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Breast Cancer Metastasis: Advances Through the Use of In Vitro Co-Culture Model Systems

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1. Introduction

Worldwide, breast cancer is the most frequent cancer diagnosed in women and is the second-most leading cause of cancer related deaths in women (Jemal, Bray et al. 2011). Death from breast cancer is most often the result of the spread of the primary tumour to distant sites, where the cancer cells lodge and develop into metastases. Depending on the site of the metastasis, the patient may live for years with reduced quality of life and needing increased health care resources. There is clearly a need for a greater understanding of the molecular events involved in breast cancer metastasis in order to improve treatment options for breast cancer patients and develop therapies aimed at preventing breast cancer metastasis.

Here we will summarize what is known about the molecular basis of breast cancer metastasis and discuss the use of *in vivo* and primarily *in vitro* model systems to study it.

2. Current knowledge

2.1 Metastasis

As early as 1889, Stephen Paget observed that some cancers metastasized preferentially to specific organs, and developed his theory of "seed and soil"(Paget 1889). The essential tenet of this theory was that cancer cells (seeds) disseminate throughout the body from their point of origin but can only develop metastatic satellites in appropriate stromal environments (soils). The many advances in our understanding of the molecular and cellular bases of breast cancer metastasis has led to a somewhat more complex picture, and the processes involved are still not completely understood. Breast cancer can spread to any secondary site in the body but metastases appear preferentially in bone, lung and liver (Rabbani and Mazar 2007). Presumably these sites provide a microenvironment favourable for the growth and development of breast cancer cells (Nguyen, Bos et al. 2009).

There are two prevailing models of breast cancer metastasis; one suggesting a linear progression and the other a parallel progression. The linear progression model advances the idea that cells in the primary tumour accumulate progressive mutations in a stepwise manner in genes regulating some aspect of cell growth and division such as oncogenes and tumour suppressor genes. Some cells eventually become able to proliferate autonomously; they expand clonally and leave the primary site to travel through lymphatic or vascular systems to a distant organ where they develop into a secondary metastatic growth. This

model implies that cells at the primary site must undergo a number of rounds of division before they become autonomous and so development of metastasis is linked to primary tumour size with metastases more likely to develop from larger primary tumours. In support of this model it has long been known that there is a close association between tumour size and the possibility of development of metastasis, and tumour size is used as part of histological classification (1983; Rakha, Reis-Filho et al. 2010). The model also suggests that cells being shed by the primary tumour are fully metastatic and that cells that have metastasized to a secondary site should also be able to leave that site to set up at a tertiary site (Klein 1998; Klein 2009). Mutations in genes such as BRCA1, BRCA2, p53 and RB and amplification of the HER-2 receptor at the site of the primary tumour have been identified as being predictive of poorer outcome for breast cancer patients, consistent with this model (Slamon, Clark et al. 1987; Ross and Fletcher 1999; Bordeleau, Lipa et al. 2007; Bosco and Knudsen 2007; Kumar, Walia et al. 2007; Baker, Quinlan et al. 2010).

The parallel progression model suggests that tumour cells may disseminate from the site of the primary tumour very early in its development and may be subsequently genetically modified in the metastatic niche where they later settle (Klein 2009). This model predicts that disseminated tumour cells in the blood or lymph should be detectable very early in development of the primary tumour and that cells at the site of metastasis could be genetically divergent from those at the site of the primary tumour. In support of this model it has been shown in a HER-2 mouse model and in women with ductal carcinoma *in situ*, that disseminated tumour cells in bone and micro metastases could be detected from the time of earliest epithelial alterations at the site of the primary tumour. The numbers of disseminated tumour cells in this study were found to be the same for small and large tumours (Husemann, Geigl et al. 2008), suggesting that shedding of cells from the tumour mass was independent of primary tumour size. In a qualitative and quantitative study of 12,423 women with breast cancer, J. Engel *et. al.* (Engel, Eckel et al. 2003) determined that systemic disease was already present at the time of diagnosis in women who went on to develop metastases, again suggesting cells left the primary tumour early during its development.

The advent of single-cell genomics has allowed comparison of the characteristics of disseminated tumour cells in the blood and lymph and cells at the site of the primary tumour and these have been found to be genetically divergent in some cases (Klein, Seidl et al. 2002; Klein 2003; Fuhrmann, Schmidt-Kittler et al. 2008; Klein 2009; Klein and Stoecklein 2009), indicating that early clonal divergence and parallel progression may occur in some breast cancers. Disseminated tumour cells may also differ genetically from cells that eventually develop into a metastasis in the same patient (Stoecklein and Klein 2010). This could reflect the requirement for the disseminated tumour cells to undergo whatever genetic changes are necessary for them to adapt and be able to successfully grow in the new microenvironment. If that is the case it follows that the genetic aberrations found in the primary tumour may not reflect those seen in the metastasis and this has been found to occur (Tortola, Steinert et al. 2001; Albanese, Scibetta et al. 2004; Gow, Chang et al. 2009; Stoecklein and Klein 2010). In colorectal cancer, mutations in B-raf, K-ras and p53 seen in the primary tumour may be absent or altered in the metastasis. In some cases mutations in the metastasis may be absent in the primary tumour (Tortola, Steinert et al. 2001; Albanese, Scibetta et al. 2004; Stoecklein and Klein 2010). In a study of non-small-cell lung cancer where EGFR mutation status is used as a determinant for treatment with tyrosine kinase inhibitors, 27% of paired primary/metastasis samples (n=67 patients) were found to be

discordant with respect to EGFR mutation status (Gow, Chang et al. 2009). This is of concern in a time of more personalized treatment, where often it is the genetic signature of the primary tumour alone on which outcome predictions or treatment options are based.

