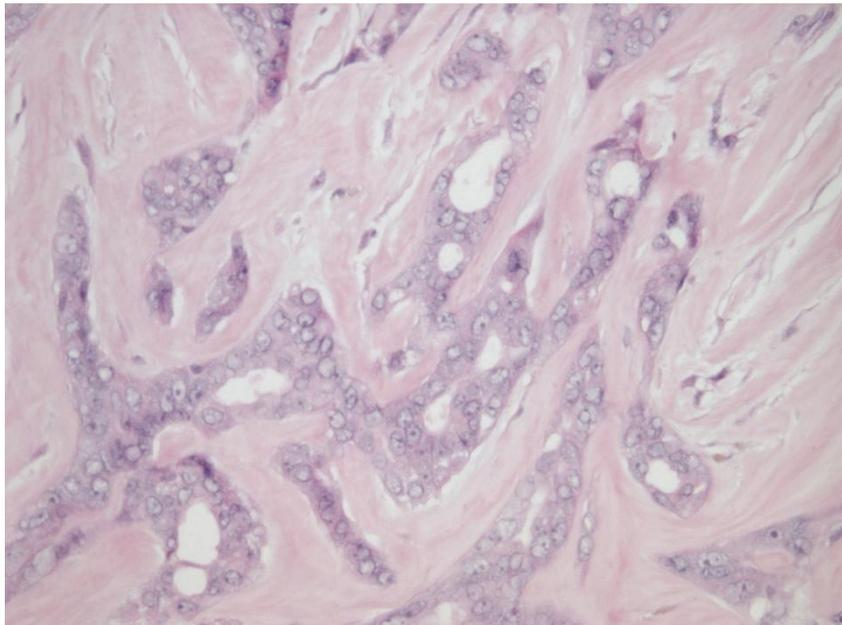




Sveriges lantbruksuniversitet
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Breast cancer stem cells- Evidence and contradictory views

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Bröstcancerstamceller-
Bevis och motstridiga åsikter

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INDEX

Summary	1
Sammanfattning	2
Introduction	3
Material and methods	3
Literature review	3
The cancer stem cell theory and breast cancer	3
Definitions	4
Properties of a cancer stem cell	4
Cell markers used to identify breast cancer stem cells	5
The origin of cancer stem cells	6
Cancer stem cells and epithelial-mesenchymal transition	6
Contradictory views	7
Recent studies indicate a progenitor origin of basal like breast cancer	7
Insufficient methods make the study results difficult to interpret	7
De-differentiation and its impact on the theory	8
Discussion	8
References	11

SUMMARY

This literature study aims to examine the existence of cancer stem cells in breast cancer. The cancer stem cell theory states that there is a hierarchical organization within a tumour, in which a small subpopulation of the cells can initiate new tumours and maintain tumour growth whilst the bulk of the tumour cannot. These tumour initiating cells have shown to possess many characteristics similar to those of adult stem cells, which is why they are often referred to as cancer stem cells. Both cell types have the capacity of asymmetric division and have shown to possess mechanisms of resistance to both apoptosis and cancer drugs. The cancer stem cell theory elucidates many biological aspects such as the heterogeneity of tumours and the relapse of many cancers after what appeared to be successful treatments. In the last decade, the first putative breast cancer stem cells were identified and further research was made which strengthened the relevance of the theory. Different markers have been used to identify the breast cancer stem cells and their tumour initiating capacity has been examined both *in vitro* and *in vivo*. However, recent studies on the existence and frequency of breast cancer stem cells have had varying results. The interpretation of these results is complicated by the difficulties in establishing a correct micro environment and the different techniques used when separating and identifying the breast cancer stem cells. There is a need for further research on the existence of breast cancer stem cells and their clinical relevance.

