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Connexins and Gap Junctions in Mammary Gland Development and Breast Cancer Progression

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Abstract The development and function of the mammary gland require precise control of gap junctional intercellular communication (GJIC). Here, we review the expression and function of gap junction proteins, connexins, in the normal mouse and human mammary gland. We then discuss the possible tumor-suppressive role of Cx26 and Cx43 in primary breast tumors and through the various stages of breast cancer metastasis and consider whether connexins or GJIC may actually promote tumorigenesis at some stages. Finally, we present *in vitro* data on the impact of connexin expression on breast cancer cell metastasis to the bone. We observed that Cx43 expression inhibited the invasive and migratory potentials of MDA-MB-231 breast cancer cells in a bone microenvironment, provided by the MC3T3-E1 mouse osteoblastic cell line. Expression of either Cx26 or Cx43 had no effect on MDA-MB-231 growth and adhesion under the influence of osteoblasts and did not result in regulation of osteogenic gene expression in these breast cancer cells. Furthermore, connexin-expressing MDA-MB-231 cells did not have an effect on the growth or differentiation of MC3T3-E1 cells. In summary, we conclude that connexin expression and GJIC are integral to the development and differentiation of the mammary gland. In breast cancer, connexins generally act as tumor suppressors in the primary tumor; however, in advanced breast tumors, connexins appear to act as both context-dependent tumor suppressors and facilitators of disease progression.

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Introduction

The mammary gland is an intricate organ that undergoes the majority of its growth and differentiation postpuberty. At birth, the gland consists of only a rudimentary collection of small ducts; and it remains somewhat quiescent until puberty, when the ducts begin to branch and elongate to fill the mammary fat pad. At pregnancy, further ductal branching occurs and the alveoli begin to develop and terminally differentiate to acquire the capability for milk production. The basic structure of the mature gland is a series of alveoli organized into lobules, each draining through a common ductal system toward the nipple. The ducts and alveoli are lined by a single layer of secretory luminal epithelial cells. A basal myoepithelial cell layer surrounds the epithelium and provides contractile strength for milk ejection during lactation. After weaning, during involution, the gland undergoes extensive apoptosis and remodeling to regain a mature prelactating structure. This cyclical developmental series occurs with each pregnancy and birth and is regulated by the cross-talk between hormonal signals, local growth factors and the interaction between the epithelial cells and the surrounding stroma (*reviewed by* Lamote et al., 2004; Robinson, Karpf & Kratochwil, 1999). However, mammary gland development and differentiation also require precise intercellular communication, one such form of which is via gap junctions. Not surprisingly then, both the function of the gap junction channels and the expression of their connexin subunits are regulated in a dynamic fashion throughout

mammary gland development and differentiation. There are 21 human and 20 mouse connexin genes, each encoding a protein that confers specific permeability, conductance and gating to the channels they form (Harris, 2001; Saez et al., 2003; Willecke et al., 2002). We will review connexin expression and gap junction function in normal mammary gland and in cancer progression, including their role in the primary tumor and metastasis. We will then provide *in vitro* data on the consequences of connexin expression in the establishment of breast cancer cells at their most common secondary site, bone tissue.

Connexin Expression in the Human Mammary Gland

In the human mammary gland, the connexin 26 (Cx26) message was first identified in normal epithelial cells by subtractive hybridization compared to cells derived from breast tumor tissue (Lee, Tomasetto & Sager, 1991). In subsequent studies, these researchers investigated the expression and function of Cx26 and several other connexins in normal mammary epithelial cells (NMECs) (Lee et al., 1992). Expression of Cx26 and Cx43 was identified by Northern analysis, while Cx31.1, Cx32, Cx33, Cx37 and Cx40 messages were absent in NMECs. In the same study, Cx26 and Cx43 proteins were visualized by immunocytochemistry at NMEC cell-cell interfaces and dye passed readily from one cell to another, proving that the gap junction channels were functional. Further *in vitro* studies identified Cx43 protein in normal human mammary fibroblasts, and dye transfer experiments revealed heterocellular gap junctional intercellular communication (GJIC) between fibroblasts and epithelial cells (Tomasetto et al., 1993). Cx43 protein was first detected in the *in situ* human mammary gland in 1992 (Wilgenbus et al., 1992). Pozzi et al. (1995) further defined its localization to myoepithelial cells by double labeling with keratin 14, although sparse Cx43 immunolabeling has also been observed between some luminal cells of gland ducts *in situ* (Laird et al., 1999). Monaghan et al. (1996) confirmed Cx43 protein expression in the myoepithelium of the ducts in the human gland and demonstrated Cx26 expression predominantly between the luminal cells of ducts, with occasional staining in the luminal cells of the alveoli. Antibodies against either Cx32 or Cx40 did not detect any expression in the *in situ* gland, and no expression of the remaining connexin family members has been reported. These authors then expanded on the existing knowledge of connexin expression in the human mammary gland and investigated gap junction function. To do this, the gland was digested with collagenase and the luminal and myoepithelial cells were separated from each other immunomagnetically and cultured *in vitro*. From this, the authors showed that Cx43 was the only connexin expressed between myoepithelial cells and that these cells had a high level of GJIC. Interestingly, cultured luminal cells retained only low levels of Cx26 intracellularly, which was not detectable by immunoblot and did not demonstrate functional GJIC by dye ransfer. Furthermore, electron microscopy only revealed gap junction plaques between myoepithelial cells. This pattern of occasional Cx26 staining in the luminal cells of the human mammary gland and strong Cx43 staining between myoepithelial cells was confirmed by subsequent studies (Jamieson et al., 1998; Kanczuga-Koda et al., 2003).

From these expression profile analyses (summarized in Fig. 1) we can speculate that in the resting human mammary gland Cx43 is required to maintain myoepithelial differentiation and, since Cx26 expression is more variable, it may have a more dynamic role in luminal cell function. Lee et al. (1992) showed that while Cx43 levels remained constant throughout the cell cycle, Cx26 mRNA levels were upregulated in G_1 and late S phases. This suggests that Cx26 may be important in luminal cell proliferation. In the endometrium, Cx26 transcription is responsive to estrogen via an estrogen receptor-dependent pathway and to progesterone (Grummer et al., 1994, 2004; Grummer, Traub & Winterhager, 1999). It is possible that Cx26 expression in the mammary gland is also regulated either directly or indirectly by hormonal factors or other microenvironmental influences, and this may account for the inconsistencies reported for Cx26 expression between various breast tissue samples.

Connexin Expression in the Developing Mammary Gland

Unfortunately, it is difficult to obtain samples of human breast tissue at all stages of differentiation, so the mouse is used to examine connexin expression throughout the development of the mammary gland, with the anticipation that findings can be extrapolated to humans. The developmental expression pattern of each connexin through virginity, pregnancy, lactation and involution is considered in detail individually below, followed by a discussion of the functional consequences of the dynamic expression profiles of the connexins. In addition to the connexins expressed in the human mammary gland, Cx26 and Cx43, two further connexin family members have been identified in the mouse gland, Cx32 and more recently Cx30 (Fig. 1).