2.2 Metastasis suppressor genes

Evidence suggests that less than 1% of breast cancer cells that enter the circulatory system are capable of generating metastatic foci (Fidler 1970; Fidler and Nicolson 1977). Often disseminated breast tumour cells that have settled in the microenvironment at the site of metastasis will lie dormant for years in patients with no evidence of disease before developing into a clinically significant metastatic focus, indicating they are capable of escaping early systemic therapies that target rapidly proliferating cells at the site of the primary tumour (Pantel, Schlimok et al. 1993; Klein, Seidl et al. 2002; Riethdorf, Wikman et al. 2008; Morgan, Lange et al. 2009). As they remain quiescent for some period of time this also suggests that they, or the cells in their microenvironment, or both undergo genetic changes which allow them to progress to a metastatic phenotype (Riethdorf, Wikman et al. 2008) (Riethdorf, Wikman et al. 2008; Klein 2009; Nguyen, Bos et al. 2009; Smith and Theodorescu 2009; Rose and Siegel 2010; Stoecklein and Klein 2010). A class of genes that has been implicated in the regulation of this process is metastasis suppressor genes (Smith and Theodorescu 2009). These are genes that inhibit metastasis but do not affect the ability of cells to produce a primary tumour, and they play key roles in invasion, dissemination, arrest, survival and colony formation. Their function must be lost or inhibited for a metastasis to develop and they represent fertile new ground for the development of anti-metastatic therapeutics.

A number of metastasis suppressor proteins have been reported to inhibit breast cancer metastasis. Reduced levels of nm23 family proteins in the primary tumour have been reported to correlate with more aggressive phenotype in breast cancer patients (Galani, Sgouros et al. 2002; Terasaki-Fukuzawa, Kijima et al. 2002; Steeg, Ouatas et al. 2003; Peihong and Perry 2007), although conflicting results have also been presented (Charpin, Garcia et al. 1998; Belev, Aleric et al. 2002; Sgouros, Galani et al. 2007). The results seen in mouse models are more straightforward, where breast cancer cells with low expression of nm23 are more metastatic than those with high levels (Leone, Flatow et al. 1993; Bhujwalla, Aboagye et al. 1999; Tseng, Vicent et al. 2001). *In vitro* models have revealed that nm23 acts by reducing breast cancer cell motility and invasiveness (MacDonald, Freije et al. 1996; Russell, Pedersen et al. 1998; Steeg, Ouatas et al. 2003; Horak, Lee et al. 2007).

For Breast Cancer Metastasis Suppressor-1 (BRMS1), the clinical data reporting it to be a metastasis suppressor protein in breast cancer tumour samples is also conflicting (Kelly, Buggy et al. 2005; Hicks, Yoder et al. 2006; Lombardi, Di Cristofano et al. 2007). Again, its role in mouse models is clearer, where higher expression in breast cancer xenografts clearly resulted in reduced metastasis (Hedley, Vaidya et al. 2008; Hurst, Xie et al. 2008; Phadke, Vaidya et al. 2008). The stage at which BRMS1 suppresses metastasis is less clear, as it appears to affect a number of steps in the process of metastasis (Stafford, Vaidya et al. 2008). At least two of its functions appear to be increasing anoikis of cells free in the vascular system and inhibition of colonization of disseminated cells (Phadke, Vaidya et al. 2008). KAI1 (CD82, Tetraspannin), has also been clearly verified as a breast cancer metastasis suppressor in clinical samples, where decreased expression correlates with poor outcome (Yang, Welch et al. 1997; Christgen, Christgen et al. 2009; Malik, Sanders et al. 2009). Similar to BRMS1, KAI1 appears to act in multiple ways to inhibit metastasis and reduce breast

cancer cell adhesion, migration and invasion *in vitro* (Malik, Sanders et al. 2009) and metastasis in mouse models *in vivo* (Yang, Wei et al. 2001). Other metastasis suppressor genes implicated in inhibiting breast cancer metastasis include KISS1 (Harms, Welch et al. 2003), MTSS1 (Parr and Jiang 2009) and alpha2beta1 integrin (Ramirez, Zhang et al. 2011), although their roles, at least in breast cancer have been less well studied.

As can be seen, the determination of the role of metastasis suppressor genes in metastasis using clinical samples is often confusing. This seemingly conflicting data may be a result of the many different experimental approaches to examining clinical samples; whether the samples are frozen or paraffin embedded and formalin fixed, whether mRNA or protein levels are the final determinant of expression (and these do not always correlate well), the type of extraction procedures used, and the source of the antibodies and staining methods for immunohistochemistry. The other difficulty with clinical samples is that they are almost exclusively derived from the primary tumour site, as biopsies of metastases are rarely carried out. Metastasis suppressor genes by definition do not inhibit events at the site of the primary tumour but must be inhibited for metastasis to take place. This inhibition may allow invasion of the circulatory system from the site of the primary tumour, survival through the process of transportation to the site of metastasis and evasion of the immune system, arrest within the metastatic niche, extravasion from the circulatory system or growth in the new environment (Kaplan, Psaila et al. 2006; Rabbani and Mazar 2007). Inhibition of expression at any step following detachment from the primary tumour would not likely be detected in the primary tumour.

3. Model systems of metastasis

It is evident that disseminated tumour cells in the blood and lymph and cells at the site of metastasis may diverge in phenotype from cells at the site of the primary tumour and from each other. Metastasis suppressor genes represent some of the genes with altered expression and one possibility of targeting metastasis therapeutically is to induce their re-expression or reiterate their function at the site of metastasis (Steeg, Ouatas et al. 2003; Stafford, Vaidya et al. 2008; Smith and Theodorescu 2009). To effectively select therapeutic targets it will be important to better understand their functions in the microenvironment of the site of metastasis. It is also known that the stromal environment surrounding the tumour cell is an active collaborator in the development of the metastasis. It is imperative to examine interactions between tumour cells and the stromal cells in the metastatic niche to understand what changes the stromal cells induce in the tumour cells and what changes the tumour cells induce in the stromal cells. *In vivo* and *in vitro* model systems have long been used as pre-clinical models to study breast cancer metastasis and ways of treating or preventing it.