SAMMANFATTNING

Denna litteraturstudie syftar till att utreda var forskningen kring bröstcancerstamceller står idag. Enligt cancerstamcellsteorin finns i tumörer en hierarkisk organisation, där en viss subpopulation av tumörcellerna har förmågan att generera nya tumörer och underhålla tumörtillväxten, medan den resterande delen av tumörcellerna inte har denna förmåga. De tumörinitierande cellerna, ofta benämnda cancerstamceller, har visat sig ha många likheter med adulta stamceller. Bland annat har båda celltyperna förmågan att dela sig asymmetriskt, samt en utvecklad resistens mot apoptos och läkemedel. Cancerstamcellsteorin klargör många frågetecken inom cancerbiologin, såsom tumörers heterogenitet samt de många fall då tumörer återkommer efter vad som verkat vara lyckade cancerbehandlingar. I början av 2000-talet identifierades de första förmodade bröstcancerstamcellerna och ytterligare forskning inom området stärkte bevisen för deras existens. Olika markörer har använts för att identifiera bröstcancerstamcellerna och deras tumörinitierande förmåga har testats både *in vitro* och *in vivo*. Senare forskning har dock lett till varierande resultat vad gäller förekomst och frekvens av cancerstamceller i olika brösttumörer. Det finns fortfarande många brister i teknikerna som används för att identifiera och isolera bröstcancerstamceller. En stor utmaning handlar om att kunna upprätta en representativ mikromiljö för att kunna undersöka cellernas tumörinitierande förmåga. Ytterligare forskning krävs inom området innan det säkert går att klargöra huruvida cancerstamcellsteorin är applicerbar på och har klinisk relevans för bröstcancer.

INTRODUCTION

In the last century, studies have shown that only a small frequency of the cells within a tumour can proliferate extensively and give rise to cancer. This fact has been explained in different ways. One theory states that every cell within a tumour could give rise to a new tumour if the cell acquires additional spontaneous mutations, but the probability for these mutations to take place is low. A second hypothesis suggests that there is a hierarchical structure within the tumour, where certain cells have characteristics that enable them to initiate the growth of tumours whilst the majority of the cells do not (Reya et al., 2001).

Initially seen in leukaemia and shortly after in many solid tumours such as breast and prostate cancers, evidence has come up which supports the hierarchy theory. Researchers have found a subpopulation of cells in many tumours that appears to possess qualities which make them tumour initiating, whilst the major part of the tumour is not capable of initiating or maintaining a tumour. These cells have many properties similar to those of adult stem cells, which is why they are often referred to as cancer stem cells (Clarke et al., 2006).

The cancer stem cell theory has revolutionized cancer research since it elucidates many biological aspects such as the heterogeneity of certain tumours and the relapse of many cancers following what has seemed to be successful therapies. It is however a controversial theory. Despite the fact that a large part of the scientific community seems to be adapting to the idea, some critics still claim that the theory should be re-evaluated. My intention with this review is to clarify where cancer stem cell research stands today, with the focus on breast cancer stem cells.

MATERIAL AND METHODS

I have used the databases Pub Med, Science Direct, Web of Science, CAB and BIOSIS. Key words used were cancer stem cell, breast cancer, tumour initiating/propagating cell, cancer stem cell theory/hypothesis, cell markers, cancer initiating and origin. The key words were combined in different ways.

From the resulting articles I chose some reviews and research articles which I deemed relevant. Upon reading these, I gained an insight into the subject and was able to utilize their references to find further related articles. My supervisor also recommended three research articles of interest.

LITERATURE REVIEW

The cancer stem cell theory and breast cancer

In 1997, Bonnet & Dick (1997) came up with evidence of a hierarchical organization in acute myeloid leukaemia which led to a huge breakthrough in cancer research. It did not take long until this organization was found in other malignant tumours as well. Al-Hajj et al. (2003) were the first to identify tumorigenic breast cancer cells propagated *in vivo*. In their study, human breast cancer cells were injected into immunodeficient mice and these cells were

identified by cell markers. The authors found a cell lineage with the surface expression CD44⁺ CD24^{-/low}, which constituted between 11% and 35% of the tumour cells. When injecting this cell lineage into the mammary fat pads of the mice, as few as 10³ cells were needed to generate tumours in all trials. In contrast, when using tumour cells which were not sorted for any markers, 5 x 10⁴ cells were needed to cause tumours in all cases. The tumours that originated from the CD44⁺ CD24^{-/low} cell line were heterogeneous and consisted of CD44⁺ CD24^{-/low} among other cells that were not tumour initiating in trials. Interestingly, the CD44⁻ cells did not give rise to detectable tumours. Further on, a fraction of the CD44⁺ CD24^{-/low} cells which also expressed the cell marker epithelial-specific antigen (ESA) showed a higher tumorigenic potential, giving rise to tumours in all cases when as few as 200 cells were injected.