Cx26

Pozzi and colleagues (1995) detected Cx26 mRNA in the mouse mammary gland only during lactation. However, Locke et al. (2000, 2004, 2006) also identified Cx26 in the pregnant gland by Northern blot, and then microarray studies revealed its presence in all stages of development from

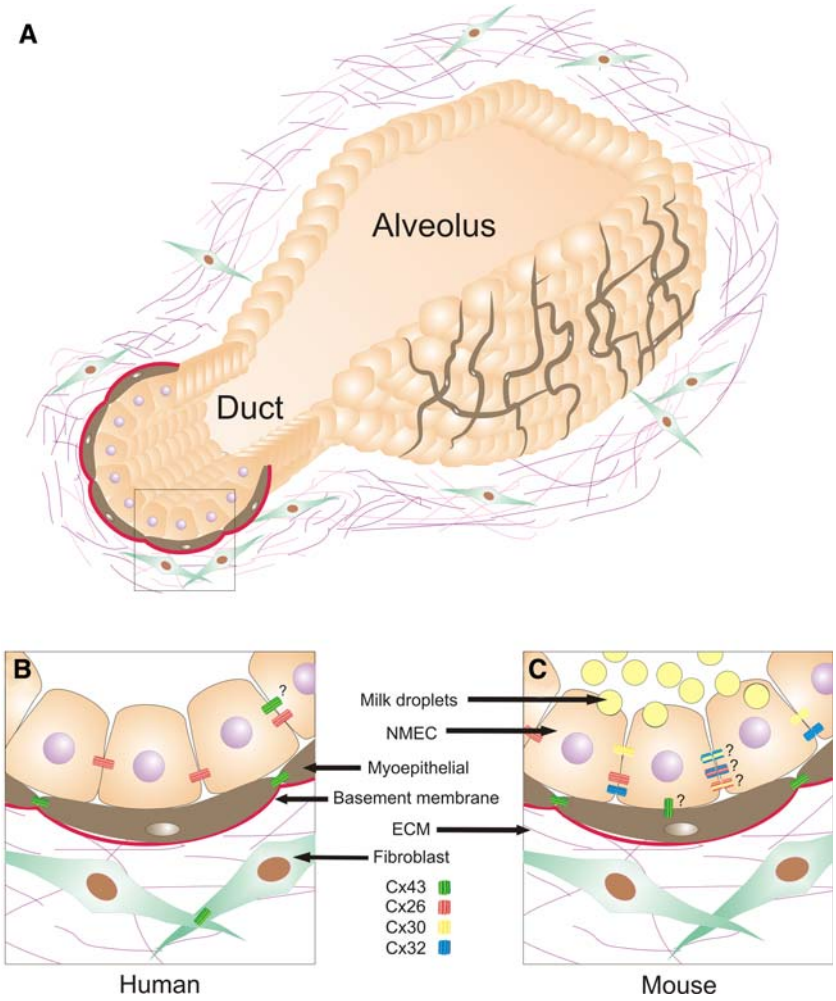

Fig. 1 Mammary gland microanatomy and connexin expression. **a** Schematic of a mammary gland duct and alveolus showing the arrangement of a myoepithelial cell layer (*brown*) surrounding the luminal epithelial cells. **b** In the human breast, Cx43 channels (*green*) are primarily localized between myoepithelial cells while Cx26 channels (*red*) connect the luminal cells. Cx43 can also be found in the fibroblasts of the stroma. **c** Similarly, in the mouse mammary gland, Cx43 channels form between myoepithelial cells and Cx26 channels are found between luminal cells; however, Cx30 (*yellow*) and Cx32 (*blue*) are also expressed in luminal cells, peaking during lactation. *In vitro* evidence suggests that Cx26, Cx30 and Cx32 can form heteromeric connexons; and one study has reported Cx43 localized to the myoepithelial-luminal cell contacts. *ECM*, extracellular matrix

virginity through involution, with expression peaking during lactation. Cx26 protein was localized between luminal cells of the ducts, at the basolateral borders, in all stages of development (Pozzi et al., 1995; Talhouk et al., 2005) (Fig. 1). Cx26 expression increases throughout pregnancy and begins to appear between the luminal cells of the alveoli as well by pregnancy day 12 (P12) (Monaghan et al., 1994). Consistent with mRNA expression, Cx26 protein levels peak at the onset of lactation and then decline in involution (Monaghan et al., 1994; Talhouk et al., 2005). The expression profile of Cx26 suggests it is important in milk production and/or secretion, but because it is also expressed in virgin mouse mammary glands and in nonpregnant human breast tissue, it likely has a role in the differentiation and tissue homeostasis of the mammary gland.

Cx30

Recently, two studies have identified Cx30 mRNA expression in the pregnant and lactating mouse mammary gland (Locke et al., 2006; Talhouk et al., 2005). Cx30 protein can first be detected by immunohistochemistry at P15, and expression appears to increase and peak at the onset of lactation and then decline in involution (Talhouk et al., 2005). Two subclones have been established from the mouse mammary cell line CID-9; SCp2 cells have an epithelial phenotype, and SCg2 cells are myoepithelium-like. Cx30 expression could only be detected in SCp2 cells, confirming that its expression is restricted to the epithelial cells (Fig. 1). Cx30 in human mammary gland tissues or cell lines has not been reported. It will be interesting to determine whether Cx30 is expressed in the human gland as it has been shown to have the ability to form heterotypic channels with Cx26 and the resulting channels have different gating sensitivities (Locke et al., 2006). It has been further postulated that heterotypic channel formation may be a mechanism to regulate milk production (Locke et al., 2006).

Cx32

Cx32 mRNA is first expressed at parturition and lactation (Locke et al., 2000; Pozzi et al., 1995). Several studies conclude that Cx32 protein is only expressed at the

basolateral region of the luminal cells in the lactating gland (Locke et al., 2000; Monaghan et al., 1994; Pozzi et al., 1995) (Fig. 1); however, Talhouk et al. (2005) were able to detect Cx32 at all developmental stages of the *in situ* gland by immunohistochemistry. In this same study, Western analysis revealed only very low levels of Cx32 in the virgin and involuting gland but high levels of expression in the lactating gland. Transcription of Cx32 cannot be detected in human breast tissue (Monaghan et al., 1996; Pozzi et al., 1995); however, there is a single report of Cx32 mRNA expression in a human breast cancer cell line, MDA-MB-435 (Saunders et al., 2001). Cx32 protein expression has not been reported in the human mammary gland *in situ*, indicating that mouse mammary glands may have a function not required in the human gland that is mediated by Cx32. Because Cx32 expression peaks at lactation and can oligomerize with Cx26, it, like Cx30, may have a role in regulating milk production. It is interesting that this level of regulation is not available in the human gland, and this suggests that different molecules mediate the gating of Cx26 channels in human lactation.

Cx43

Cx43 mRNA expression is also regulated in mammary gland development. Northern analysis and quantitative polymerase chain reaction (PCR) reveal that Cx43 is downregulated at mid-pregnancy and almost disappears during lactation but is reexpressed after involution (Lambe et al., 2006; Talhouk et al., 2005). At the protein level, Cx43 is expressed at the cell surfaces between the basal myoepithelial cells (Pozzi et al., 1995) (Figs. 1 and 2). In the study by Talhouk et al. (2005), it appears that Cx43 is also localized to the myoepithelial-epithelial junction at several developmental stages. This is somewhat controversial as it is not clear what the connexin contributor would be from the adjacent luminal cell. While indirect evidence suggests that Cx43 and Cx30 localize within the same gap junction plaque in astrocytes (Altevogt & Paul, 2004) and may even interact, there is no direct evidence that Cx43 can form heterotypic channels with Cx26, Cx30 or Cx32. Therefore, if Cx43 is truly a constituent of myoepithelial-epithelial gap junction channels, it would require that Cx43 itself or another connexin be expressed in the luminal cells; but this scenario cannot currently be substantiated. Interestingly, while total Cx43 protein levels may not fluctuate considerably, a shift to the more highly phosphorylated species is evident in the last days of pregnancy and at the onset of lactation (Talhouk et al., 2005), a phenomenon also seen in the rat mammary gland (Yamanaka et al., 1997). Since Cx43 is expressed throughout development, it must play a role in the growth and differentiation of myoepithelial cells; but it appears that it may even be essential to myoepithelial function during lactation.