3.1 In vivo mouse models

The use of mouse models in studying human breast cancer metastasis has the very great advantage of being able to study the entire process of metastasis from development of the primary tumour to the final development of the metastasis. It is possible to label the tumour cells with a variety of probes including green fluorescent protein (GFP) or luciferase and there are many excellent imaging techniques such as magnetic resonance, computed tomography and ultrasound available for live animal imaging to follow the progress of the tumour cells in the mouse. Live imaging is an advantage as progress can be monitored in one mouse over a period of time rather than sacrificing a number of mice at different time

points. One of the major disadvantages in using mouse models to study human cancer metastasis is that mice are not human, and there is no guarantee that the metastasis will develop in them in a way that recapitulates what happens in a human body. Mice do develop breast cancer as a heterogeneous disease, similar to humans (Andrechek and Nevins 2010), but there are significant differences between mice and humans in the capacity of the primary cells for transformation, the size of tumours, expression of hormone receptors and preferential sites of homing for breast cancer. For researching metastasis of human cells, immunodeficient mouse strains need to be used, taking the model a further step away from what happens in a human host. In addition human breast cancer cell lines may not accurately reflect the biological characteristics of in-vivo breast cancer such as natural evolution and tumor diversity. Given that caveat, mouse models are very important for testing pre-clinical data before moving on to clinical trials or human tumour tissue samples.

There are many technical issues to take into account when considering the use of a mouse model to study breast cancer metastasis. Those are beyond the scope of this chapter but are very fully reviewed by Danny Welch (Welch 1997). Mouse models have been particularly useful in identifying molecules important for a number of steps in metastasis, such as epithelial to mesenchymal transition (EMT), invasion, extravasation and intravasation (Vernon, Bakewell et al. 2007). One approach to modeling metastasis is to use xenografts, where human tumour cells are injected subcutaneously or into the mammary fat pad of a mouse and allowed to develop a primary tumour that spontaneously metastasizes. A second approach is to inject tumor cells directly into the venous system, using tail vein injection or cardiac puncture. Tail vein injection results primarily in metastasis to the lung, but cardiac puncture results preferentially in bone metastasis. This approach obviates the need for development of the primary tumour but is not useful for studying some of the early steps of metastasis. The artificial injection of tumor cells directly into the venous system may produce pseudometastasis through a process of embolization rather than true physiological metastasis. A third approach is to utilize genetically engineered mice that have had a tumour suppressor gene deleted or an oncogene activated in an organ specific manner.

Xenograft models and venous injections most generally use breast cancer cell lines, many of which are maintained and sold by the American Type Culture Collection (ATCC). These cell lines have a variety of gene expression profiles that identify them as similar to luminal, basal A or basal B [subtypes initially defined in tumour samples in 2006 (Fridlyand, Snijders et al. 2006)] and they show a variety of receptor and p53 profiles (Neve, Chin et al. 2006). Gene expression in these cell lines can be modified by over expression or deletion and the effect of the altered gene on metastasis can be monitored following injection. Although the resulting tumours are considered to metastasize "spontaneously", the injected cell lines are an artificial starting material as they have been cultured for long periods of time *in vitro* and do not resemble a spontaneously arising tumour. One of the advantages of xenograft models is that the cells of the primary tumour must interact with the stromal cells surrounding the tumour and must also interact with the stromal cells at the site of the metastasis for a productive metastasis to develop. A great deal of information about the interactions of human tumour cells and stromal cells has been accumulated by injecting human tumour cells and human mesenchymal stem stromal cells together in xenograft models [reviewed in (El-Haibi and Karnoub 2010)]. By using the same cell lines and different routes of injection it is possible to determine whether a gene is necessary for early steps of metastasis or whether it is involved in later steps (Chabottaux, Ricaud et al. 2009).

Genetically engineered mice, whether transgenic or gene knockout animals, have an advantage over xenograft models in that they illustrate the metastasis of tumours that arise in the mouse mammary gland as a result of internal genetic changes and not exogenously injected cells. This is more representative of how tumours develop from the very beginning of the metastatic process. To limit expressed genes to the breast of the transgenic mouse a breast-specific promoter such as the Mouse Mammary Tumour Virus (MMTV) promoter or the Whey Acidic Protein promoter (WAP) is generally utilized (Kim and Baek 2010). As the tumour arises from mouse cells, immunocompetent mice can be used and the effects of an intact immune system on the process of metastasis can be determined. There are many strains of mice available with well defined genetic backgrounds, enabling researchers to study the effects of differing genetic backgrounds on the development of metastasis arising from gain or loss of the gene of interest (Husemann and Klein 2009). Mice carrying different transgenes or knockouts can be crossed with each other to determine if there is an additive or synergistic effect of the different genes on development of metastasis (Vernon, Bakewell et al. 2007). Gene expression or deletion can be temporally regulated, using an MMTV promoter either alone or directing expression of the Cre/loxP system for somatic deletion. The MMTV promoter becomes active only after puberty, preventing the oncogene or tumour suppressor gene of interest from causing embryonic lethality. One of the drawbacks to using a genetically engineered mouse model is in testing therapeutic compounds. These mice develop subtypes of breast cancer similar to, but not identical with the subtypes seen in human breast tumours. Also, most tumours arising in genetically engineered mice lack expression of the estrogen receptor and thus fail to recapitulate human tumours that are estrogen receptor positive. Cytogenetic and genetic backgrounds are different between mice and humans as well and this could lead to misinterpretation of the usefulness and safety of a therapeutic compound (Kim and Baek 2010).