Two years later Ponti et al. (2005) confirmed that the CD44⁺ CD24⁻ cell lineage identified by Al-Hajj et al. (2003) possessed some qualities similar to those of stem or progenitor cells. The study of Ponti et al. (2005) showed that CD44⁺ CD24⁻ cells from breast cancer maintained their tumorigenic capacity when multiplied *in vitro*. When injected in immunodeficient mice, these cells could cause tumours in three of five cases when injecting 10³ cells. In comparison, 10⁶ cells were needed from the control group, to generate a tumour.

Ben-Porath et al. (2008) used 13 gene sets to investigate if regulatory pathways known to exist in the embryonic stem cell were also to be found in tumour cells. The study indicated that the embryonic stem cell networks observed were over-expressed in poorly differentiated cancers. In breast cancer, the expression of the gene sets varied significantly between different subtypes, but basal-like tumours showed a very similar signature to that of embryonic stem cells.

Definitions

Ever since the theory emerged, there has been confusion concerning the term “cancer stem cell”. Some interpret it as a mutated stem cell, which is misleading since the origin of cancer stem cells is so far unknown. In 2006 the American Association for Cancer Research held a workshop on cancer stem cells. As an attempt to reduce this confusion, the attendants defined a cancer stem cell as “a cell within a tumor that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor” (Clarke et al., 2006).

Other terms which have been used in the literature are tumour initiating, tumour propagating or cancer initiating cell. In this review the term cancer stem cell (CSC) will be used, since this is the term most commonly used in the literature today. When referring to breast cancer stem cells, the abbreviation bCSCs will be used.

Properties of a cancer stem cell

Many of the known properties of CSCs are similar to those of adult tissue stem cells. To begin with, both adult stem cells and CSCs can undergo asymmetric division. This means that when dividing, a CSC can give rise to one daughter cell which is a copy of itself, and another

daughter cell which is a progenitor cell that divides a limited number of times and will give rise to finally differentiated cells. Likewise, CSCs have been shown to obtain properties which make them resistant both to apoptosis and to drugs. Like adult stem cells, CSCs are believed to exist in either a dormant or a proliferative state. The dormant phase might be another explanation to CSCs supposed drug resistance, since most cytostatics today target dividing cells (Soltanian, S. & Matin, M., 2011).

Regardless of the similarities of adult stem cells and cancer stem cells, it is important to emphasize that there are major differences between these cells. The division and acts of adult stem cells are tightly controlled by signals from their environment, whilst the cancer stem cells do not respond to similar signals. Instead, they can continue to propagate the tumour and initiate tumours in new locations (Soltanian, S. & Matin, M., 2011).

Cell markers used to identify breast cancer stem cells

Different markers have been used to identify the breast cancer stem cells (bCSCs). The cell surface pattern CD44⁺ CD24⁻ identified by Al-Hajj et al. (2003) has been explored further and been used in many studies (Ponti et al., 2005; Wright et al., 2008). However, the exact nature of the association between this surface pattern and bCSCs has been debated due to the fact that the expression has varied considerably in different studies. Honeth et al. (2008) found that CD44⁺ CD24⁻ cells existed in only 31 % of 240 analyzed tumours, and the frequency within each tumour differed from close to 100% to very few. The expression was most common in basal-like breast cancers, whilst it was very rare in some other tumours. It was shown to be highly associated (found in 94% of the tumours) with inherited tumours with a mutation in the tumour-suppressor gene Brca1.

Today CD44⁺ CD24⁻ cell lines are often used together with other markers, such as aldehyde dehydrogenas 1 (ALDH1) and epithelial-specific antigen (ESA). ALDH1 is an enzyme involved in detoxifying xenobiotics and some endogenous aldehydes (Ginestier et al., 2007). This enzyme may also be involved in early cell differentiation because of its oxidative capacity. Ginestier et al. (2007) examined its role in stem cells and neoplastic human mammary epithelium, finding it to be a marker for a tumorigenic cell fraction with the properties described for CSCs. Expression of this cell marker was associated with a poor clinical outcome. The ESA profile has also been associated with high tumorigenic activity in studies (Al-Hajj et al., 2003; Fillmore & Kuperwasser, 2008). In a study by Fillmore & Kuperwasser (2008), the results indicated that the CD44⁺ CD24⁻ profile is not alone specific enough to be able to identify bCSCs. However, when sorting the cells on the basis of a CD44⁺ CD24⁻ ESA⁺ profile, as few as 100 cells were needed to generate a tumour. According to this study, CSCs constitute, depending on tumour subtype, between 0, 01 -2, 5% of the tumour cells.