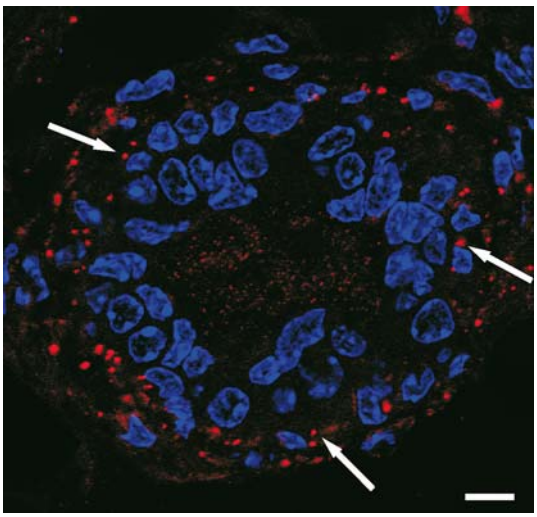

Fig. 2 Mouse mammary gland expresses Cx43. Adult mammary gland was removed, fixed, embedded and cryosectioned prior to immunolabeling with a Cx43 antibody (Sigma, Oakville, Canada; 1:500). Confocal imaging revealed Cx43 (*red*) localized to the periphery of the duct (*arrows*), consistent with the myoepithelium. Nuclei are stained with Hoechst (*blue*). Bar = 10 μm

These numerous studies of connexin expression throughout mammary gland development have uncovered conflicting subtleties in terms of their temporal regulation. The discrepancies between the various reports have been attributed to differences in mouse strain and in experimental techniques and tools. We can conclude that Cx26, Cx30, Cx32 and Cx43 are expressed in mouse mammary gland and Cx26 and Cx43 are expressed in humans (Fig. 1); but we cannot discount the expression of other connexins as they have not all been rigorously evaluated. Northern analysis of human breast tissue ruled out the transcription of Cx31.1, Cx32, Cx33, Cx37 and Cx40 (Lee et al., 1992); and immunohistochemistry of the mouse glands failed to reveal Cx40 (Monaghan et al., 1994). Also, despite efforts, no Cx32 or Cx40 protein could be detected in the human gland (Monaghan & Moss, 1996). Beyond these studies, however, there has not been an appraisal of the presence of other connexins in the mammary gland.

Connexin Function in the Mammary Gland

The developmental expression profiles of the various connexins in the mouse mammary gland suggest they have distinct functions. We now know that channels constructed from different connexin members have the ability to facilitate the movement of distinct secondary messengers and metabolites (Bevans et al., 1998; Goldberg, Moreno & Lampe, 2002; Locke et al., 2004; Ma & Dahl, 2006). Let us

first consider the luminal cell connexins. When the gene encoding Cx26 is conditionally suppressed in the mammary epithelium before puberty, development of the lobuloalveolar structure of the gland and its lactation are impaired (Bry et al., 2004). However, a conditional knockout of Cx26 in the mammary epithelium during pregnancy has no developmental or functional outcome, and mammary glands of Cx32-null mice appear to develop and function normally, suggesting that Cx26 and Cx32 can compensate for each other at lactation or these connexins serve no essential role. Conditional double knockouts have not been done, but findings from such a transgenic would provide further insight into this issue. Interestingly, Locke and colleagues (2000, 2004, 2006) have presented extensive studies using several different techniques that conclude that Cx26 and Cx32 co-oligomerize within the same connexon and within the same channel in the luminal epithelial cells of the mouse mammary gland. By purifying native channels from the mammary gland at parturition and throughout lactation and reconstituting them in liposomes, this group determined that the stoichiometry and corresponding permeability of the heteromeric connexons change throughout this period (Locke et al., 2004). At the onset of parturition, when Cx32 is first expressed, it is only found in heteromeric channels containing predominantly Cx26. The Cx32 ratio within the connexons increases throughout lactation to eventually achieve homomeric Cx32 connexons. Cx32 channels are wider than Cx26 channels and display very distinct transjunctional permeabilities with intermediate permeability characteristics for various stoichiometric configurations of heteromeric Cx26-Cx32 channels (Bevans et al., 1998; Cao et al., 1998; Locke et al., 2004; Weber et al., 2004). Interestingly, an increase in Cx32 ratio would be expected to lead to the passage of larger molecules, including cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). The authors postulate that since the cellular ratio of cAMP/cGMP aids in the regulation of mammary growth and differentiation, the stoichiometry of Cx26-Cx32 connexons continually alters to accommodate the biological need. Furthermore, Cx32-dominated channels are increasingly insensitive to taurine-induced closure compared to Cx26 channels. Interestingly, taurine is an amino acid used for osmolytic balance during milk protein synthesis. These studies were extended to include Cx30 after it was identified in the mammary gland and it was shown that both Cx26 and Cx32 could form heteromeric connexons with Cx30 but that any channel containing Cx30 was insensitive to taurine (Locke et al., 2006). Unfortunately, development and function of the Cx30-null mammary gland have not been investigated to date. We can postulate that in the early development of the gland Cx26 plays a role in epithelial proliferation and patterning of the gland but its function changes in later stages of development, when it cooperates with Cx32 and Cx30 to finely regulate the production of milk by the secretory cells. Presently, there is a considerable human population of patients that harbor mutations in Cx32 which cause the X-linked form of Charcot-Marie-Tooth disease (Abrams et al., 2000), a peripheral neuropathy, or Cx26 mutations linked to congenital deafness (Kelsell et al., 1997). Some of these mutant connexins cannot localize to gap junctions properly, and others can form channels but with compromised function. Since Cx26 is found in breast tissue, it is remarkable that the development, differentiation and function of breasts in patients harboring Cx26 mutations have not been systematically examined. It would be interesting to evaluate lactation efficiency in these patients as we would hypothesize that the regulation of milk production may be compromised.

Cx43 knockout mice die at birth (Reaume et al., 1995), and a conditional knockout in the mammary gland has not been generated; however, an innovative knockin approach sheds some light on Cx43 function in the myoepithelium. Heterozygous Cx43KI32 mice, in which the Cx43 gene has been replaced with Cx32, develop normal mammary glands and produce milk but seem to have impaired milk ejection (Plum et al., 2000). Mutant mothers were unable to nourish their pups through to weaning, while pups had better survival rates when fostered to a wild-type mother. Considering that Cx43 phosphorylation is often linked to its localization at the cell surface and acquisition of channel function (*reviewed by* Lampe & Lau, 2004) coupled with the Cx43KI32 mouse phenotype, the increase in Cx43 phosphorylation in the mammary gland at parturition suggests that Cx43 has an essential role in providing contractile force to the myoepithelial cells for milk ejection. A similar phenomenon is observed in the endometrium, where Cx43 expression first increases prior to birth but accumulates in the cytoplasm. At parturition, Cx43 is transported to the plasma membrane and assembled into gap junction plaques, allowing for the synchronization of electrical activity and smooth muscle contraction required for delivery (Hendrix et al., 1992). A study in which GJIC was blocked in mammary epithelial cells grown *in vitro* and secretion of the milk protein β-casein was inhibited supports the overall conclusion that GJIC may be required for milk secretion as well as ejection (El-Sabban et al., 2003). These studies highlight the idea that Cx43 may not be essential at all stages of development as other connexins may compensate for its loss of function. However, due to the precise biophysical nature of each type of connexin channel, one connexin can only compensate for another in certain microenvironments and at specific developmental time points. Furthermore, a new class of putative gap junction proteins has been identified (*reviewed by* Panchin,

2005), and members of this pannexin family may also be able to compensate for loss of connexin function.

