewal and the generation of partially differentiated progenitor cells [35]. The involvement of these pathways in human breast cancer has not yet been investigated in sufficient detail. However, the functions of several distinct signalling pathways in stem cell biology are well established. Most of these pathways have initially been discovered by genetic analysis in Drosophila and have subsequently been investigated in mammals. Their deregulation in mice has been shown to cause mammary tumours [1,36] and their activation has been discovered in human tumours.

The concept of CSCs postulates that upon asymmetric cell division a cell with stem cell properties is retained and a second cell partially differentiates and gradually loses its repopulation potential. These processes must be controlled by signalling pathways which govern stem cell self-renewal and differentiation. A number of candidate genes and pathways have been taken into consideration. The HER-2 receptor is a member of the epidermal growth factor receptor family and very strongly expressed in about 25% of human breast cancers. HER-2 overexpressing tumours grow aggressively and are frequently associated with metastasis formation and an unfavourable prognosis.

HER-2 overexpression is significantly correlated with the expression of the stem cell marker ALDH1 and increases the proportion of stem cells [21]. Treatment of responsive tumours with the monoclonal antibody trastuzumab reduces the stem cell population [37]. HER-2 may regulate the stem-cell population in breast tumours by preventing the progression of stem cells into ER^+ progenitor cells.

Another pathway which is frequently deregulated in cancer cells originates with the activity of phosphoinositide-3 kinase and results in the induction of the Akt kinase and mTOR. An intermediate pathway component is PTEN, a phosphatidylinositol phosphatase, an enzyme which is functionally impaired in about 40% of the breast cancer cases. A correlative observation concerns the self-renewal of haematopoietic and neuronal stem cells. They are regulated by PTEN [1] and it is therefore reasonable to assume that inhibitors of Akt and mTOR might become useful therapeutics in breast cancer treatment.

The Wnt ligands activate a signalling pathway and induce transcription factor activities which regulate cell fate decisions, cell proliferation, morphology, migration, apoptosis and differentiation [38]. Wnt signalling can influence mammary gland growth and differentiation and possibly involution [39] and exerts an effect on the self-renewal and differentiation of stem cells. Transgenic mice expressing a MMTV-Wnt oncogene develop mammary tumours which strongly express stem cell markers [36].

Notch signalling regulates cell-fate determination, survival and proliferation and is involved in normal mammary development and differentiation [17]. Transgenic mice expressing a constitutively active form of Notch4 fail to develop normal mammary glands, but develop mammary tumours. Notch signalling promotes self-renewal and proliferation of early progenitor cells and restricts myoepithelial lineage specific commitment and proliferation. It also promotes branching morphogenesis, but has little effect on terminally differentiated mammary epithelial cells. Notch signalling may prevent terminal differentiation and maintain mammary epithelial cells in a proliferative state [33]. Notch3 signalling is particularly important for the proliferation of HER-2 negative breast cancer cells [40]. Finally, hedgehog signalling regulates the self-renewal of both normal and malignant human mammary stem cells. The pathway is mediated through the action of the polycomb protein Bmi-1 [41].

Targeted tumour therapy relies on genetic and biochemical distinctions between normal and tumour cells. The identification of specific signalling pathways and signalling components active in CSCs opens new possibilities for therapeutic exploitation. CSCs could possibly be targeted and these agents might improve conventional treatment results. CSCs, however, initially have properties which make them more resilient against therapy. They are more resistant to chemotherapy and ionising radiation. New therapeutic agents which target the genuine stem cell properties have to be designed.

Agents could be developed which induce the differentiation of cancer cells, equivalent to all trans retinoic acid (ATRA) which promotes promyelocytic differentiation and is being used in the treatment of acute promyelocytic leukaemia [42]. Alternatively, the elimination of CSCs can be envisaged by targeting crucial regulatory genes and signalling pathways. The pathways regulated by PTEN, Wnt, hedgehog and Notch are involved in the self-renewal of both normal mammary stem cells and CSCs and constitute promising candidates. The hedgehog pathway can be inhibited with cyclopamine [43] and is essential for maintenance of CSCs in myeloid leukaemia. Inhibition of the Notch pathway prevents CSC self-renewal and inhibits tumour growth [44]. Many of the signalling components involved in these pathways are conventionally considered as "non-druggable", i.e. they do not have enzymatic activities or small molecular weight compound binding pockets. New approaches based on the inhibition of protein-protein interactions or protein DNA interactions have to be considered to target pathway endpoints [45,46].

CSCs are most likely maintained in a particular cellular environment and this niche might lend itself as a drug target [5]. This is particularly important for CSCs in the breast and the frequent resistance to endocrine therapy [47]. In normal breast tissue, the stem cells have a basal phenotype and do not express ER. If this is also the case in breast cancer, CSCs may be endocrine resistant and treatment responses are relayed by paracrine effects emanating from neighbouring ER^+ tumour cells. Normal breast epithelial stem cells are also dependent on EGFR activation and other growth factor receptors. Increased growth factor receptor activation in endocrine resistant breast cancers is accompanied by an increased proportion of stem-like cells. This might be a consequence of the endocrine therapy. Epigenetic regulation of gene expression and influences of the stromal microenvironment might also be responsible. Epigenetic reprogramming agents might become useful by downregulating growth factor receptor expression and increasing the proportion of ER expressing cells [48].