CD133 is a cell marker identified in tumours of other organs such as the brain and the prostate. Wright et al. (2008) identified this cell marker in some Brca1 tumour cell lines and found these cells to have many properties similar to those of CD44⁺ CD24⁻ cell lineage, such as resistance to xenobiotics and tumour initiating capacity. The study indicated that the

expression of many stem cell associated markers such as Oct4 was enriched in both CD44⁺ CD24⁻ and CD133⁺ cells. However, the presence of the cell markers varied between different Brca1-deficient tumours.

The origin of cancer stem cells

The origin of CSCs is yet unknown. One hypothesis is that they arise from adult stem cells. Stem cells are long-lived cells, which increases the possibility of the mutations required for malignancy to occur. In addition to this, they already have the capacity of self-renewal described for CSCs. Another possibility is that CSCs originate from progenitor cells that have obtained a more efficient self-renewal capacity (Reya et al., 2001). A third hypothesis is that CSCs are derived from well differentiated cells that de-differentiate and so acquires the characteristics of stem cells (Soltanian S. & Matin, M., 2011).

Possibly, more than one origin of CSCs may exist, depending on the location of the tumour and its phenotype (Hanahan & Weinberg, 2011). It is unclear whether CSCs are actually the cells receiving the first oncogenic hit, or if the first neoplastic cells acquire stem cell like properties after several additional mutations during the carcinogenesis, which results in CSCs (Clarke et al. 2006; Hanahan & Weinberg, 2011).

Cancer stem cells and epithelial-mesenchymal transition

Recent studies have indicated a correlation between epithelial-mesenchymal transition (EMT) and cancer stem cells (Mani et al., 2008). EMT is a multi-step process needed for remodelling and building up new tissue formations, both under embryogenesis and later in life. It involves alternating expressions of many cell interactions, such as adhesion or loss of adhesion to other cells and reorganization of the cytoskeleton. This signalling program has earlier been associated with cancer progression and metastasizing (Creighton et al., 2010).

Mani et al. (2008) found in their study that when forcing immortalized non-tumorigenic human mammary epithelial cells to undergo an EMT, these cells acquired properties similar to those of mammary stem cells. In addition to this, the study showed that a major part of these EMT-induced cells appeared to have acquired the CD44^{high} CD24^{low} expression pattern found in earlier studies (Al-Hajj et al., 2003). This cell marker shift was not seen among the control cells (Mani et al., 2008). Further tests indicated the CD44^{high} CD24^{low} cells to have a self-renewal capability *in vitro* and the ability to give rise to cells from both luminal and basal lineages. Mammospheres formed by these cells could differentiate and form secondary structures, with a heterogeneous cell population. The conclusion was that the CD44^{high} CD24^{low} cells behaved like bipotential precursor cells.

Moreover, immortalized neoplastic human mammary epithelial cells, forced to undergo EMT, showed to form markedly more tumour spheres *in vitro* than did the control group. It was also shown that cells that were given insufficient EMT-inducing signals developed back towards an epithelial phenotype. Mani et al. (2008) then injected neoplastic human mammary epithelial cells with constitutive EMT-inducing signals into immunodeficient mice. The result

was that when injecting 10^3 of these cells, tumours were formed in most of the mice. Cells that had not undergone EMT needed as many as 10^5 cells to see any tumour formation, and tumours only appeared in three out of five cases.

These results indicate that differentiated cells when influenced by certain signals could be able to gain stem like properties, i.e. de-differentiate.

Contradictory views

Recent studies indicate a progenitor origin of basal like breast cancer

Recently, Molyneux et al. (2010) found that deleting *Brcal* in mouse luminal progenitors led to a tumour phenotype clearly associated with the one seen in human *Brcal* basal-like breast cancer. In contrast, deleting *Brcal* in basal stem cells led to a high frequency of aggressive malignant adenomyoepithelioma. The results indicate that while malignant adenomyoepitheliomas may originate from mutated stem cells, basal-like breast cancer seems to originate from luminal progenitors. These cells do not have the same characteristics as those seen in CSCs, for example they are $CD24^{+high}$ unlike CSCs which in most studies are identified by their $CD24^{low}$ expression (Molyneux et al., 2010).