As with Cx32 and Cx26, there is a population of humans that carry mutations in Cx43 which cause oculodentodigital dysplasia (ODDD) (Paznekas et al., 2003). This is an autosomal dominant disorder, and the patients present with a range of phenotypes including craniofacial anomalies, vision problems, skeletal malformations and syndactyly. The Cx43 mutants localize to the cell surface for the most part; but their channel function is severely compromised, and the mutants are dominant-negative to wild-type Cx43 (McLachlan et al., 2005; Roscoe et al., 2005; Shibayama et al., 2005). ODDD patients, therefore, are expected to have <50% of normal Cx43 gap junction function. While many patients have children, there has not been any systematic investigation into breast function of women with ODDD, which could shed further light on the role of Cx43 in the breast.

Connexins in Breast Cancer

Cx26 was first deemed a breast cancer tumor suppressor in 1991 (Lee et al., 1991), and now almost two decades later the importance of connexins in cancer progression remains unclear and even controversial. Lee et al. described connexins as class II tumor suppressors because the genes encoding connexins are not mutated while the protein expression levels are frequently altered, presumably due to the mutation of an upstream regulator or other factors. It is debatable though how far downstream connexins are from the primary assault and whether their regulation is a key player in carcinogenesis or simply a distant secondary effect. If we side with the position that connexins have a significant role in carcinogenesis, the question remains as to whether they are truly tumor suppressors at all stages of carcinogenesis or whether they even act to promote distinct stages of cancer progression in the breast. We will consider the evidence for each, first in the primary breast tumor and then in metastasis and secondary site establishment.

Primary Tumor

Several studies involving breast cancer patient tissue samples reveal a loss of overall connexin protein expression or a relocalization of the connexin to intracellular compartments, resulting in a predicted loss of GJIC compared to matched normal or benign breast tissue (Kanczuga-Koda et al., 2003, 2005; Laird et al., 1999; Lee et al., 1991; Wilgenbus et al., 1992). Multiple *in vitro* studies corroborate the loss of connexin and/or GJIC in breast cancer cells. Early studies showed that Cx26 and Cx43 were downregulated at the mRNA level in primary cells derived from human breast tumors (Lee et al., 1992) as well as in rat mammary tumors (Laird et al., 1999) and breast cancer cell lines (Laird et al., 1999; Singal et al., 2000). The Cx26 promoter is located within a CpG island, which is a region with higher than expected CG content; and it is speculated that methylation of these sites could repress expression of the gene. This notion led to an investigation into whether hypermethylation may cause downregulation of Cx26 (Singal et al., 2000; Tan, Bianco & Dobrovic, 2002). Singal et al. (2000) showed that Cx26 in MCF-7 breast cancer cells was hypermethylated but that inhibition of a DNA methyltransferase did not induce expression of the gene. Tan et al. (2002) found that only one in eight breast cancer cell lines tested was hypermethylated in the Cx26 promoter region but that, in this case, it correlated with a complete loss of mRNA that was recovered after treatment with a DNA methyltransferase inhibitor. Furthermore, the Cx26 promoter was found to be methylated in >50% of patient tissue samples tested, albeit heterogeneously. These conflicting results suggest that Cx26 may indeed be a tumor suppressor that is inactivated by methylation, but this is likely not the only mechanism to downregulate expression.

Contrary to a tumor-suppressive role for connexins, there are a few reports of connexin upregulation in breast cancer tissue. First, Cx43 was detected in normal and benign tissue but also in myoepithelial cells of ductal carcinoma *in situ* (DCIS) and in the stroma of all invasive carcinomas, where stroma from normal breast tissue did not typically express Cx43 (Jamieson et al., 1998). Furthermore, 50% of invasive carcinomas were positive for Cx43 in the carcinogenic cells, although the majority of the staining was cytoplasmic. Moreover, Cx26 was absent in normal tissue samples but intracellular expression was detected in 75% of DCIS and >50% of invasive carcinomas (Jamieson et al., 1998). A more recent study also reported upregulation of phosphorylated forms of Cx43 in both myoepithelial cells and transformed luminal cells of *in situ* carcinomas and in all cells of invasive breast carcinomas, although the specific localization of Cx43 to gap junctions could not be confirmed (Gould et al., 2005). Kanczuga-Koda et al. (2005) report that the level of Cx43 expression, which was cytoplasmic in 90% of the tumors, was positively correlated with advanced histological grade of the tumor. A new study reports that a higher level of Cx26 expression positively correlates with larger tumor size and advanced histological grade with poor prognosis (Naoi et al., 2007). This study also showed increased lymph vessel invasion of Cx26-positive tumors. These results challenge the notion of connexins as tumor suppressors, yet they do not clearly point to a role for connexins in promotion of cancer progression either. While levels of connexin expression may be upregulated in some breast

tumors, the connexins are typically retained within intracellular compartments and removed from their cell surface site of normal function. Furthermore, we cannot assume that this dysregulation of connexin expression and localization is directly linked to carcinogenesis, but rather, it may simply be a secondary effect of the dedifferentiation of the cells. In fact, a substantial body of *in vitro* studies offer mechanistic evidence that connexins do indeed promote differentiation and inhibit primary tumorigenesis.

Extensive studies involving the reconstitution of connexin expression in GJIC- deficient breast cancer cell lines have shed some light on their role in cell growth control and differentiation. Reexpression of either Cx26 or Cx43 in MDA-MB-435 cells resulted in growth suppression, a regained ability to differentiate into three-dimensional (3D) structures in the presence of a basement membrane and, importantly, reduction of tumor growth in mice (Hirschi et al., 1996). Similarly, studies in our laboratory together with our collaborators have shown that overexpression of either Cx26 or Cx43 in MDA-MB-231 cells results in decreased tumor growth in mice but surprisingly has no effect on *in vitro* cell proliferation (Qin et al., 2002). However, when connexin-expressing cells were cultured in a 3D matrix, they partially redifferentiated toward an alveolar-like organoid, whereas control cells were not able to form differentiated structures (McLachlan et al., 2006) (Fig. 3). What is especially intriguing about the latter studies is that the effects were produced with only minimal rescue of GJIC; instead, the connexins were typically diverted to lysosomes. A recent study by Conklin et al. (2007) also reported an effect of Cx43 upregulation by the drugs genistein and quercetin and inhibition of cell proliferation that was GJIC-independent. Connexin activity unrelated to communication is corroborated in a variant of MDA-MB-435 cells, where the expression of both functional and nonfunctional Cx26 variants was able to inhibit cell proliferation while a Cx26 mutant that was retained in an intracellular compartment had no effect (Kalra et al., 2006). Moreover, all three Cx26 species were able to inhibit anchorage-independent growth. Coupled with the studies in MDA-MB-231 cells, this suggests that Cx43 and Cx26 may need to be localized to the plasma membrane to provide putative adhesive effects that may be responsible for growth control, while the Cx26 molecule itself is enough to elicit partial tumor-suppressive properties in MDA-MB-435 cells.