3.2 In vitro co-culture systems

3.2.1 Three-dimensional co-culture

Normal breast epithelial cells grown in three dimensional cultures will spontaneously aggregate to form hollow, cyst-like acini. The cells develop apicobasal polarization and are tightly regulated with respect to growth and proliferation thus reiterating several important features of glandular epithelium *in vivo*. For this reason these models represent a physiologically relevant system that is a reasonable alternative to expensive *in vivo* experimental systems. Some breast cancer cell lines such as MDA-MB-435 also form acinar structures in three dimensions (Glinsky, Huflejt et al. 2000) but others (DU4475) form clusters and cords (Langlois, Holder et al. 1979).

For cells to grow as aggregates in three dimensions they need to be in an environment where the adhesive forces between the cells are greater than their affinity for the substrate they are plated on. Some of the commonly used techniques include embedding the cells completely in a reconstituted basement membrane substrate such as Matrigel or collagen I, or growing them on a thin layer of solidified reconstituted basement membrane in a dilute solution of basement membrane in medium (liquid overlay) (Hebner, Weaver et al. 2008). Three dimensional aggregates can also be obtained using spinner culture flasks, where they are maintained in suspension by constant rotation. Some of the recently developed methods include growing the cells on pre-fabricated scaffolds of extracellular matrix that recreate the natural structure of a living tissue, and a NASA developed Rotary Cell Culture System

where the cells are grown in simulated microgravity in liquid medium (Kim, Stein et al. 2004).

Monotypic three-dimensional cell cultures have been the primary model used in the study of human breast cancer. These studies have revealed a great deal about the functions of oncogenes, tumour suppressor genes, reversion of tumour phenotypes, how cells escape from proliferative arrest, invasive and migratory behaviour and epithelial to mesenchymal transition [reviewed in (Weaver, Fischer et al. 1996; Debnath and Brugge 2005)]. Fewer researchers have used heterotypic co-culture models in three dimensions. Some of the approaches are summarized here.

Some studies have concentrated on the relationship between breast tumour cells and stromal fibroblasts as it has long been known that alterations in the stroma can alter tumour cell behaviour and disease progression. A research group at the Lawrence Berkeley Laboratory in Berkeley, California used three dimensional co-cultures to determine the origin of myofibroblasts in breast cancer. These are interstitial cells frequently found in the stroma of breast neoplasias that were, at that time, of uncertain origin. They isolated fibroblasts, vascular smooth muscle cells and pericytes from normal stroma and grew them in collagen gels with MCF-7 or HMT-3909 S13 breast cancer cell lines in co-culture for fourteen days. They found that it was primarily the fibroblasts that were converted to myofibroblasts and that only five percent of the fibroblasts closest to the spherical colonies made by the tumour cells were converted, suggesting a concentration gradient of factors released by the tumour cells was responsible for the conversion (Ronnov-Jessen, Petersen et al. 1995). A second group in Regensburg, Germany grew tumour cell lines and normal, breast tumour derived or skin fibroblasts as separate spherical colonies in three dimensional liquid overlay co-cultures. Interestingly, only two of the breast cancer cell lines tested, MCF-7 and SK-BR-3 cells, could infiltrate either the breast or the skin fibroblast spheroids under these experimental conditions. MCF-7 cells are normally considered to have low metastatic potential and only occasionally invaded the fibroblast spheroids whereas SK-BR-3 cells are highly metastatic and extensively infiltrated the fibroblast spheroids. Induction of the myofibroblastic phenotype by the tumour cells was only induced in the normal or tumour-derived fibroblasts, and not the skin fibroblasts (Kunz-Schughart, Heyder et al. 2001).

Another research group at Universitat Halle in Halle, Germany investigated the properties of mesenchymal stem cells in three dimensional co-cultures with MCF-7 or MDA-MB-231 breast cancer cell lines. In their experiments they found that within two hours of plating mesenchymal stem cells with MCF-7 spheroids or MDA-MB-231 aggregates the mesenchymal stem cells could invade the cancer cell masses. Using a Transwell assay, with breast cancer cell lines grown in the bottom well, they were able to show that the breast cancer lines attracted the mesenchymal stem cells indicating they were secreting a chemoattractant (Dittmer, Hohlfeld et al. 2009).

Researchers at The Pennsylvania State University have developed a specialized bioreactor for long term (up to ten months) co-culture of MDA-MB-231 breast tumour cells with murine osteoblasts. They have determined that the osteoblast cultures develop over time in the same way as in natural bone including development of ossification and phenotypic transformation into osteocytes. They differentially labelled the bone cells and the breast cells with green fluorescent protein and Alexa Fluor 568 respectively and were able to follow the real-time cancer cell invasion and colonization of the osteoblast tissue. They observed that important pathologic events such as cancer cells infiltrating the bone cells in single file and microtumour formation that are seen clinically were reproduced their *in vitro* system. They

also observed that breast cancer cell colonization of the bone cells depended strongly on the maturity of the osteoblastic culture (Dhurjati, Krishnan et al. 2008; Mastro and Vogler 2009; Krishnan, Shuman et al. 2010).

A novel approach to three dimensional co-culturing of cells was developed by researchers at the University of Wisconsin-Madison in Madison, Wisconsin. They used a ninety-six arrayed single channel microchannel plate for co-culturing cells in 2ul collagen matrices and compared their results to conventional co-culturing of cells in collagen in six-well tissue culture plates. T47D breast cancer cells were co-cultured with human mammary fibroblasts and growth properties and inhibition of growth by small molecule inhibitors were compared between the two systems and found to be the same. The microchannel model has a number of advantages over conventional three dimensional co-culture systems in that it requires fewer resources, uses fewer cells, creating the possibility of using patient samples, and it is amenable for using high throughput screening of potential therapeutics (Bauer, Su et al. 2010). It will be interesting to follow future developments in the use of three dimensional heterotypic co-cultures in breast cancer research as this model system appears to have great potential.

