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Breast cancer stem cells: An overview

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ABSTRACT

The theory that cancer may be originated and sustained by a small proportion of stem-like, self-renewing cells (termed 'cancer stem cells') has gained support in recent years. Breast cancer stem cells have been identified as $CD44^+CD24^-$ breast tumour cells and have recently been isolated and propagated in vitro. It has been demonstrated that these cells exclusively retain the ability to form new tumours in mouse models and that they display stem/progenitor cell properties. The ability to identify breast cancer stem cells in vivo and to propagate them in vitro provides the means to compare them with normal cells, in order to investigate from which cell they originate, which molecular alterations critically affect them, and how they interact with the microenvironment. Elucidation of these critical points is essential to develop new therapeutic strategies and to improve diagnosis and prognosis for breast cancer patients.

1. Mammary stem cells

Terminal duct lobular units (TDLUs) are the basic functional/structural components of the branching ductal–alveolar system in the resting human mammary gland of premenopausal women, where luminal/ductal epithelial cells line the inner surface and myoepithelial cells form the outer basal layer.

There is robust evidence that both luminal and myoepithelial cell types originate from a common multipotent progenitor cell. It was demonstrated decades ago that fragments of mouse mammary gland could reconstitute an entire mammary tree upon serial transplantation into the cleared fat pad of female mice.[1] More recently, clonal analysis revealed that single mammary epithelial cells retained the ability to give rise to a complete mammary gland structure.[2] Finally, analysis of the X-chromosome inactivation pattern demonstrated that the breast epithelium is organised in patches of mature cells derived from a common stem/progenitor cell.[3]

Mammary gland stem cells have been described as undifferentiated pale or light-staining cells,[4] showing two distinct forms: small light cells (SLC) with a basal location and undifferentiated large light cells (ULLC), which localise in an intermediate position between the basal and the luminal layer, are larger in size than SLC and exhibit larger nuclei than other epithelial cells.[5,6] Radio-labelling experiments indicated that a proportion of label-retaining cells (LRCs) in the breast epithelium is similar in morphology to previously described ULLC, expresses the putative stem cell markers $p21^{CIP1}$, Musashi-1 and Keratin 19, displays a 'side-population' phenotype and is enriched for steroid receptor expression.[7]

Purification and in vitro propagation of human breast stem/progenitor cells has been achieved using different experimental strategies. Gudjonsson and colleagues identified and isolated bipotent luminal epithelial cells expressing epithelial-specific antigen (ESA) and negative for sialomucin (MUC-1), capable of giving rise to fully-differentiated luminal and myoepithelial cells, in addition to other MUC^-/ESA^+ cells. MUC^-/ESA^+ cells retained the ability to generate entire TDLUs when cultured in three-dimensional reconstituted basement membrane, as well as when transplanted into nude mice.

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Moreover, they have been reported to express keratin K19 and to occupy a suprabasal position in vivo.[8]

More recently, Dontu and colleagues developed a culture system to isolate and propagate in vitro human breast multipotent stem/progenitor cells in an undifferentiated state as non-adherent spherical clusters, termed 'mammospheres'. Mammospheres have been demonstrated to be clonally derived from single self-renewing cells, which are entirely comprised in a Hoechst-excluding side-population; moreover, they encompass undifferentiated precursors capable of differentiating along the three cell lineages of the mature mammary gland epithelium (luminal, myoepithelial and alveolar cells) in reconstituted three-dimensional culture systems.[9]

Recent studies suggest that there is a cellular hierarchy in the mammary gland epithelium, with its origin in long-term oestrogen receptor-negative (ER^-) stem cells, which rarely divide to generate a population of short-term oestrogen receptor-positive (ER^+) stem cells; these latter divide more frequently than do primitive stem cells, to produce transit amplifying progenitors, which are committed to undergo terminal differentiation after a limited number of cell divisions.[8–10]