Insufficient methods make the study results difficult to interpret

Critics have questioned both the *in vitro* systems and the xenograft models used to examine the tumorigenic potential of CSCs (Hill, 2006; Visvader & Lindeman, 2008; Gupta et al., 2009). The environment has a significant impact on processes such as self-renewal, differentiation and tumour propagation. A solid tumour consists of not only cancer cells but of inflammatory and other cells, surrounded by a complex matrix and multiple factors interacting with the tumour growth. When separating a tumour cell from its original site to an *in vivo* environment this could have a huge impact on the cell's tumour initiating capacity. Can one be sure that cells grown in an *in vitro* culture have the same ability to generate tumours as the ones grown at an actual tumour site would have (Hill, 2006)?

The xenograft model contains some readily debated issues. A foreign environment with murine signalling systems to which a human cell do not respond, could lead to an underestimation of the frequencies of tumour initiating cells. Even though it is necessary when using xenograft models, the immunocompromission of the mice has been criticised, since the immune system normally has an influence on the tumour growth. On the other hand, some claim that the presence of natural killer cells in NOD-SCID to have an impact on the result (Visvader & Lindeman, 2008). Studies using allografts and non immunocompromised mice have indicated that much higher frequencies of the tumour cells, if not all, are capable of initiating tumours (Yoo & Hatfield, 2008). To use allograft models instead of xenografts exclude some biases associated with the species differences, however, it is debatable whether syngeneic mice models are actually representative of human cancers (Kennedy et al., 2007).

The techniques used to isolate and identify the cells are advanced and the methods used vary between the researchers. One must bear in mind that factors such as enzyme treatment during

the process and the transfer of the cells may change the expression of different cell markers and cells might even lose their viability (Visvader & Lindeman, 2008). As to flow cytometry, used by Al-Hajj et al. (2003), it is a critical step to keep the cells viable. The instruments are most often developed for sorting out blood components, and bigger, fragile cells within organs are not as easy to deal with as are blood components with this method. When separating the cells there is also a risk that cells stick to each other, which is why the separation of single cells should always be controlled in a microscope (Clarke et al., 2006). Matrigel, a basement membrane-like substance used in many of these procedures, for example by Al-Hajj et al. (2003), has been shown to enrich the tumour initiating capacity of the cells transplanted. Due to the importance of the microenvironment, the site of transplantation may well have a great impact on the frequency of tumour initiating cells (Visvader & Lindeman, 2008).

De-differentiation and its impact on the theory

Recent studies have supported the possibility that differentiated cancer cells can de-differentiate into stem cell like cancer cells (Mani et al., 2008). If non-CSCs can rapidly de-differentiate into CSCs and there is equilibrium between the amount of CSCs and the non-CSC cells, this could question the applicability of the theory. However, if this can in fact happen within a tumour site is yet to be proven (Gupta et al., 2009).

DISCUSSION

None of the cell surface patterns used to identify putative bCSCs today seem to be exclusively expressed on bCSCs, and markers believed to sort out bCSCs in one study do not overlap with those that seem to do so in another (Honeth et al., 2008; Wright et al., 2008). This can be due to technical problems, such as varying antibody preparations and difficulties in sorting out tumour cells in solid tumours, which leads to impure populations with a mix of cells (Visvader & Lindeman, 2008). Alternatively, it could indicate that these cell surface expressions are improper markers for bCSCs. Another possibility is that this variety in cell markers indicates that there might be different subtypes of bCSCs, even within the same breast tumour subtypes and that the frequency of the bCSCs differs widely both between and within breast tumour subtypes. It is worth noting that none of the first studies supporting the existence of breast cancer stem cells (Al-Hajj et al., 2003; Ponti et al., 2005) excludes the possibility that the frequency of the cells can vary between and within different tumours. On the other hand, the clinical applicability of the theory could be questioned if the major part of the tumour cells seems to be CSCs. However, the absence of selective markers for bCSCs could as well be due to the fact that the CSC theory is not applicable on breast cancers. To summarise, there are still many uncertainties when it comes to the cell markers of bCSCs.

