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Title Page

Human glandular organoid formation in murine engineering chambers after collagenase digestion and flow cytometry isolation of normal human breast tissue single cells

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Keywords

Breast cancer; xenograft; biochambers; collagenase digestion; FACS; mammary stem cell.

Abbreviations
BC: breast cancer
BMI: body mass index
CK: cytokeratin
ECM: extracellular matrix
FACS: fluorescence-activated cell sorting
H&E: haematoxylin and eosin
HMD: high mammographic density
LMD: low mammographic density
MD: mammographic density
MFP: mammary fat pad
MaSC: mammary stem cell
SEM: standard error of mean

Abstract

Women with high mammographic density (MD) are at increased risk of breast cancer (BC) after adjustment for age and body mass index. We have developed a murine biochamber model in which both high (HMD) and low MD (LMD) tissue can be propagated. Here we tested whether cells isolated by collagenase digestion and fluorescence-activated cell sorting (FACS) from normal breast can be reconstituted in our biochamber model, which would allow cell specific manipulations to be tested.

Fresh breast tissue was collected from women (n=7) undergoing prophylactic mastectomy. The tissue underwent collagenase digestion overnight, and in some cases, additional FACS enrichment to obtain mature epithelial, luminal progenitor, mammary stem, and stromal cells. Cells were then transferred bilaterally into biochambers in SCID mice (n=5-7) and incubated for 6 weeks, before harvesting for histological analyses, and immunohistochemical staining for cytokeratins (CK), vimentin, Ki-67 and murine macrophages.

Biochambers inoculated with single cells after collagenase digestion or with flow cytometry, contained glandular structures of human origin (human vimentin-positive), which expressed CK-14 and panCK, and were proliferating (Ki-67-positive). Glandular structures from the digested tissues were smaller than those in chambers seeded with finely-chopped intact mammary tissue. Mouse macrophage infiltration was higher in the chambers arising from digested tissues.

Pooled single cells and FACS fractionated cells are viable in the murine biochambers and formed proliferating glandular organoids of human origin. This is among the first report to demonstrate the success of formed human glandular organoids from isolated primary mammary cells in the murine biochamber model.

1. Introduction

Breast cancer (BC) is the leading cause of cancer morbidity and mortality for women in both developed and less developed countries. It was estimated by the World Health Organisation that that over 508,000 women worldwide died as a result of BC in 2011 (Organisation, 2015). Important risk factors for BC are aging, body mass index (BMI), parity, genetic mutations, family history or personal history of BC, alcohol intake and mammographic density (MD), which refers to the extent of opaque appearance of dense breast tissue on a mammogram (Couto et al. , 2012, Linton et al. , 2013, Quandt et al. , 2015). When adjusted for age and BMI, women with breasts that have ≥ 75% MD are 4-6 times more likely to develop BC than women with breasts that have <10% dense tissue (Boyd et al. , 1995). MD is not uncommon in the community; a study of 1,353 women found 42% of the 40-59 age group and 25% of the 60-79 age group have breasts that are at least 50% dense (Stomper et al. , 1996). Duss and colleagues used a heterotypic co-culture system to study the differentiation potentials and gene expression profiles of human breast epithelial and mesenchymal precursors isolated from reduction mammoplasty tissue (Duss et al. , 2014). Others have employed a human-in-mouse model to implant human mammary epithelial or stromal cells in murine mammary fat pads (MFP) (Proia and Kuperwasser, 2006), and Eaves *et al.* described the utilisation of kidney capsule to grow dissociated normal human epithelial cells (Eirew et al. , 2010).