2. Breast cancer stem cells

That cancer may be a stem cell disease is not a new concept; a stem cell origin for cancer was proposed and subsequently abandoned decades ago, probably because research facilities available at that time in the field of stem cell biology were not adequate to support the theory with convincing experimental evidence.[11–13] It was only in the last few years that technological improvements, together with a critical reappraisal of data collected previously, have made scientists reassess the biology of cancer using a stem cell-based hierarchical model. The basic arguments supporting the idea that tissue stem cells may be primary targets for transformation can be summarised as follows: (i) stem cells are long-lived, slowly dividing cells that persist in tissues long enough to accumulate multiple genetic alterations required for neoplastic transformation, while somatic cells are constantly replaced through periodic cell turnover; moreover, long-lived cells are exposed to genotoxic insults much longer than are short-lived ones; (ii) molecular pathways, which play a critical role in governing stem cell self-renewal (i.e. Wnt, Notch, Sonic Hedgehog, PTEN)[14] are often deregulated in a number of tumours; (iii) normal stem cells and tumour cells share a number of phenotypic features, such as: a relatively undifferentiated state, the ability to self-renew, the activation of cytoprotective mechanisms (i.e. telomerase activity, overexpression of anti-apoptotic proteins, increased trans-membrane molecule efflux capability) as well as a remarkable competence for migration. Definitive evidence that tumours arise from and are sustained by mutated stem/progenitor cells has been achieved recently in blood, brain and breast cancers.[15–19] Indeed, it was demonstrated that only a small proportion of cancer cells (termed 'tumour-initiating cells' or 'cancer stem cells') retains the ability to form new tumours after transplantation in immunodeficient mice; moreover, tumour-initiating cells (T-ICs) display stem/progenitor cell properties: specifically, competence for self-renewal and capacity to re-establish tumour heterogeneity.

2.1. *Identification and isolation of breast cancer stem cells*

In a study reported by Al-Hajj and colleagues,[19] breast cancer-initiating cells (BC-ICs) have been identified prospectively based on the expression of unique cell surface antigens. In that study, authors fractionated lineage-negative human breast cancer cells from nine patients with respect to a combination of three additional markers: CD44, CD24 and ESA. $Lin^-CD44^+CD24^{-/low}$ cells were able to form palpable tumours when a few hundreds of cells were transplanted into the mammary fat pad of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice, while breast cancer cells expressing diverse combinations of these markers did not, even when 10,000 or more cells were injected. Moreover, in three out of nine cases it was found that among $CD44^+CD24^{-/low}$ cells, ESA-positive cells were enriched in tumour-initiating potential compared with ESA-negative ones. Interestingly, injected $CD44^+CD24^{-/low}$ cells generated additional $CD44^+CD24^{-/low}$ cells as well as phenotypically distinct cells; this provides evidence that breast cancer stem cells can undergo asymmetric division to self-renew and generate heterogeneous populations of non-tumourigenic cells that form the bulk of the tumour.

Since stem/progenitor cells of the normal human mammary gland have the distinguishing ability to grow in selective culture conditions as non-adherent spherical clusters of cells, we reasoned that the stem cell-like nature of the aforementioned tumourigenic breast cancer cells could be exploited to isolate and propagate them in vitro. Accordingly, we have recently provided evidence that this is the case.[20] Tumour-derived single cells showed the ability to proliferate and form clonally derived mammospheres when cultured in serum-free medium supplemented with bFGF, EGF and insulin. Long-term mammosphere cultures were comprised of undifferentiated, self-renewing tumour cells, which could differentiate into epithelial-like cells expressing markers of the mature mammary gland epithelium, thus demonstrating they were endowed with stem/progenitor cell properties. Most importantly, breast cancer cells propagated in vitro as floating mammospheres were found to be positive for CD44 and negative for CD24 and retained the ability to generate tumours in immunodeficient mice when as few as 1000 cells were injected. Taken together, these data demonstrate that we have isolated and propagated breast cancer-initiating cells, otherwise termed breast cancer stem cells.