3.2.2 Two-dimensional co-culture

By far the most commonly used *in vitro* co-culture model in the study of breast cancer metastasis is two-dimensional. Cells of various origins are cultured directly with breast cancer cells or in separate layers, as in Transwell plates. The measured outcomes in two dimensional co-cultures relate to breast cell growth, proliferation, adhesion, colony formation, migration and invasion. Signalling between cell types can be modified using gene overexpression or knock-down assays, or by adding inhibitory or stimulatory antibodies or other soluble compounds or drugs to the assay system. Some of the many and varied approaches are outlined below.

Researchers in Munster and Witten, Germany were interested in the role played by the HER-2 receptor in extravasation from the primary tumour through the venular wall. They modeled the venular wall using human umbilical vein endothelial cells grown on porous membranes coated with basement membrane extracellular matrix. They co-cultured these calls with breast cancer cell lines and with disaggregated tumour cells from twenty-three patients. They found that cell lines or patient samples with higher levels of HER-2 expression were significantly more invasive than cells with lower HER-2 expression. Interestingly, they also noted that there were subpopulations within individual breast cancers that had high HER-2 expression, and presumably high metastatic potential (Roetger, Merschjann et al. 1998).

A study was carried out in Milan, Italy, to investigate the interactions between hormone-dependent MCF-7 and ZR75.1 cells and hormone-independent MDA-MB-231 or BT20 breast cancer cells. Using a modified Transwell plate and measuring cell growth in the bottom well under serum-free conditions, they determined that the hormone-independent cell lines were capable of inducing cell growth in the hormone-dependent cells, in the absence of estrogen. Growth of the hormone-dependent cell lines could be further stimulated by the addition of transforming growth factor alpha to the medium. Their results confirmed the importance of paracrine interactions between cells in heterogeneous tumours and suggested an important role for transforming growth factor alpha in these interactions (Cappelletti, Ruedl et al. 1993).

Two dimensional co-culture systems are amenable to the use of primary tumour cells. A research group in Manchester, UK used primary epithelial cells from tumorous, benign or normal breast tissue in co-culture with human bone marrow or mammary fibroblasts from normal or malignant breast tissue. They found that breast epithelial cells from tumour tissue adhered preferentially to bone stroma over breast fibroblasts. The epithelial cells from normal or benign breast showed no preference for any of the stromal substrates. Interestingly, although breast tumour epithelial cells adhered preferentially to bone cells, this stromal environment did not provide a preferential growth platform (Brooks, Bundred et al. 1997). A similar study was carried out in Marseilles, France to determine the effect of stromal and epithelial cells from normal and tumorous breast tissue on growth of breast cancer cell lines. Fibroblasts from normal breast tissue but not conditioned medium from normal breast tissue were able to inhibit the growth of MCF-7 cells suggesting complex paracrine interactions between the two cell types. Normal fibroblasts did not inhibit the growth of immortalized S2T2 cells. Normal breast epithelial cells or the conditioned medium from them could inhibit a number of breast cancer cell lines suggesting that both fibroblasts and epithelial cells could have growth regulatory roles in the breast.

Many researchers co-culture breast cancer cell lines with bone-derived mesenchymal stem cells (MSCs) as these have been shown to have a profound effect on breast cancer metastasis. These cells were first observed by Friedenstein in 1976 (Friedenstein, Gorskaja et al. 1976) and have come to be defined as non-hematopoietic cells derived from bone stroma that are spindle-shaped and can be separated from other bone stromal cells by their tendency to adhere to plastic tissue culture plates. They have the stem cell characteristics of being able to differentiate into multiple cell lineages such as osteoblasts, chondrocytes, adipocytes and myoblasts and they express a consistent set of marker proteins on their surface (Brooks, Bundred et al. 1997; Pittenger, Mackay et al. 1999). Mesenchymal stem cells have been used in a number of laboratories in co-culture experiments with breast cancer cell lines or primary tumour cells and have been found to influence breast cancer cell adhesion, morphology, gene expression, proliferative capacity and growth characteristics (Brooks, Bundred et al. 1997; Hombauer and Minguell 2000; Fierro, Sierralta et al. 2004; Oh, Moharita et al. 2004). They have been shown *in vivo* to be able to migrate to sites of tissue damage and to primary tumour sites, and to modify the ability of breast cancer tumours to metastasize to other organs, making them potentially interesting vehicles for cell-based anti-tumour agents (Ferrari, Cusella-De Angelis et al. 1998; Hall, Dembinski et al. 2007; Rhodes and Burow 2010). They have also been shown in one research study to stimulate epithelial to mesenchymal transition in breast cells which may make them less suitable for use in drug delivery (Martin, Dwyer et al. 2010).

We use MSCs in a two dimensional co-culture model designed to determine factors that affect breast cancer cell behaviour in a microenvironment resembling breast cancer metastasis to bone; one of the most common sites of breast cancer metastasis. Our source of bone cells is from reamings from hip and knee replacement surgeries that are carried out on a regular basis in local hospitals rather than the more commonly used bone marrow aspirates that are more difficult to obtain. We wanted to determine that bone marrow cells that we derived from bone reamings resembled bone cells that were normally biologically involved in breast cancer metastases to bone. Breast cancer bone metastases are frequently characterized by the presence of a desmoplastic response, where normal haematopoietic tissue is replaced by activated fibroblastic cells. Adherent fibroblastic cells were isolated from both hip and knee bone reaming samples with a

successful recovery rate of approximately 62% (8/13 patients). Microscopically, recovered cells that grew as a monolayer were observed to be morphologically heterogeneous, spindle shaped and fibroblast-like in appearance (Figure 1A) similar in appearance to mesenchymal stem cells previously reported in the literature (Wagner and Ho 2007; Wagner, Roderburg et al. 2007).