To study the biology behind MD, we have adapted a murine biochamber model developed for tissue engineering studies (Morisson, 2015) by inserting paired samples of HMD and LMD human breast tissue into bilateral silicone chambers in the mouse groin. The normal HMD and LMD breast tissue remains viable in the chambers and maintains key histological differences associated with MD status for at least 6 weeks (Chew et al. , 2012). The chamber material is responsive to endogenous pregnancy related hormones and exogenous oestrogen and Tamoxifen, respectively (Chew et al. , 2013, Chew et al. , 2014). In comparison to another type of *in vivo* vascularized chamber in rats where an arteriovenous loop needed to be surgically created from the femoral vessels (Debels, 2015), our study utilised the inferior epigastric pedicle that was mobilised and inserted into the biochamber,

resulting in well vascularised chambers without gross infiltration of mouse tissue, as demonstrated in our previously published studies (Chew, Huang, 2013, Chew, Huang, 2012, Chew, Huo, 2014). As we are focussed on identifying the cell types in the breast that mediate MD and MD-associated BC risk, we aimed here to determine if the biochamber model could be used to grow human samples that had been collagenase digested or FACS sorted. Recombining FACS-isolated mammary cell populations may ultimately allow us to reconstruct and compare HMD *versus* LMD tissue scenarios and further allow us to manipulate gene expression in specific cellular populations to test potential causes and consequences of HMD in the future.

2. Method

2.1 The study population

This study was approved by both the Peter MacCallum and St Vincent's Hospital Human Research Ethics Committee (#08/21) and St Vincent's Hospital Animal Ethics Committee (049/09). It was conducted in line with the Australian National Statement on Ethical Conduct in Human Research (2007). Between 2013 and 2014, informed consent was obtained from ten women undergoing prophylactic mastectomy at St Vincent's Hospital or Peter MacCallum Cancer Centre to participate in this study. The reasons for mastectomy were a known deleterious BRCA mutation, a strong family history of BC, or a personal history of BC in the contralateral breast. Women who had suspicious breast lesions on imaging were not considered for this study.

2.2 Tissue accrual

Slices of breast tissue were accrued using sterile techniques by pathologists immediately after mastectomy. Specimens were kept viable and sterile, and (Lin et al. , 2011) tissues were then partitioned for mincing prior to implantation into chambers (intact control), collagenase digestion, and in some cases flow cytometry sorting after collagenase digestion. Additional regions of intact human breast specimens were randomly selected and fixed separately in 10% neutral buffered formalin for comparison with chamber materials.

2.3 Intact tissue, collagenase digestion and FACS

Intact tissue controls: as previously, breast tissue was finely minced with a scalpel and then 0.3g of minced tissue was mixed 1:1 with Matrigel™ (a murine tumour extract rich in basement membrane proteins; BD Biosciences, Bedford, MA) and supplemented with FGF-2 (an angiogenic / vasculogenic growth factor; 1ug/ml, Sigma-Aldrich, Sydney, Australia) in a sterile environment, as previously described (Chew, Huang, 2013, Chew, Huang, 2012, Chew, Huo, 2014). Collagenase digested tissue: finely chopped breast tissue was incubated with a collagenase/hyaluronidase digestion mixture for 12 hours and then digested to single cells, as described previously (Lim et al. , 2009). FACS isolated tissue: was prepared as per the collagenase group, but also underwent FACS the next morning to isolate viable, Lin- (CD235a, CD45, CD31) cells, as described previously (Lim, Vaillant, 2009). The resultant cells, representing all of the epithelial cell populations as well as the mesenchymal stromal cells, were then pelleted and the proportion of live cells was counted by an automated cell counter (Invitrogen, Countess™) before mixing 1:1 with Matrigel and FGF-2 for chamber implantation. Each chamber was implanted with 1.5-2 million live cells of either HMD or LMD tissue origin.

2.4 The murine biochamber model

The biochambers were cylindrical silicone chambers that were sustained by mouse epigastric pedicles in the groin in SCID mice, as described in detail previously (Chew, Huang, 2012, Lilja et al. , 2013) and illustrated in Figure 1. Material was harvested from mouse chambers after 6 weeks for histological examination.