Al-Hajj and colleagues reported that $ESA^+CD44^+CD24^-$ cells are enriched in tumour-initiating potential compared with $ESA^-CD44^+CD24^-$ cells.[19] Assuming that tumourigenicity is related to self-renewal, one may speculate that such an enrichment may result from an enhanced purification of the self-renewing subpopulation: if so, it would imply that $CD44^+CD24^-$ cells are comprised of self-renewing cells together with other cells lacking this property, or, alternatively, of long-term and short-term self-renewing cells. Accordingly, we observed that sphere-forming ability is a property of a minor proportion of cells propagated in vitro (10–20% of total cells in culture). In contrast to BC-ICs directly sorted from tissue samples, we could not detect ESA expression in BC-ICs propagated in vitro; however, it is worth pointing out that ESA expression was detected in metastatic pleural effusions

of breast carcinoma, while we derived our long-term cultures from solid lesions. Therefore, it is possible that one or more markers, different from ESA, are required to identify self-renewing cells in solid lesions, although it cannot be excluded that modifications in antigen expression occurred because of prolonged in vitro expansion. Further investigations are required to elucidate this point, which may have profound clinical implications. For instance, in a study by Abraham and colleagues,[21] the prevalence of $CD44^+CD24^{-/low}$ cells in breast cancer was estimated to be $\leqslant$10% in 78% of 122 tumours, $\geqslant$10% in the remainder, and found to be unrelated to the clinical outcome; such a conclusion might be different if the proportion of self-renewing cells in breast cancer should represent a minor subpopulation of the examined cells.

As stated previously, there is increasing evidence that normal mammary stem cells may be primary targets of transformation and that alterations in the Notch signalling pathway may play a relevant role in this process. In support of this theory, Clarke and colleagues reported that Musashi-1 and Notch1 expression was completely lost in ER^+ breast cancer lesions.[7] Since both proteins act as positive regulators of mammary stem cell asymmetric division, authors proposed that ER^+ tumours may occur as a consequence of abnormal predominance of symmetric on asymmetric division in ER^+ stem/progenitor cells.[7] In keeping with this view, Notch agonists have been demonstrated to induce a 10-fold increase in the percentage of self-renewing cells and to promote proliferation and survival of normal mammary stem/progenitor cells propagated in vitro as non-adherent mammospheres.[22] Similarly, we have observed recently that BC-ICs propagated in vitro constitutively display a remarkable sphere-forming ability, which has been postulated to be expected when symmetric, rather than asymmetric, self-renewal division prevails. On this basis, it is likely that a detailed characterisation of Notch signalling cascade in BC-ICs may improve our current knowledge about the impact of alterations in this pathway on breast carcinogenesis.

2.2. *What about the cell of origin?*

The term cancer stem cell (or tumour-initiating cell) is essentially an operational definition: it refers to that relatively small proportion of cancer cells that can long-term repopulate the bulk of the tumour and retain the ability to regenerate themselves continuously, thus mimicking their normal counterparts, albeit abnormally. In other words, as normal stem cells sustain organogenesis in normal tissues, so cancer stem cells do in cancer. However, while in normal tissues self-renewal and long-term repopulating ability are distinguishing properties of stem cells, this may not be the case for cancer stem cells which, by their very nature, arise as a consequence of genetic mutations. Accordingly, gain-of-function mutations occurring in transit amplifying or even in committed progenitors may produce a cancer stem cell phenotype, when self-renewal is the newly bestowed property; moreover, stem cells may silently carry genotypic alterations, which become phenotypically evident only in their progeny, leading the latter to act as cancer stem cells. Consequently, there is not an univocal correspondence between cancer stem cells and normal stem cells, as the former may originate from both an actual stem cell and from downward progenitors. On these bases the idea is founded that different tumour histotypes may be generated according to the cell type in which initiation occurs.