The stability and plasticity of CSCs and their clinical significance

If stem cells and CSCs are dependent on signal transduction pathways triggered by external cues, it is reasonable to assume that they are located in a specialised regulatory microenvironment. The cells constituting this environment probably contribute the components which control the fate specification of stem and progenitor cells. The bone marrow, for example, can function as such a niche and provides the proper architecture composed of osteoblasts, osteoclasts, bone marrow endothelial cells, stromal cells, adipocytes and extracellular matrix proteins (ECM). The elements of the microenvironment regulate survival, growth and differentiation of diverse cell lineages, through the provision of cytokines, chemokines, proteolytic enzymes and adhesion molecules. Since the environment can change and exert different influences on stem cells, it is not unreasonable to suspect that stem cells are not fixed entities, but that they can emerge and disappear as a function of changing conditions in their surroundings [49].

Important observations have been made which suggest that transitions between cell types can occur and that these transitions have a role in tumour progression. Epithelial and mesenchymal states are not absolutely stable and transitions from one to the other contribute to tumour progression and intratumoural heterogeneity. The epithelial mesenchymal transition (EMT) is triggered by growth factor signalling, tumour stromal cell interactions and hypoxia and mediated by transcription factors and reprogramming gene expression patterns. EMT can change cancer cells to cells with stem cell-like characteristics and increase the potential to invade surrounding tissues and metastasise [50,51]. The mesenchymal cells express stem cell markers and are related to stem cells in normal tissue and to CSCs. This includes the transcription factors Snail and Slug and the activation of the TGFβ signalling pathway [52]. The cells also lose epithelial markers and acquire basal-mesenchymal properties. Breast cancer cells of luminal, intermediate and basal phenotypes have an increased fraction of CSCs, i.e. $CD44^{+}/CD24^{low}/ESA^{+}$ expressing cells, when compared to hormone sensitive luminal cancers [28]. ER suppresses the expression of transcription factors regulating EMT [53].

The suspected roles of CSCs in the maintenance of tumours, their metastatic potential and their drug sensitivity suggest numerous therapeutic implications. It would be most advantageous if these parameters could be exogenously manipulated [54]. The increased resistance of CSCs to drug treatment might be a function of their relative quiescence. The majority of CSCs are probably not dividing and thus escape many of the chemotherapeutic agents dependent upon DNA synthesis. The expression of ABC transporters in CSCs might contribute to this resistance phenotype. Targeted approaches to CSC functions should spare normal stem cells to avoid collateral damage. Inappropriately activated kinases, such as Akt and mTOR, might be suited.

The CD44 molecule is a marker observed on the surface of many stem cells. The interaction of CD44 with a specific monoclonal antibody has been exploited to eradicate acute myeloid leukaemic stem cells [55]. This target molecule may also become valuable for the treatment of breast cancer. Genetic programmes and signalling pathways differentially utilised in normal stem cells and CSCs might provide the best options. The targeting of Wnt signalling in skin tumours [56] and BMP expression in glioblastomas, causing the depletion of the $CD133^{+}$ cell fraction and the induction of a more differentiated phenotype, might become beneficial.

Although the CSC niche is conceptually attractive and might offer possibilities to disrupt the cellular communication required for CSC maintenance, the components involved are still poorly defined. The CSC niches themselves might have aberrant properties due to their adaptation to the requirements of CSCs. The simultaneous interference with the autonomous tumour cell pathways driving their growth and survival and the interference with the supportive microenvironment seems like a promising strategy. Chemotherapeutic and anti-angiogenic agents might cooperate effectively [57].

Treatment of tumours with chemotherapeutic and cytotoxic drugs or hormone antagonists puts selective pressure on these cells and might yield the outgrowth of new variants. The emergence of drug and hormone resistance of tumours is a well known consequence in many patients and might be linked to CSC phenotypes. Tamoxifen resistant ER^{+} breast cancers exhibit a more basal phenotype with a reduction in E-cadherin expression. They also have an enhanced motility, with upregulation of src kinase, NF-κB activation and CD44 expression [58]. CSCs in colorectal cancers are substantially enriched following chemotherapy [59]. Drug resistance is often acquired through the enhanced expression of ATP-binding cassette transporters (ABCG2 and ABCG5) and multidrug resistance protein 1 (MDR1). These proteins are also strongly expressed in CSCs and contribute to chemotherapy resistance.

Conclusions

The presence and the molecular characteristics of CSCs have interesting implications for the understanding of tumour aetiology, the response to treatment and the development of novel therapeutics [52]. If CSCs are more resistant to conventional chemotherapeutic drugs and radiation than the majority of the cells present in solid tumours, and if they are the source of residual cells which cause recurrence of tumour growth after therapy, strategies and targeted drugs have to be designed which are preferentially aimed at their communication with their microenvironment and their eradication. The targets of such drugs could be components of the pathways which maintain breast cancer stem cells, such as the Notch, hedgehog, and Wnt pathways. Since many of these components are not susceptible to conventional drug action, new classes of drugs might have to be discovered and developed.

Conflict of interest statement

None declared.

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