2.5 Histological and immunohistochemical assessment

Paraffin-embedded tissue blocks were sectioned at 5µm thickness. Conventional haematoxylin and eosin (H&E) staining was used to assess the basic histological morphology. Human-specific pan-cytokeratin (CK) (DAKO clone, AE1/AE3) and CK-14 (Cymbus Biotech, CBL197) double immunofluorescent staining was carried out to identify human epithelial cells. Human breast tissue and mouse tissues were used as positive and negative controls, respectively. In order to further assess the basal and luminal epithelial layers, human-specific CK-14 (Merck Millipore, clone LL002, cbl

197) and CK-19 (ThermoScientific, MS-198-P1) were used for additional immunofluorescent staining. Slides were stained with isotype matched secondary antibodies and with 4', 6-diamidino-2-phenylindol (DAPI) (Sigma, St Louis, USA) before being mounted in fluorescent mounting medium (Dako, Glostrup, Denmark). All slides were stored at 4^{o}C in the dark until image capture.

2.6 Proliferation, macrophage infiltration and apoptosis assessment

Cellular proliferation was determined using Ki-67 antibody (DakoCytomation, Clone MIB-1, code 7240). The abundance of mouse macrophages invaded into the human tissue was examined using a pan-mouse macrophage marker F4/80 (eBioscience, Clone: BM8) that does not stain human macrophages, using previously published methods and a primary antibody dilution of 1:500, as previously published (Sun et al. , 2013). All stained slides were reviewed by blinded consultant pathologists and photographed with digital microscopy (AxioCam MRc5, Zeiss). In addition, the level of apoptosis was assessed by immunohistochemical staining using an antibody to Cleaved Caspase-3 (Asp175) antibody (Cell Signaling #9661) at 1:200 dilution overnight at 4 ^{o}C. Bright field microscopy was then performed on an Olympus BX-51 microscope to generate 40x magnification images, which were scored in MetaMorph software. For scoring of Caspase-3 staining, separate regions of interest (ROI) were drawn around ductal structures, and the stromal areas surrounding these, or around mixed structures. Mixed structures were defined by the presence of epithelium and stromal cells in part organisation but lacking ductal morphology. The number of cells positive for caspase cells were normalised against the standard nuclei count within in each ROI. The overall experimental method is outlined in Figure 2.

3. Results

3.1 The study sample

All specimens were reviewed by a consultant pathologist to confirm that they were free of malignancy. Out of the ten women consenting, two were excluded due to possible ductal carcinoma in-situ (DCIS) recognised peri-operatively, and the tissue sample from one lacked HMD and LMD contrast as assessed by breast radiologist on

X-ray (homogeneous MD throughout the specimen). Hence a total of 7 women were eligible for this study. The demographic characteristics of the women are described in Table 1.

3.2 Histological comparison between donor tissue specimens and intact tissue implants of HMD and LMD origin

Consistent with previously published work (Chew, Huang, 2012), the intact control mammary tissues implanted in murine chambers (without enzymatic digestion) for 6 weeks generally remained viable and maintained the histological appearance of the original human breast tissue. Out of 7 women, the intact mammary tissue from 5 women (Participant 1, 2, 3, 6, 7) showed preservation of human stromal and epithelial components (Table 2). This was evident on H&E staining (Fig 3). However, the intact tissue explants from two participants (Participant 4 and 5) showed scarce epithelium, which was in keeping with the histology of their corresponding original human tissue (Table 2). Manual counting of entire tissue sections stained with H&E found that the average number of glandular structures was 5.89 +/-1.00 (Mean+/-SEM, n=2-3 mice/woman) per specimen in the intact tissue chamber group.

3.3 Histological assessment of biochamber reconstituted tissues derived from collagenase digested or FACS isolated cells

Human mammary tissue was digested overnight with collagenase down to single cells, and several samples were further subjected to FACS before implantation into murine biochambers (1.5-2 million cells/biochamber). The chambers were incubated for 6 weeks, and in the majority of the cases contained small glandular structures on H&E staining (Fig. 4). Overall, only one gland-like structure was observed from each biochamber explant. Human specific epithelial markers CK-14 and panCK revealed that they were of human tissue origin (Fig. 5), and Ki-67 staining indicated cellular proliferation (Fig. 7). We did not observe any difference in the structures formed between high and low MD breast tissue in this study.