Evidence collected by some authors appears to agree in indicating that ER^+ breast cancers probably originate from ER^+, suprabasally located ULLC, which have been demonstrated to comprise mammary stem/progenitor cells.[7,8] Others have suggested that both ER^+ and ER^- lesions may derive from mutations occurring in a primitive, ER^- stem cell, according to different types of mutations variously affecting their differentiation.[10] In this respect, we preferentially obtained long-term cultures of BC-IC mammospheres from ER^+ lesions. Though we cannot state at present whether this occurred because of technical or biological reasons, and a number of cases larger than ours needs to be tested to come to any meaningful conclusion, it is worth pointing out that these BC-ICs showed a reduced ERα expression (as demonstrated by immunocytochemistry, unpublished data), which is outwardly in keeping with the model proposed by Dontu and colleagues.[10] In our opinion the possibility that ER^- breast tumours may arise from ER^- luminal progenitors should be also considered. Indeed, it was demonstrated recently that granulocyte-macrophage progenitors (GMP) may act as leukaemic stem cells in blast-crisis chronic myelogenous leukaemia (CML), following abnormal activation of β-catenin-dependent self-renewal; this would lead to impaired differentiation in cells belonging to the myeloid lineage, resulting in abnormal amounts of circulating blasts.[23] This study provides evidence that activation of specific pathways promoting stem cell self-renewal and maintenance of an undifferentiated status may result in more aggressive tumour histotypes as they occur in lineage-committed progenitors. This is not surprising if one considers that molecular alterations affecting differentiation of lineage-committed as well as transit amplifying precursors are likely to involve a greater number of cells than those occurring in primitive stem cells. Similarly, genetic modifications affecting ER^- luminal precursors may involve a large number of cells, preventing them from differentiating normally; this would result in a poorly differentiated phenotype, which is commonly associated with ER^- breast cancers. Despite these considerations, the cell of origin in breast cancer remains to be established. This point can be elucidated by comparing phenotypic features as well as gene expression profiles of normal and cancer stem cells, which are both currently under investigation.

2.3. *The niche and the microenvironment*

It is a fact that tissue specificity occurs, despite virtually all cells in an organism sharing the identical genome. It is also well established that interactions among different cell types are responsible for correct tissue morphology and functionality. The clearest evidence for this is provided by cell culture systems, where cells rapidly lose their distinguishing properties unless appropriate environmental requirements are satisfied. Thus, the microenvironment plays a key role in ruling cell positioning, proliferation and differentiation.

There is a good deal of evidence that the microenvironment exerts a critical influence on tumour development and

progression.[24,25] It has been demonstrated that a normal microenvironment can hold in check potentially tumourigenic cells carrying severe genetic injuries. For instance, retrospective studies on women, who had been exposed to toxic radiation in their youth, revealed that they developed breast tumours with a higher frequency than expected from the disease incidence in the overall female population; notably, tumours developed many years after the genotoxic insult, suggesting that potentially transformed cells were held in check by their normal neighbours. Conversely, alterations affecting stromal cells have been shown to promote formation of epithelial tumours,[26,27] and local modifications of tissue homeostasis induced by chronic inflammation may result in tumour development. Moreover, it is well established that tumours display a tissue-specific pattern of dissemination; for instance, it is known that breast carcinomas metastasise preferentially in bone, liver, lung and soft tissues. Finally, recent experiments strongly support the notion that a malignant genome can be reprogrammed to exhibit a normal-like phenotype as transferred into a new biological context.[28]

Stem cells, as other cells in the tissue, are not autonomous, self decision-making entities. On the contrary, their activity is minutely regulated by a few adjacent cells that create the strictly defined microenvironment in which they reside, usually termed 'niche'. In bone marrow, osteoblasts support and control haematopoietic stem cells, neural stem cells interact with both astrocytes and endothelial cells, so epithelial stem cells do with fibroblasts/myoepithelial cells. These few sup-