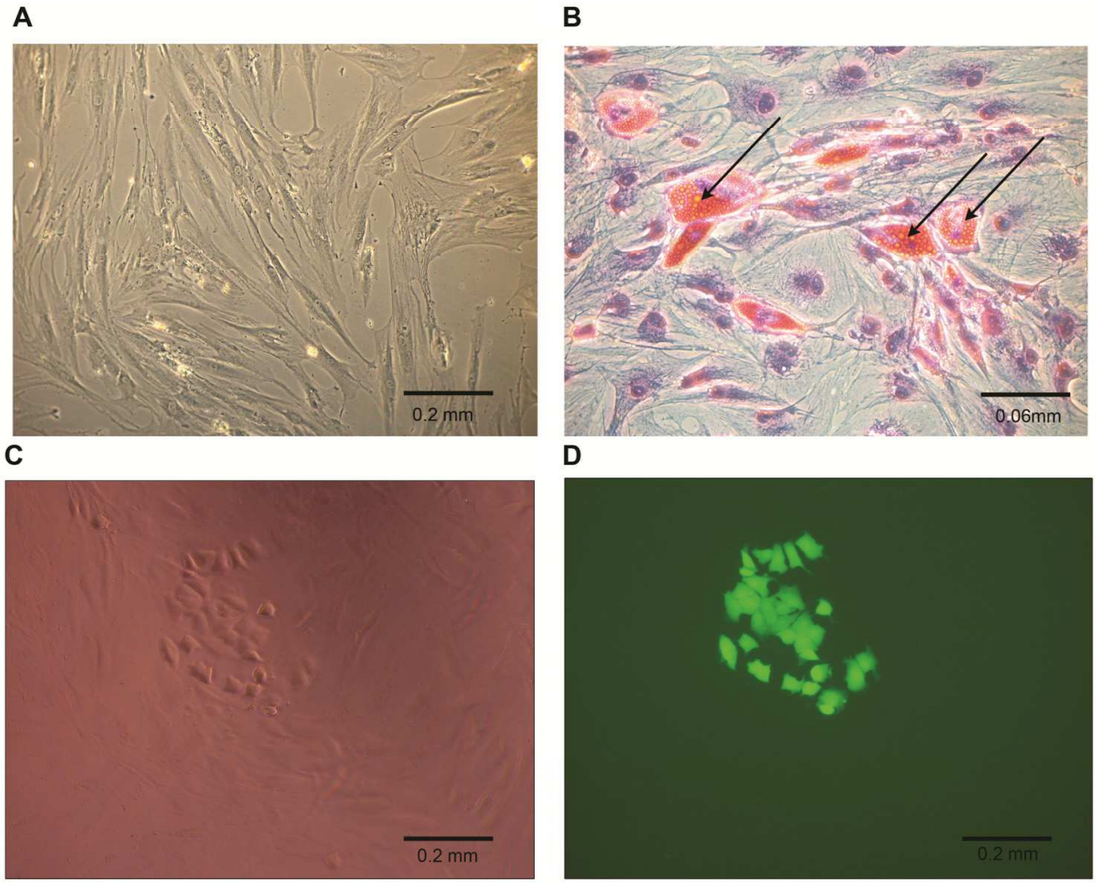

Fig. 1. Characterization of mesenchymal stem cells (MSCs) and breast cancer cell colonies plated on MSCs. MSCs growing as a monolayer are morphologically heterogeneous, spindle-shaped and fibroblast-like (A). MSCs can be induced to differentiate into adipocytes (B). Arrows indicate accumulations of lipid-rich vacuoles. Colonies of breast cancer cells growing on a lawn of MSCs can be visualized by light microscopy by their different refractive index (C) or, if they are stably transfected with GFP can be visualized using fluorescent microscopy (D).

To determine the multipotent potential of the bone cells adipogenic differentiation was induced in the isolated bone marrow derived cell cultures by treatment with Adipogenic Differentiation Medium (Fisher Scientific, SH3088602) according to the manufacturer's instructions. Induction was apparent by the accumulation of lipid-rich vacuoles within cells (Figure 1B). The content of the observed vacuoles was stained with Oil Red O dye and was localized to inside the cells where cell nucleus and membrane were counterstained with haematoxylin. This is consistent with our bone cell cultures having some of the multipotent characteristics of mesenchymal stem cells and we will refer to them as MSCs.

One of the usual characteristics to measure when breast cancer cells are grown on any stroma includes the ability of the breast cancer cells to form colonies on that stroma cell type. This can be determined using limiting dilution analysis. We used statistical analysis for limiting dilution assays adapted from the method described by Lefkovits and Waldmann (Lefkovits and Waldmann 1999). Limiting dilution analysis software developed by P. Rovenksy, J. Rubes, and T. Beran and included with the Lefkovits and Waldmann textbook was used for chi square and frequency calculations.

A modified limiting dilution analysis (LDA) method was used to evaluate the frequency of a given event in a population. We evaluated proliferation/survival of individual cancer cells, where binomial colony formation events were defined as 1) a *positive event* being the presence of colony ≥ 8 cells in size after the indicated time (days) period, and a 2) a *negative event* being the absence of any colonies or a colony <8 cells after the indicated time (days) period.

For co-cultures, 1000 cells/well of substratum cells were seeded in 96-well plates and allowed to attach and grow over 2 day period. After 2 days, the wells were washed twice using PBS and various dilutions of the breast cancer cell lines (1, 2, 4, 5, 7, 10 cells/well) were added in 100uL volume of serum-free Opti-MemI media per well (Gibco cat. #31985). The plates were incubated for an indicated time period at 37^{o}C and 5% CO_2. Each well was analysed for the presence of colonies using an inverted microscope (100X magnification). Breast cancer cells were identified by morphology and a different refractive index when compared to the large flattened MSCs. An example is presented in Figure 1C and D.