Connexins may have a GJIC-independent role in the regulation of apoptosis, as suggested from the correlation of intracellular Cx26 and Cx43 expression with the presence of the proapoptotic factor Bak (Kanczuga-Koda et al., 2005). In addition, there is evidence that GJIC-independent connexin expression favors an epithelial, rather than a mesenchymal, phenotype, as revealed from the upregulation of cytokeratin 18 and downregulation of vimentin upon Cx26 and Cx43 overexpression in MDA-MB-231 cells (McLachlan et al., 2006). Furthermore, SIP-1, a molecule associated with epithelial to mesenchymal transition, was shown to repress the Cx26 promoter in MCF-7 cells (Vandewalle et al., 2005). Finally, there is evidence that Cx26 and Cx43 inhibit angiogenesis independently of cell communication as both functional and nonfunctional Cx26 in MDA-MB-435 cells mediated the transcriptional and translational upregulation of the angiogenesis inhibitor thrombospondin-1 (TSP-1) and downregulated proangiogenic connective tissue growth factor (Qin et al., 2003). Conversely, when Cx43 expression was inhibited by RNAi in breast cancer Hs578t cells, resulting in increased aggressiveness of the cells, TSP-1 expression was reduced while expression of proangiogenic vascular endothelial growth factor was increased (Shao et al., 2005). A similar shift in the balance toward antiangiogenic factors was observed in MDA-MB-231 cells overexpressing Cx26 and Cx43, and conditioned media from these cells inhibited *in vitro* endothelial cell tubulogenesis and migration (McLachlan et al., 2006). Additionally, xenografts of Cx43-overexpressing MDA-MB-231 cells showed reduced tumor angiogenesis.

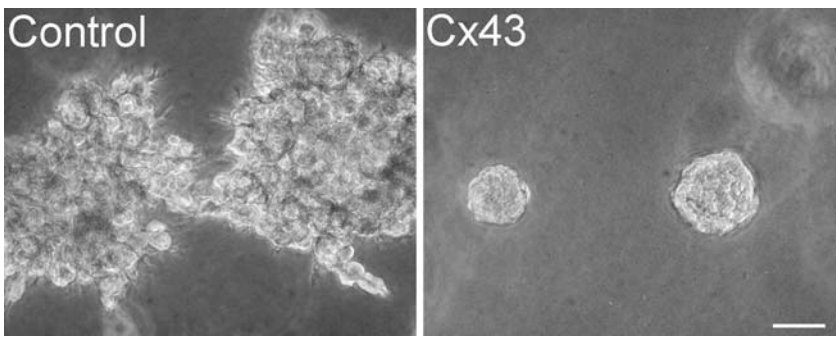

Fig. 3 Cx43 promotes mammary cell organoid growth in 3D culture. MDA-MB-231 cells were cultured for 7 days in BD Matrigel™ Matrix Growth Factor Reduced (BD Biosciences, Mississauga, Canada). Control cells form undifferentiated structures, while cells that stably overexpress Cx43 form partially differentiated alveolar-like organoids. Bar = 50 μm

Collectively, many cell culture models support a role for Cx43 and/or Cx26 in regulating tumorigenesis; however, it is important to temper these findings in the context that cultured environments are far from equivalent to *in vivo* conditions. When assessing the generic role of connexins in breast tumorigenesis, the cell type differences, genetic backgrounds, cell growth conditions and degree of connexin overexpression or silencing must all be considered relevant and taken into account. There is no doubt that the literature on this topic is complex and even conflicting, owing in large part to differences in cell lines. Above all, we have yet to fully understand the possible consequences of expressing connexins in excess of normal physiologically relevant levels or to obtain and investigate suitable animal models that reflect the human condition. It is notable that no increase in human breast cancer has been reported in patients harboring loss-of-function Cx43 or Cx26 mutations, but the question remains whether anyone has really performed such a study. While this is negative evidence for the role of connexins in breast carcinogenesis, it could be considered the most relevant as it may most accurately reflect the human condition.

Metastasis

Successful breast cancer metastasis requires that cells dissociate from the primary tumor and intravasate through the endothelium to travel through the circulation. To establish a secondary tumor, the tumor cells must then extravasate across the endothelium and proliferate within a new microenvironment (Fig. 4). These processes require modulation of both cell-matrix interactions and cell-cell interactions (Cairns, Khokha & Hill, 2003). There have been far fewer studies on the role of connexins in breast cancer metastasis than connexin studies that attempt to mimic the primary tumor. Exogenous expression of the breast metastasis suppressor 1 gene in MDA-MB-435 cells led to upregulation of Cx43 and restoration of GJIC, providing

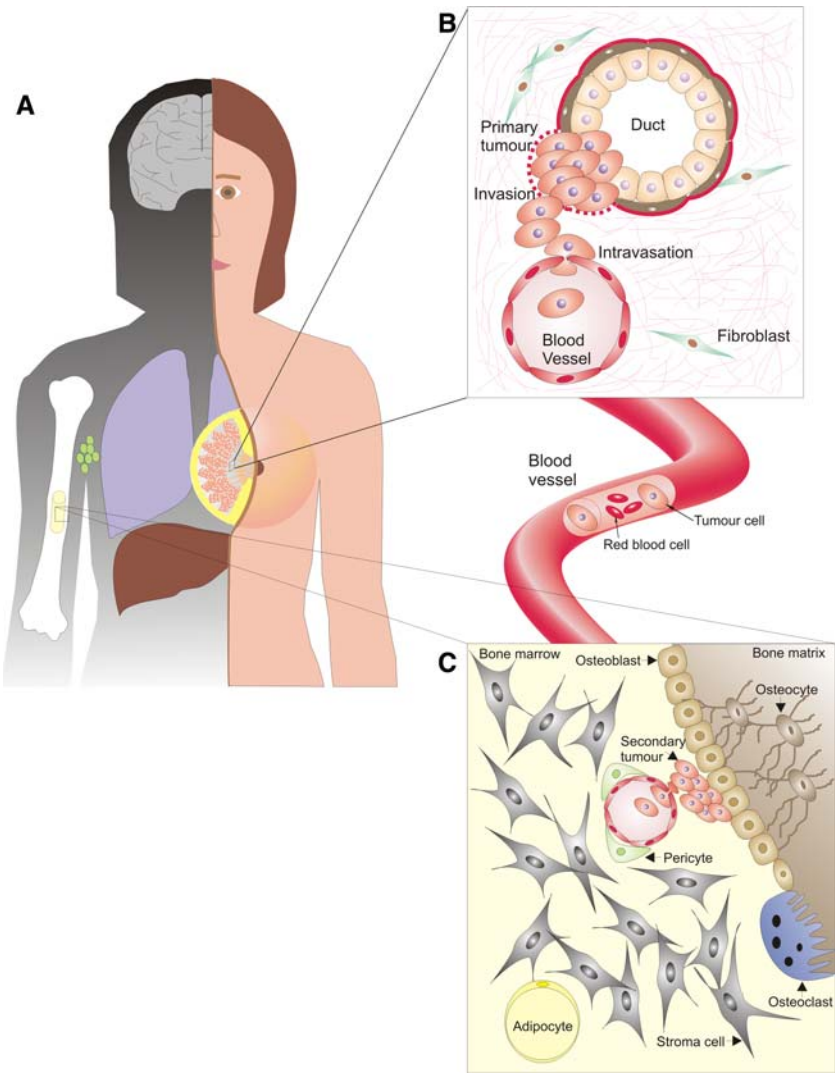

Fig. 4 Breast cancer metastasis. **a** Breast cancer most commonly metastasizes to the bone, lymph nodes, liver, lung and brain. **b** Tumor cells are depicted dissociating from the primary breast tumor and invading into the surrounding tissue en route to lymph or blood vessels. The tumor cells must then intravasate through the endothelium to enter the lymph or bloodstream. **c** Breast tumor cells that enter the blood vessels are carried toward other organs such as the bone. The tumor cells extravasate and enter the bone marrow, adhere to the tissue and begin to influence the microenvironment to promote bone catabolism