During the workshop on CSCs held by the American Association for Cancer Research, Clarke et al. (2006) defined the cancer stem cell as “a cell within a tumor that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor” (Clarke et al., 2006). However, this definition reveals nothing about the frequencies and the origin of CSCs and the interpretation of the CSC concept still differs markedly between

researchers. Some researchers seem to have the approach that CSCs are indeed mutated stem cells, and the focus lies on the origin of primary tumours. Others state that the term does not refer to the cell of origin but to the cell which drives the cancer progress and can give rise to secondary tumours. In this view, the focus lies more on cancer propagation and treatment strategies. It is worth noting that putative bCSCs studied today are identified in already existing tumours, and it is their potential to generate secondary tumours which has been examined so far. The study by Molyneux et al. (2010) provides evidence against that mutated stem cells should be the origin of basal like breast cancer. However, I do not see that the study excludes the second approach of the CSC concept, i.e. that CSCs exist, but that they do not need to be the cells receiving the first oncogenic mutation. Basal-like breast cancer has a phenotype more similar to that of basal stem cells than that of luminal progenitors (Molyneux et al., 2010). It might then be possible that basal-like breast cancer originates from luminal progenitors, but that cells with stem cell like characteristics, i.e. CSCs, arise during the carcinogenesis. However, this possibility is yet to be proven.

The CSC model is very appealing since it elucidates question marks in tumour biology, such as tumour heterogeneity and relapses of tumours, and opens the door for new treatment strategies. Nevertheless, breast cancer is a multifactorial disease and I believe both genetic and environmental factors to play a major part in its development. There is plenty evidence for the existence of a subpopulation of cells in some breast tumours which is tumour initiating and have stem cell like properties (Al-Hajj et al., 2003; Ponti et al., 2005; Ben-Porath et al., 2008). However, in my opinion the study results so far do not exclude that the cells identified as CSCs might have gained their tumorigenic potential from mutations and not inherited them. Perhaps, the CSC theory can be relevant for some breast cancers but not for others.

As for all scientific studies, one should keep in mind the biases which complicate the interpretation of the results. Ben-Porath et al. (2008) collected gene sets seen to be expressed by stem cells in earlier studies. What techniques the previous researches used, how the authors interpreted their results and whether these gene sets are adequate for the purpose have significant impact on the result. It is obvious that the microenvironment has a huge impact on the result of the study. Today, there are no ideal assays for examining CSCs but according to Clarke et al (2006), serial transplantation in animal models is the best existing assay for studying self renewable and lineage capacity. One must keep in mind the biases concerning species-species differences, however, some murine growth factors to which human cells respond are known, which indicates that the human cells might get adequate signals from the microenvironment after all (Visvader & Lindeman, 2008).

If the CSC theory would turn out to be applicable on breast cancer, this could have a major impact on the development of new treatment strategies. The focus should lie on targeting the fuel of the tumour, i.e. the CSCs, instead of attacking the bulk of the tumour, which is not tumour initiating and will finally differentiate (Clarke et al., 2006). Naturally, the CSC's supposed drug resistance must be acknowledged when developing such medicines. If it is possible to find markers that are specific for bCSCs the impact upon other cells, a major issue in contemporary cancer treatment, would be reduced. However, as Gupta et al. (2009) brings

up, the therapy aspect is complicated by recent studies, such as that of Mani et al. (2008), which indicate that CSCs might arise from differentiated cells that have the ability to de-differentiate. If this balance is held in place by certain signals within the tumour environment, it would not help solely to focus the treatment on CSCs, since new ones might arise at the same rate that old ones are killed. That is, while further evaluating the theory, one must take in focus the treatment of both CSCs and the bulk of the tumour (Gupta et al., 2009).

To further evaluate the veracity and applicability of the CSC theory on breast cancer more research is necessary. There is a need for more specific ways to identify the putative bCSCs, including both cell surface patterns, genetic markers and the behaviour of the cells in assays. The same implies for further developed methods and assays, both *in vitro* and *in vivo*, to reduce biases such as differing microenvironments. The origin of CSCs is an area where more research is needed. Are they in fact the cells which get the first “oncogenic hit” or do they originate from other neoplastic cells which are transformed during the carcinogenesis? It would also be interesting to further examine whether CSCs differ in frequency in the same tumour over time. This would provide a greater knowledge of whether de-differentiation takes place within tumours, and which mutational and/or environmental factors that can turn non-CSCs into CSCs. The connection between EMT and cells obtaining stem cell properties has much left to be understood. Since EMT is also associated with metastasis, further research within this area could lead to new understanding of the connection between CSCs and metastasis. To gain further evidence for the theory’s clinical applicability, the relation between bCSCs, the tumour phenotype and the clinical outcome should be further investigated. If a correlation were to be found, more knowledge of the similarities and differences between mammary stem cells and bCSCs could help developing new cancer drugs with bCSCs as target.

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