Each well was scored as positive or negative based on the above established criteria. The data was tabulated and frequencies were determined using a Poisson distribution:

$$F_r = \frac{\mu^r}{r!} e^{-\mu}$$

Frequencies were calculated using the aforementioned LDA software package with a linear regression through the origin. Graphical representations of the distributions were also plotted on the μ vs. -lnF_0 graphs. The accuracy of the fitted line was evaluated using a chi-squared test for goodness of fit

$$(X^2 = \frac{df \times V}{\sigma^2})$$

based on a 95% confidence interval of accepting the null hypothesis that the best line of fit accurately represents the observed data. The null hypothesis was accepted (line of best fit accurately represents the data that follows single-hit kinetics) when the p-value was greater than 0.05.

Inter-trial frequencies were compared based on the overlap of the 95% confidence limits of the slopes based upon evaluation of the reliability of the regression line estimates. Confidence limits of the slope (*a*) were calculated using the following equations,

$$a_{upper} = a + t_{\frac{\alpha}{2},n-1} \sqrt{\frac{1}{(n-1)(\frac{\sum y_i^2}{\sum x_i^2} - a^2)}} \ ,$$

$$a_{lower} = a - t_{\frac{\alpha}{2},n-1}\sqrt{\frac{1}{(n-1)(\frac{\sum y_i^2}{\sum x_i^2} - a^2)}}$$

so that new slopes (a_{lower}, a_{upper}) define the boundaries of the fan. The values for the area α or α/2 were obtained from the Student's *t*-test table. The overall frequency for each cell line was calculated using pooled data from experiments using the above described analysis.
Using limiting dilution analysis we determined, for example, that one MCF-7 cell in every fourteen could develop a productive colony on MSCs but only one MCF-7 cell in every thirty could develop a colony on HS68 fibroblasts, indicating that bone stroma was the preferential stroma to colonize (Figure 2).
Another characteristic very often measured in two dimensional co-cultures involves the ability of one cell type or conditioned medium from a cell type to influence the migratory capacities of another cell type. This is sometimes done with a wound healing assay where a confluent culture of one type of cells is disrupted by scratching cells off the tissue culture plate surface in a straight line and then measuring how long it takes the "wound" to fill in with new cells under conditions of differing types of conditioned media.

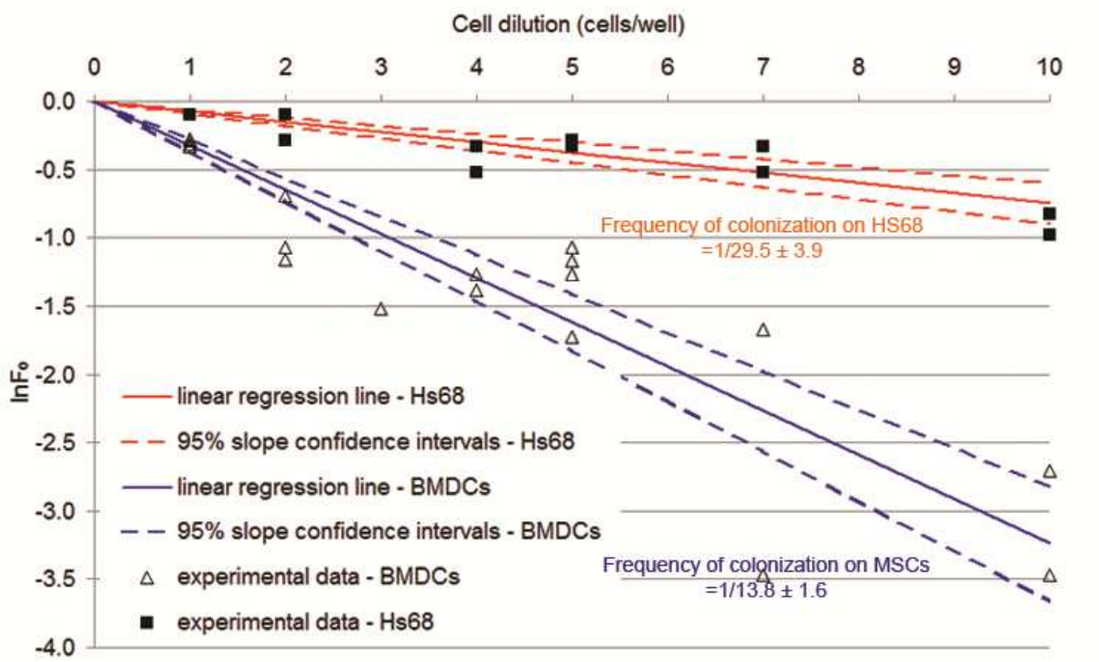

Fig. 2. Limiting Dilution Analysis (LDA) of MCF-7 Breast cancer cells grown on MSCs (blue) or HS68 fibroblast cells (red) indicates that the breast cancer cells can colonize wells having MSCs as a substrate at a significantly higher frequency than they can colonize wells in which HS68 cells have been plated as a substrate.

An alternative and more quantitative way to measure cell migration is in a Transwell or Boyden Chamber assay. Here, cells of interest are placed in the lower well of a Transwell plate and allowed to grow for some time to provide conditioned medium (Figure 3A). Cells to be tested for migratory capacity are placed on a porous membrane in the upper chamber

and are allowed to migrate through the membrane for a given period of time. An example of MCF-7 breast cancer cells migrating through pores in response to MSCs or HS68 fibroblasts is given in Figure 3, where it can be seen that the breast cancer cells migrate preferentially in response to bone stromal cells.

A variation on the Transwell migration assay is an invasion assay where the cells must invade through a Matrigel layer before migrating through the pores. Usually an invasion assay and a migration assay are carried out at the same time under the same conditions and invasion is measured as a percentage of number of cells invading/migrating.