evidence that connexins act as tumor suppressors in metastasis (Saunders et al., 2001). Functional *in vitro* studies have observed that Cx26 and Cx43 expression leads to a decrease in the cell's migratory potential and ability to invade through a basement membrane matrix (Momiyama et al., 2003) accompanied by a slight reduction in matrix-metalloproteinase activity (Kalra et al., 2006). One interpretation of these findings could suggest a connexin-driven inhibition of cancer cell invasion into the surrounding tissue and inhibition of intravasation and extravasation through the endothelium, thereby hindering metastasis. In one *in situ* study, Cx26 and Cx43 expression was examined in primary tumors and lymph node metastases. Interestingly, cytoplasmic Cx26 protein was detected in 53% of the primary tumors, whereas the other 47% were negative for Cx26 staining. Cx43 was also expressed in 82% of tumors, and the majority of the staining was intracellular, although some cell surface plaques were detected. Finally, Cx26 was expressed in 88% of the lymph node metastases examined and Cx43 was expressed in 96% of metastases (Kanczuga-Koda et al., 2006). As seen in cases where primary breast tumors express connexins, the connexins were predominantly localized to the cytoplasm, though cell surface localization of both connexins could be detected in some metastases, and the number of metastases that showed Cx43 at the cell surface was higher than that reported for primary tumors. When these authors evaluated matched pairs for changes in Cx26 or Cx43 expression levels as the cancer progressed from primary tumor to metastasis, a significant increase in the expression of both connexins was observed in the lymph node metastasis compared to the corresponding primary tumor. Furthermore, almost 80% of the connexin-negative primary tumors expressed connexin in the metastases they developed, although it is not clear whether the connexin formed gap junctions (Kanczuga-Koda et al., 2006).

One of the key steps in metastasis is the movement of cancer cells across the endothelial cell barrier as they move in and out of the blood vessels. Direct investigation into this process has provided some insights into the role of connexins in tumor cell vascular intravasation and extravasation. In one study, GJIC was reduced in endothelial cells when they were cocultured with breast cancer cells, presumably weakening cell-cell contacts and making it easier for the cells to cross the endothelial barrier during both intravasation and extravasation (Cai, Jiang & Mansel, 1998). Another study showed that overexpressing Cx43 in GJIC-deficient HBL100 breast cancer cells allowed them to form heterocellular junctions capable of dye transfer with cells from a human microvascular endothelial cell line that expresses Cx43 and that this increased tumor cell diapedesis (Pollmann et al., 2005). This increase in transendothelial migration was blocked when the endothelial culture was pretreated with GJIC inhibitors or when the breast cancer cells expressed a membrane localizing, nonfunctional chimeric mutant of Cx43 (Pollmann et al., 2005). This study implies that both homocellular GJIC in the endothelium and heterocellular GJIC between breast cancer cells and the endothelium facilitate transendothelial migration.

It appears that connexins act as context-dependent tumor suppressors or, in some cases, as facilitators of metastasis. One can imagine that connexins are tumor-suppressive at the onset of metastasis, when the cells must dissociate from the primary tumor and invade and migrate through the surrounding tissue toward the circulation. However, connexins may promote tumor progression during intravasation and extravasation, when their expression is required for heterocellular communication with the endothelium to facilitate their transendothelial migration. However, whether extravasation plays a regulatory role in tumor progression is controversial. The role of GJIC in the endothelium itself is also an area of some debate. The downregulation of gap junction plaque formation may be important in reducing adhesion between the cells to allow passage of cancer cells, or perhaps GJIC is required to transmit a signal between endothelial cells that in turn will decrease the transendothelial resistance. We postulate that after extravasation, connexin expression would again be detrimental to breast cancer cells as they migrate toward secondary tumor sites. This model would require that connexin expression and/or gap junction function be dynamically regulated throughout cancer progression. The majority of the studies performed to date address only one stage of metastasis and typically rely on connexin overexpression systems; thus, formulating a cohesive model of the role of connexin expression and function in breast tumor cell metastasis is difficult. In the future, it will be necessary to establish better dosage controlled models where breast tumor cell progression and metastasis can be followed entirely in a common animal model system.

Metastasis to the Bone

Breast cancer cells preferentially metastasize to the bone (Coleman, 1997), often resulting in osteolytic lesions due to a skewing of the balance between the bone cells, osteoblasts and osteoclasts, thereby disrupting the bone matrix microenvironment. Breast cancer cells accomplish this through various mechanisms (*reviewed by* Guise et al., 2005; Rose & Siegel, 2006), one of which is by taking on osteomimetic properties to become better "seed" for the bone tissue "soil." A recent study examined the expression profile of genes important to osteoblast differentiation that are expressed in breast cancer cells. The researchers compared a parental MDA-MB-231 cell line with a highly

bone metastatic variant, B02, and identified multiple genes that were differentially regulated, resulting in an expression pattern in the B02 cells that mimics that of differentiating osteoblasts (Bellahcene et al., 2007). Interestingly, Cx43 mRNA was expressed almost fivefold higher in the B02 cells, and this was also reflected in the protein level quantification. Furthermore, when the B02 cells were injected into mice and allowed to form metastases, those found in the bone expressed high levels of Cx43 while those in the liver did not express detectable levels of Cx43. To date, we know that breast cancer cells can form functional gap junctions with the bone marrow stroma *in vitro* (Moharita et al., 2006).

We began a series of *in vitro* studies to determine if connexin expression may regulate any of the common molecules and mechanisms involved in establishing breast cancer cells in the bone microenvironment. For this we used MDA-MB-231 human metastatic breast cancer cells overexpressing either of the mammary connexins, Cx26 or Cx43, as previously described (McLachlan et al., 2006), and compared them to wild-type and mock-infected (empty vector) controls. To provide the bone microenvironment, we used MC3T3-E1 mouse osteoblastic cells (ATCC, Rockville, MD) grown in α-minimal essential medium (Invitrogen, Burlington, Canada). We also used conditioned media collected from confluent cultures of both cell types to simulate the corresponding cellular microenvironment influences.

Bone-derived diffusible factors can influence breast cancer cell proliferation (Goren et al., 1997), so we began by testing whether secreted factors from osteoblasts might alter the growth rates of breast cancer cells depending on their connexin expression profile. We plated 1×10^4 cells of each of the four MDA-MB-231 cell lines (wild type, empty vector control, Cx43- and Cx26-overexpressing) and cultured them in conditioned media collected from osteoblasts for 8 days. We sampled and counted the cells every 2 days and found that proliferation was equal in cells overexpressing either Cx26 or Cx43 compared to controls (Fig. 5a). Since breast cancer cells must adhere to the cells at the secondary tumor site, we next tested if expression of either Cx26 or Cx43 altered the ability of the MDA-MB-231 cells to adhere to an osteoblast monolayer. We show that a similar number of both Cx26- and Cx43-overexpressing cells adhered to the osteoblasts over 4 h as the control cells (Fig. 5b). Next, given that bone cells and matrix can release diffusible factors that induce breast cancer cell migration and invasion (Giunciuglio et al., 1995), we performed two types of coculture assays to assess if there were differential effects on connexin-expressing cells *vs.* controls. In the first, we tested MDA-MB-231 cell migration over a 24-h period through filter inserts toward chemoattractants released by an established