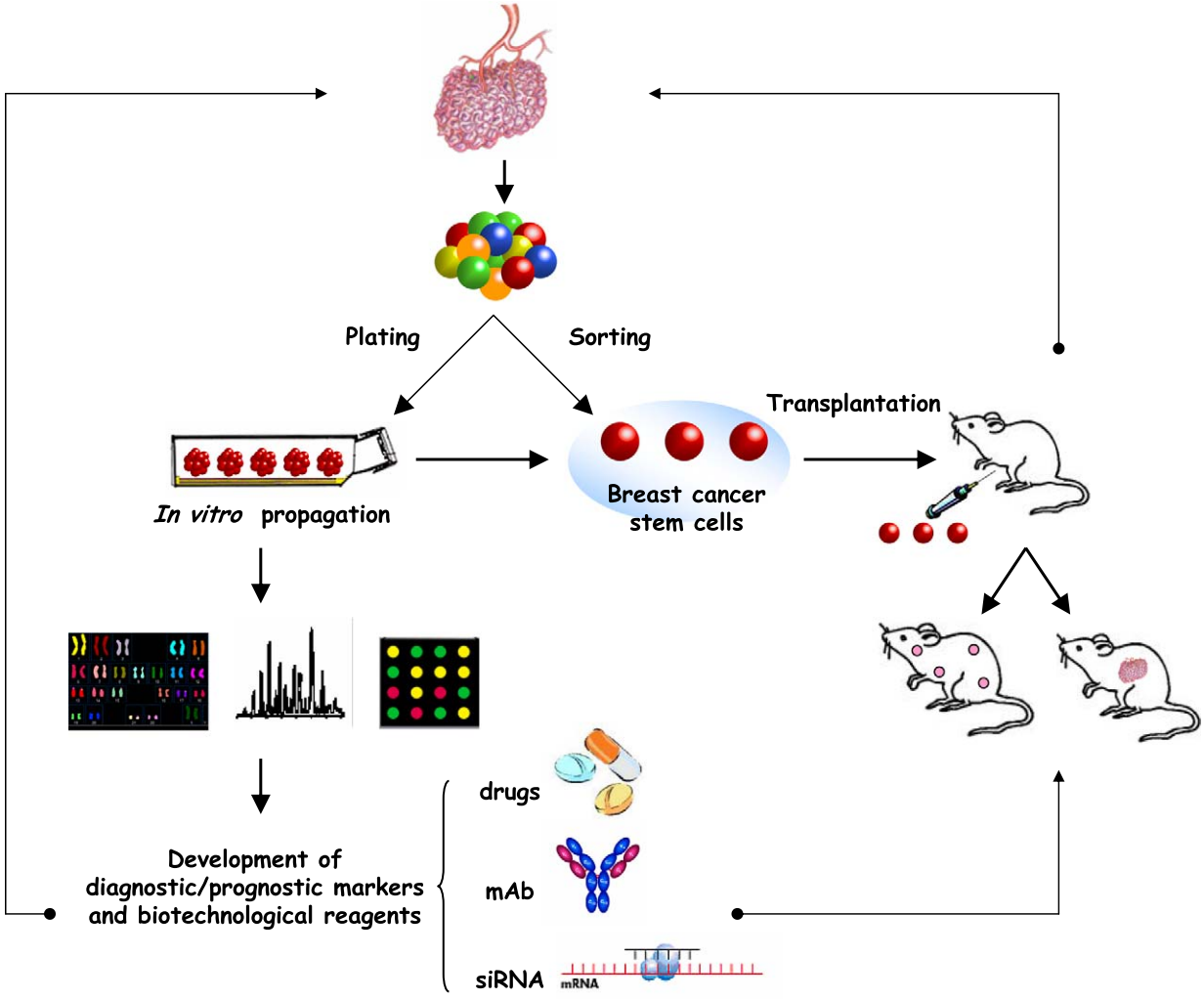

Fig. 1 – Culture systems to long-term propagate both normal and tumour breast stem/progenitor cells, which, in addition to appropriate animal models for functional transplantation studies and to high through-put technologies, represent a complete panel of experimental tools to perform integrated studies on both normal and tumour breast stem cells. Sorting followed by functional transplantation provides a powerful strategy for identification of breast cancer stem cells and will allow determination of whether there is a correlation between the dissemination pattern of breast carcinomas and preferential sites for homing of breast cancer stem cells. Reliable animal models are essential to test and validate in vivo results coming from in vitro models. In vitro models provide an important contribution, by allowing, for instance, production of a large amount of cells for profiling genetic and proteomic expression, which may lead to the development of new therapeutics to be tested in vivo. Furthermore, new diagnostic and prognostic markers may be identified, which could be validated on large numbers of collected breast cancer lesions/tissues.

port cells exert a critical role in maintaining stem cells as undifferentiated and quiescent. In addition, they act as a hub in orientating dividing stem cells, to hold one daughter cell in the niche (which therefore maintains its stemness), while the other one exits the niche and undergoes transit amplification followed by differentiation. The effectors mediating heterotypic cell interactions within the niche are comprised of a number of soluble factors and cell-surface receptors; interestingly, some of these molecules, such as Wnt, Notch, TGF-β, bone morphogenetic proteins (BMPs) and others, are known to be involved in tumour development. Far from being passive players in the tissue context, stem cells can in turn influence their microenvironment. Neural stem cells have been demonstrated to give rise to endothelial cells in addition to mature neural cells, showing that they are able to generate their own microenvironment;[29] similar findings have been reported for epithelial stem cells.[30] Interestingly, tumour cells also have the well-established ability to interact with their surrounding environment and to influence it profoundly; examples of this are neo-angiogenesis, recruitment of immune cells, and modification of tissue architecture.