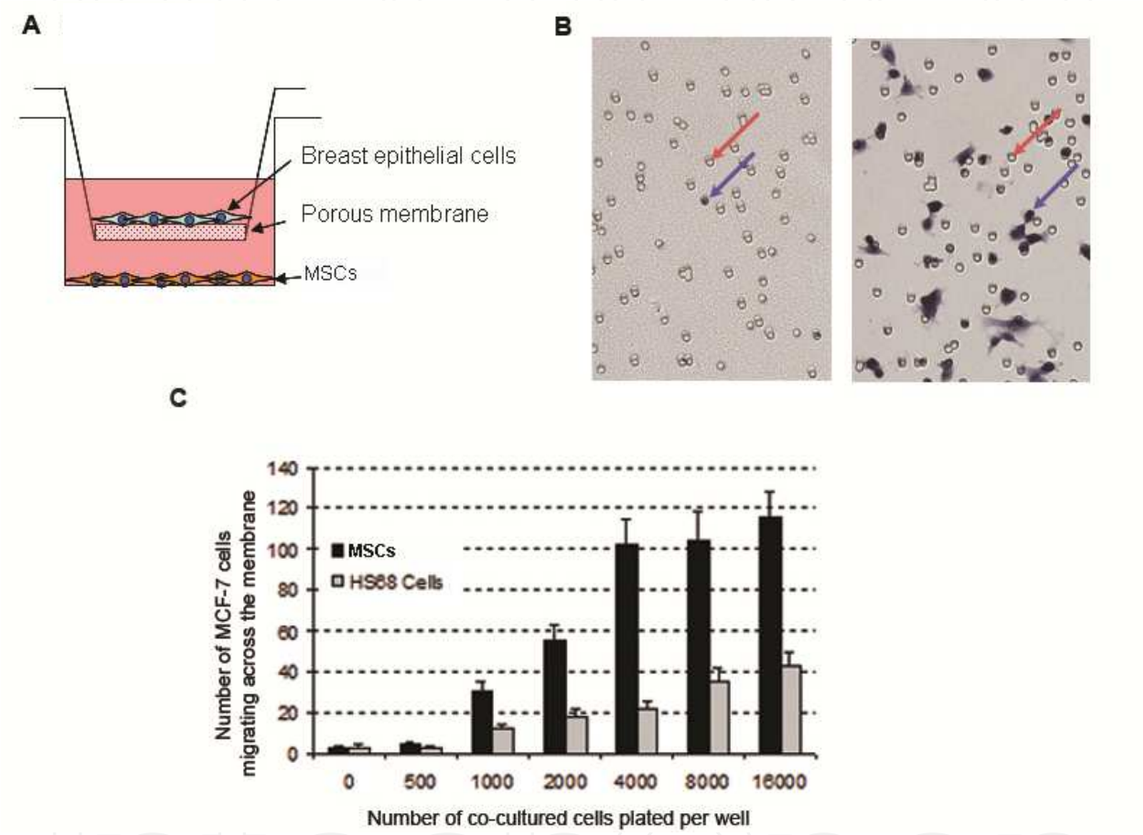

Fig. 3. Use of a Transwell Assay to determine the migration of MCF-7 breast cancer cells in response to MSCs or HS68 cells. A schematic diagram to illustrate the assay design (A). The Transwell membrane when stained and photographed from an inferior aspect has 8um pores (red arrows) and cells that have migrated through the pores can be visualized and counted (blue arrows). More cells migrate through the Transwell membrane in response to MSCs than in response to HS68 cells (C). Error bars indicate standard error of the mean.

One of the advantages of using a two dimensional co-culture system using Transwell plates is the ability to separate the cells after exposure to each other for analysis by western blot, PCR analysis or microarray analysis of differentially expressed genes. Another advantage is the ability to separate cells to determine which cell type is expressing a factor that regulates invasion or migration. For example, the bone remodeling protein Osteopontin is produced by bone cells and breast cancer cells. There are a number of reports in the literature suggesting Osteopontin produced by breast cancer cells regulates their migratory properties

and contributes to the aggressiveness of the disease (Sharp, Sung et al. 1999; Chakraborty, Jain et al. 2008; Hedley, Welch et al. 2008; Patani, Jouhra et al. 2008; Ribeiro-Silva and Oliveira da Costa 2008). In our co-culture model we found, at least in the breast/bone microenvironment, that it was Osteopontin produced by the bone cells, not the breast cells that increased breast cancer cell migration (Koro, Parkin et al. 2010).

4. Future directions

It is becoming evident that gene expression at the site of breast cancer metastasis may not be the same as at the site of the primary tumour and we need better ways to treat metastases. It will likely be important to biopsy more metastatic tissue to provide the type of designer therapeutics aimed at pathways known to be targetable at the site of the metastasis as we currently do with the primary tumour. Currently, biopsies of metastases are rare. As stroma is known to be an active contributor to the metastasis we also need to develop therapeutic approaches aimed at targeting the stroma. The recent development of new technologies for capture and analysis of circulating tumour cells may help to improve our understanding.

5. Conclusions

Breast cancer is a complicated disease and progression to metastasis may occur by clonal expansion or parallel progression. Changes in gene expression may occur between the primary tumour and the site of metastasis and development of therapeutics aimed at either the breast or stromal cells at the site of the metastasis will likely be needed to develop better therapeutics against breast cancer metastasis. Some of these new therapeutics may be aimed at reconstituting the expression of breast cancer metastasis inhibitor genes and much research is being done in this field. *In vivo* and *in vitro* model systems have contributed in many ways to our understanding of breast cancer metastasis and will surely continue to do so.

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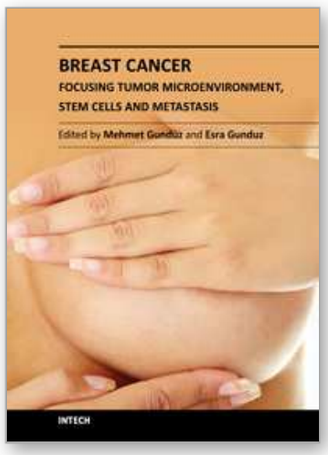

Breast Cancer - Focusing Tumor Microenvironment, Stem cells and Metastasis

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed characteristics of breast cancer cell, role of microenvironment, stem cells and metastasis for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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