As shown in Table 2, tissues from 5 cases out of the 7 total underwent collagenase digestion only (without flow cytometry) down to a mixed preparation of single cells. Compared to intact control tissues, single cell preparations from 4 out of 5 women were observed to have formed glandular structures. Tissues from 5 women underwent collagenase digestion as well as further FACS isolation, and of those implanted single cells from 4 of the 5 women demonstrated formed epithelial organoids.

3.4 Analysis of epithelial structure formation in harvested biochamber material

These structures expressed the basal epithelial marker CK-14, and were pan-CK positive (Fig. 5). However, as shown in Figure 5, some ducts were positive for pan-CK but not CK-14, and the glandular structures formed were not identical to the normal bilayer structures that were found in normal breast glands: some of the organoids were small, lack the epithelial bilayers or a complete glandular lumen as illustrated in Figure 5. However, immunofluorescent double staining using a combination of the luminal marker CK-19 and the basal marker CK-14 did reveal distinct luminal and basal bilayers in some of the formed organoids (Fig. 6). Manual point counting showed that the proportion of glandular organoids with formed bilayers and a complete lumen was higher in the collagenase group compared with that of the FACS group, suggesting a higher degree of alteration in reformed structures in the FACS group than that of the collagenase group. After correlating the histological appearances of the original human tissue and intact tissue chamber (controls), there was no direct relationship between the amount of epithelium found in the intact tissue chambers and the number of organoids formed by single cell suspensions of the same MD tissue origin from the same woman. For example, original breast tissue of Participant 4 and Participant 5 showed scant epithelium, yet single cells post collagenase digestion from these tissues were both capable of forming glandular structures (Table 2).

3.5 Analysis of macrophage infiltration and apoptosis in harvested biochamber material

H&E staining showed substantial cellular infiltration in the collagenase and FACS groups compared with the intact tissue chamber group (Fig.4). Consistent with the possible inflammatory infiltration indicated by H&E staining, mouse macrophage staining demonstrated marked infiltration of macrophages across whole tissue sections in both collagenase digested and FACS sorted chamber specimens, compared to intact tissue chambers (Fig. 8). With regards to the level of apoptosis (see Figure 9), the proportion of cleaved caspase-3 positive cells was approximately 2.5% for all three experimental groups. For the collagenase group, the percentage of Cleaved Caspase-3 positive cells was elevated by approximately 2% within mixed structures characterised by regions enriched with glandular epithelia but lacking in mature ductal architecture. Due to the scarcity of outgrowths in biochambers seeded from the FACS group, we were unable to assess Caspase-3 staining in ductal structure or their surrounding stroma.

4. Discussion

4.1 Models for studying human mammary tissue

The existence of self-replenishing, multipotent human mammary stem cells (MaSC) has been well established, and demonstrated by transplantation studies into mammary fat pads in rodents (Daniel et al. , 1971, Kim et al. , 2000). In order to examine the property of MaSCs, isolated mammary epithelial single cells post enzymatic and mechanical digestion have been shown to grow into mammospheres in 3D Matrigel™ culture *in vitro* (Dontu et al. , 2003, Soule and McGrath, 1986). Since we hoped to not only study the stem/progenitor cell type, but also the characteristics and functionality of all other mammary cell types *in vivo*, we sought to develop a model where these single cells could maintain their viability and regenerate. Our results showed that collagenase digestion and FACS isolation allowed human mammary glandular organoids to form from a mixture of single, Lineage negative mammary cells.

Others have previously studied the formation of human mammary tissue in model hosts. Proia and colleagues (Proia and Kuperwasser, 2006) have used the incorporation of human stromal fibroblasts into the cleared mammary fat pads of mice one month prior to transplantation of normal breast tissue to test tissue viability and growth. Eirew and colleagues (Eirew, Stingl, 2010) similarly grew dissociated normal human mammary tissue under the kidney capsule when co-suspended with fibroblasts in collagen gels. Our method allows us to simultaneously add the differently isolated cell types against a reference of fresh tissue, which has been directly isolated and propagated in mice. Our dissociated samples have previously been shown to not only maintain the normal architecture of the human breast, but also to maintain the breast density differential of the input tissue (Chew, Huang, 2013, Chew, Huang, 2012). In this study, we further demonstrated the feasibility of using the murine engineering biochamber model to regenerate human glandular organoids from single cells isolated from normal human breast tissue. Furthermore, the silicone biochamber provides a sealed container for implanted materials which can be very liquid after FACS isolation of single cells, whereas in MFP or the renal capsule liquid can more easily accidentally escape before sealing, compromising the accuracy of cell quantity implanted.