Taken together, these observations clearly indicate that, if we accept the cancer stem cells paradigm, then we must also take the microenvironment into serious consideration. Accordingly, gene and proteomic profiles of purified breast cancer stem cells should be integrated with those of surrounding stromal cells. Moreover, a great deal of information may derive from in vitro co-culture systems as well as from co-transplantation experiments. Defining the molecular basis underlying cancer stem cell interactions with their normal or mutated microenvironment could throw new insights into the oncogenic process and, consequently, provide the basis for development of diagnostic reagents for detection of early-stage lesions and for design of new therapeutic strategies.

3. Conclusion

A growing body of evidence supports the notion that breast cancer may arise from mutated mammary stem/progenitor cells, which have been termed 'breast cancer stem cells' because of their exclusive ability to sustain tumour formation and growth. Breast cancer stem cells have been identified based on the expression of their $CD44^{+}CD24^{-/low}$ membrane phenotype and they have been demonstrated to have stem/progenitor cell properties. More recently, breast cancer stem cells have been isolated and long-term propagated in vitro as non-adherent clusters of undifferentiated cells, in a similar way to normal mammary stem/progenitor cells.

Culture systems for the isolation and expansion of both normal and tumour mammary stem cells, along with the availability of appropriate animal models for in vivo studies and of high through-put technologies (i.e. genomic and proteomic arrays), provide a complete panel of experimental tools (Fig. 1) to elucidate some crucial points that are still unclear and can be summarised as follows:

- From which cell do tumours arise?
- Which are the critical molecular alterations affecting breast cancer stem cells?
- How do they interact with the microenvironment?

The answers to these questions will probably provide useful information, which could be exploited to improve the diagnosis of breast cancers and our ability to predict clinical outcome and response to current therapies. At the same time, they would allow the development of therapeutic strategies aimed at selectively targeting breast cancer stem cells and sparing normal stem cells, which at present represent the ultimate common goal of research in this field.

Conflict of interest statement

None declared.

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Note added in proof

After the submission of this manuscript, a paper demonstrating the reconstitution of a complete mouse mammary tree in vivo by a single epithelial stem cells was published.[31]

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