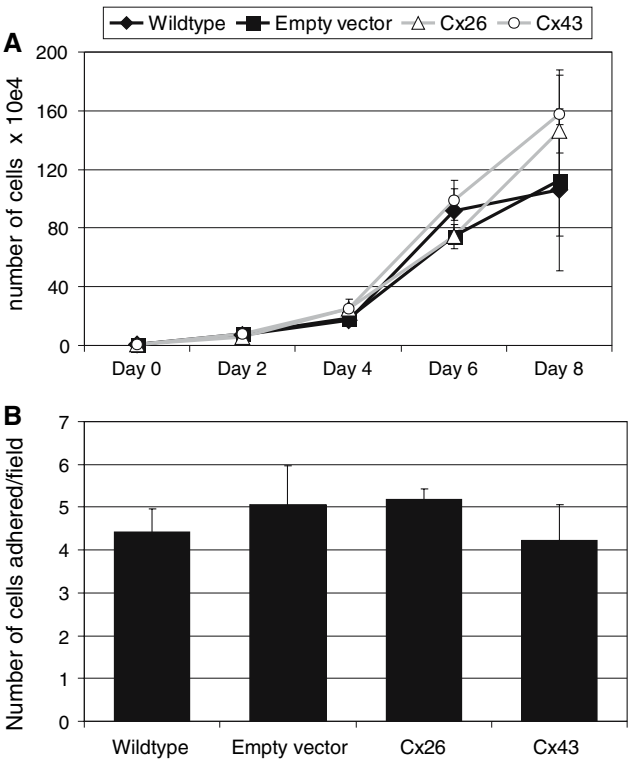

Fig. 5 Connexin expression does not alter MDA-MB-231 growth or adherence in an *in vitro* osteoblastic environment. **a** Cells overexpressing Cx26 or Cx43 grew at the same rate as wild-type or control cells when cultured in medium previously conditioned by MC3T3-E1 osteoblast-like cells. **b** DiI-labeled MDA-MB-231 cells expressing Cx26 or Cx43 were seeded onto a confluent monolayer of MC3T3-E1 cells, and nonadherent cells were washed away after 4 h. The cultures were imaged on a Leica (Richmond Hill, Canada) DM IRE2 inverted epifluorescent microscope and the fluorescent cells counted. Connexin expression did not alter the ability of any of the cell variants to adhere

culture of osteoblasts growing in the well (Fig. 6a). Cx43 expression in the breast cancer cells significantly inhibited their migration toward the osteoblasts. Cx26-expressing cells also showed some migratory inhibition, but it was not statistically significant. In the second test, we allowed MC3T3-E1 cells to reach monolayer confluence on a filter insert and then seeded fluorescently prelabeled MDA-MB-231 cells at low density on top of these and quantified their invasion through the osteoblast layer after 24 h (Fig. 6b). Again, Cx43-expressing cells were unable to invade through the osteoblast layer to the same degree as control and Cx26-expressing cells.

In the next set of experiments, we investigated whether connexin expression led to the regulation of any osteogenic genes in the breast cancer cells. Runx2 is a transcription factor and master regulator of bone development (Komori, 2002). It is expressed in osteoblast lineage cells and regulates the transcription of several key molecules involved in differentiation (Schroeder, Jensen & Westendorf, 2005). Interestingly, Runx2 is also expressed in normal mammary

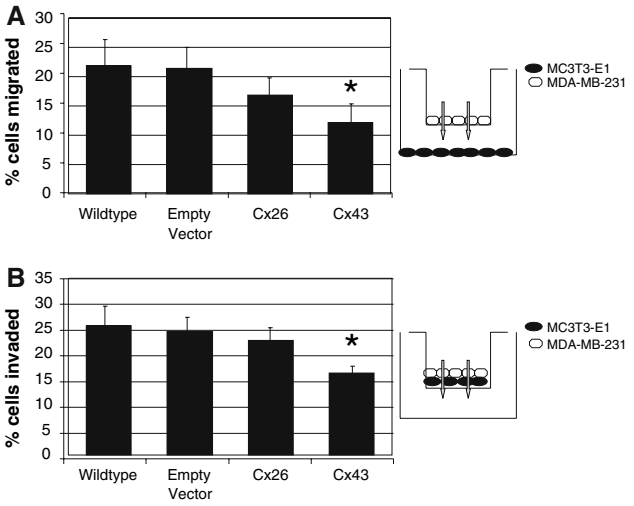

Fig. 6 Cx43 overexpression alters MDA-MB-231 migration and invasion in an *in vitro* osteoblastic environment. **a** MDA-MB-231 cells prelabeled with diI were allowed to migrate through HTS FluoroBlok Insert filters (Becton Dickinson, Franklin Lakes, NJ) toward a layer of confluent MC3T3-E1 cells and their conditioned medium for up to 24 h. Cells that migrated to the bottom of the filter were counted and compared to the number remaining on top to determine the percentage of cells that migrated. Migration of Cx43-overexpressing cells was significantly inhibited. **b** Filter inserts were seeded with MC3T3-E1 cells, and once they reached confluence, diI-prelabeled MDA-MB-231 cells were incubated on top and allowed to invade through the osteoblasts for 24 h. Percentage of invading cells was determined as in **a**. Invasion of Cx43-overexpressing cells was inhibited by approximately 40% compared to controls $^{*}p < 0.05$

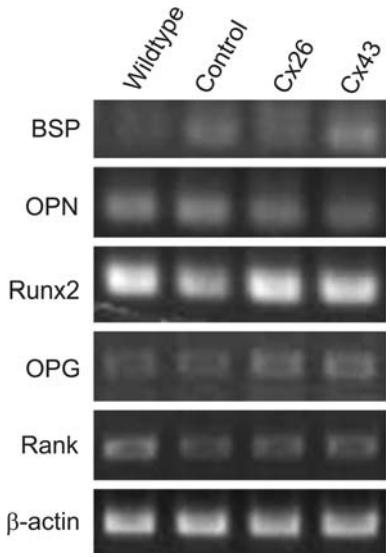

Fig. 7 Connexin expression does not regulate the expression of osteogenic genes in MDA-MB-231 cells. Total RNA was collected from cells overexpressing Cx26 or Cx43 and controls for RT-PCR to determine if connexin expression led to the regulation of osteogenic genes. There were no detectable differences in mRNA expression of RANK, OPG, Runx2, OPN or BSP compared to control cells. β-Actin was used as a template control. See primer sequences in Table 1

epithelium and often overexpressed in breast cancer cells (*reviewed in* Pratap et al., 2006). We therefore decided to begin by assessing the levels of Runx2 as well as osteopontin (OPN) and bone sialoprotein (BSP), molecules regulated by Runx2 and known to be expressed in mammary epithelial cells. In addition, we examined the expression of osteoprotegerin (OPG) and receptor activator of nuclear factor κB (RANK), important molecules in balancing osteoblast-osteoclast activity. Semiquantitative reverse transcriptase (RT) PCR did not reveal differences in the expression levels of any of these genes in the Cx26- or Cx43-expressing MDA-MB-231 cells compared to controls (Fig. 7).

Previously, a series of studies on the effects of metastatic breast cancer cells on osteoblast differentiation was performed with MDA-MB-231 and MC3T3-E1 cells that showed inhibition of osteoblast differentiation by conditioned medium from breast cancer cells (Mercer & Mastro, 2005; Mercer, Miyasaka & Mastro, 2004). Expression of the important differentiation markers alkaline phosphatase (ALP), BSP and OPN as well as the degree of mineralization were reduced when the MC3T3-E1 cells were grown under the influence of secreted factors from MDA-MB-231 cells. We executed the same type of study using MDA-MB-231 cells that overexpressed Cx26 or Cx43 to determine if they had a different influence on osteoblast growth and differentiation. We plated 2×10^4 MC3T3-E1 cells and sampled them every 2 days by counting; the cells grew at the same rate whether they were cultured with conditioned medium from control or connexin-overexpressing breast cancer cells (Fig. 8a). Next, we cultured osteoblasts in a 1:1 mix of breast cancer cell conditioned medium and osteoblast differentiation permissive medium for up to 28 days. We compared the expression levels of differentiation markers among osteoblasts treated with conditioned medium from Cx26- or Cx43-overexpressing cells or control breast cancer cells and osteoblasts grown without the influence of breast cancer cell conditioned medium (MC3T3-E1). Interestingly, there was no difference in ALP activity (*data not shown*) or in the expression levels of Runx2, OPN, BSP or collagen I, an early-stage differentiation marker (Fig. 8b). There was, however, an overall decrease in the expression of the late-stage differentiation marker osteocalcin (OCN) in osteoblasts treated with breast cancer cell medium compared to untreated MC3T3-E1 cells, consistent with a negative influence of breast cancer cells on osteoblast differentiation; but there were no differences among the breast cancer cell line conditioned media treatments. This suggests that connexin expression does not regulate the expression of any osteoblastic molecules tested in this culture environment.

The above experiments examined key molecules and mechanisms associated with metastasis to the bone but

Table 1 RT-PCR primers used at a 53°C annealing temperature

Gene	Species reactivity	Sequence
BSP	Human	F- CCA GAG GAA GCA ATC ACC AAA
BSP	Human	R- TTG AGA AAG CAC AGG CCA TTC
OPN	Human	F- ACA GCC ACA AGC AGT CCA GAT T
OPN	Human	R- TGC TCA TTG CTC TCA TCA TTG
Runx2	Human	F- AAC CCA CGA ATG CAC TAT CCA
Runx2	Human	R- CGG ACA TAC CGA GGG ACA TG
OPG	Human	F- GGG GAC CAC AAT GAA CAA GTT G
OPG	Human	R- AGC TTG CAC CAC TCC AAA TCC
Rank	Human	F- GGG AAA GCA CTC ACA GCT AAT TTG
Rank	Human	R- GCA CTG GCT TAA ACT GTC ATT CTC C
β-actin	Human/mouse	F- AAG AGA GGC ATC CTC ACC CT
β-actin	Human/mouse	R- TAC ATG GCT GGG GTG TTG AA
OCN	Mouse	F- GGA CCC TGG AGC CTC TTG T
OCN	Mouse	R- CGT AGA TGC GTT TGT AGG C
BSP	Mouse	F- TCT GCA TCT CCA GCC TTC TTG
BSP	Mouse	R- GAA AAT GGA GAT GGC GAT AGT T
OPN	Mouse	F- GTG AGG TCC TCA TCT GTG GCA TC
OPN	Mouse	R- GGA TGA ATC TGA CGA ATC TCA C
Runx2	Mouse	F- GAA CCA AGA AGG CAC AGA CA
Runx2	Mouse	R- AAC TGC CTG GGG TCT GAA AA
Collagen I	Mouse	F- TCT CCA CTC TTC TAG TTC CT
Collagen I	Mouse	R- TTG GGT CAT TTC CAC ATG CT

failed to show any obvious role of connexins in promoting or inhibiting breast cancer establishment in the bone microenvironment. While Cx43 expression did inhibit both migration and invasion of breast cancer cells in an *in vitro* bone microenvironment, it also did so in an unstimulated environment (McLachlan et al., 2006); thus, we cannot conclude that the inhibition is a direct effect resulting from the influence of the bone tissue. This is not to say that connexins do not play a part somewhere in breast cancer establishment in the bone as there are countless other pathways and mechanisms involved in metastasis that could be examined. It is also important to note that we only examined the interaction between breast cancer cells and osteoblasts and not between breast cancer cells and osteoclasts. Osteoclasts are responsible for osteolysis, so it remains possible that Cx26 or Cx43 has a role in promoting or inhibiting osteoclast stimulation in bone metastases.

Conclusions

It is apparent that connexins have multifaceted functions in both normal mammary gland development and homeostasis, as well as in cancer progression. From the mouse models we know that Cx26 is integral to the early development and differentiation of the mammary gland and, along with Cx32 and Cx30, likely regulates lactation after parturition. Cx43 almost certainly has a role in milk ejection during lactation in the mouse and likely is also important for the proper differentiation of the gland during development. We await a mammary gland-specific knockout of Cx43 to verify this *in vivo*. In humans, Cx26 and Cx43 are expressed in the resting mammary gland and likely provide some level of protection as tumor suppressors in the prevention of breast cancer onset and/or progression of the primary tumor. Their role in metastasis, however, seems to be highly variable and stage-dependent as connexins may even facilitate the advancement of the tumor. For example, during intravasation and extravasation, connexin expression and/or GJIC seem to promote cancer progression. Yet, some *in vitro* models support the notion that connexins inhibit the tumorigenic steps of migration and invasion, but this remains to be conclusively demonstrated in better-designed animal models. Lastly, it is becoming established that connexins have GJIC-dependent and –independent cellular effects, raising the complexity of the roles connexins play in carcinogenesis even further. Additional innovative studies will need to be done to dissect out the role of connexins at each stage of breast cancer progression if connexins are to ever be considered as a therapeutic drug target.

Acknowledgment We thank Isabelle Plant for providing the image for Figure 2 and Crystal Lounsbury for generating the artwork pre-

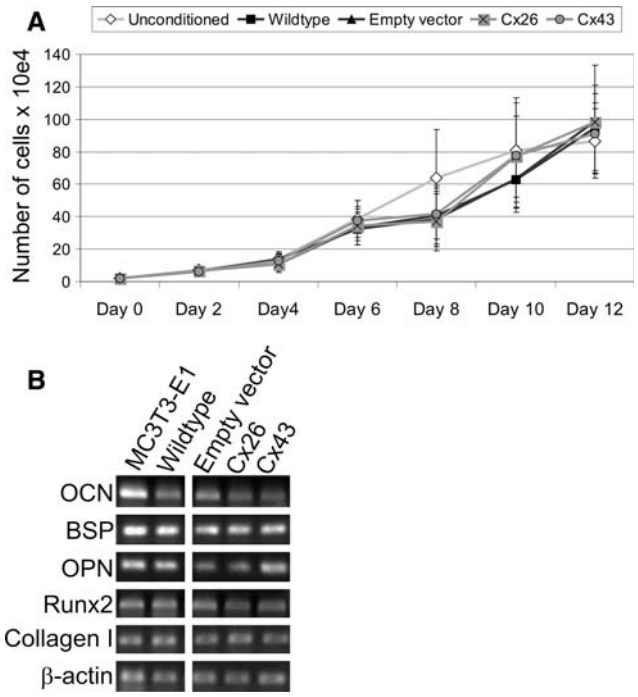

Fig. 8 The *in vitro* growth and differentiation of MC3T3-E1 cells was not altered under the influence of connexin-expressing MDA-MB-231 breast cancer cells. **a** MC3T3-E1 cells grew at the same rate regardless of whether they were incubated in conditioned medium from control or Cx26- or Cx43-overexpressing MDA-MB-231 cells. **b** MC3T3-E1 cells were cultured in a mixture of differentiation-permissive α-minimum essential medium containing 50 μM ascorbic acid and 5 mM β-glycerol phosphate and RPMI conditioned by control or connexin-overexpressing MDA-MB-231 cells for 28 days, and then total RNA was collected for RT-PCR. OCN expression was reduced in cells grown in conditioned medium from any of the MDA-MB-231 cell lines; however, no changes were seen in the expression of the early- and late-stage osteoblast differentiation markers when the cells were under the influence of connexin-expressing breast cancer cells. See primer sequences in Table 1

sented in Figures 1 and 4. This study was funded by the Canadian Institutes of Health Research and the Canadian Breast Cancer Research Alliance. E. M. holds a Terry Fox Foundation Studentship through the National Cancer Institute of Canada.

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