4.2 The viability of cells post collagenase digestion and FACS

Although we ensured 1.5-2 million live cells for each chamber implantation, the processes of collagenase digestion and FACS may reduce the viability of live cells, as these processes lead to the loss of organisation and extracellular matrix. In a large study on the cytoarchitecture and property of cancer stem cells, Pece *et al.* used FACS to isolate cells from normal human mammary glands and found the epithelium reconstitution efficiency to be approximately 4% (one in 10-66 cells) at highest, even when injected at 10^5 cells/transplant concentration. They reported that this value was consistent with the maximum expected value of reconstitution, based on the kinetics of their cells used (Pece et al. , 2010). Prater and colleagues observed that the colony-forming efficiency in mammary epithelial cells was reduced to around 50% by flow cytometry-sorting (Prater et al. , 2014). In keeping with this finding, a study on the mammosphere reforming ability of breast cancer stem cells in MCF-7

cells showed that enzymatic digestion and FACS reduced their quality and quantity (Yi et al. , 2013). In our study, the transplant outgrowths from collagenase and FACS groups were relatively small and only one was observed per typical tissue sample, suggesting a low degree of engraftment and expansion within the 6-week incubation period. By contrast, the intact tissue chamber group showed on average 6 glands per tissue sample.

4.3 Epithelial maturation as shown by cytokeratin staining

Some of the formed organoids in this study were positive for CK-14 and pan-CK, while others were only pan-CK positive (Figure 5), suggesting that their degree of organisation or maturation was altered. We believe that the structures that co-express CK-14 and pan-CK may represent cells that have not matured into their luminal and basal fates. This is supported by studies on human mammary gland development showing expression of the basal epithelial cell marker CK-14 in the luminal epithelium of the infant breast (Anbazhagan et al. , 1998, Purkis et al. , 1990). Similarly mammary outgrowths generated from human fetal mammary stem cells exhibited co-expression of luminal (CK-8) and myoepithelial keratins (CK-14) *in vivo* (Spike et al. , 2012). In mouse mammary gland development, CK-14 was detected in scattered luminal cells in addition to its unequivocal expression in basal epithelium in pre-pubertal and pubertal glands (Mikaelian et al. , 2006). As cells co-expressing luminal and basal keratins are suggested to represent stem cells or bi-potent cells with intermediate phenotypes (Anbazhagan, Osin, 1998, Bocker et al. , 2002), we believe in our study they represent incompletely matured cell types. Despite this, separate basal and luminal epithelial layers were detected using human-specific CK-14 and CK-19 markers for the organoids (see Figure 6), indicating that a higher degree of epithelial maturation and organisation was able to occur, albeit not to the same extent as in the intact tissue chambers shown in Figure 5.

4.4 Macrophage infiltration and apoptosis in murine biochambers

Compared to chambers containing intact tissues, chambers containing collagenase digested tissue, with or without FACS, were highly populated with mouse macrophages, as revealed by immunostaining for mouse-specific macrophages.

Macrophages are known for their diverse functions in innate and adaptive immunity and in the mammary gland they promote both proliferation of mammary epithelial cells associated with early alveolar development, and phagocytosis of dying epithelial cells during alveolar regression (Chua et al. , 2010). Macrophages are a component of the MaSC niche, and this interaction may be essential for normal mammopoiesis (Shackleton et al. , 2006, Stingl et al. , 2006). Null mutation for the cytokine colony stimulating factor 1 (CSF1), which results in greatly reduced macrophage abundance, is associated with diminished mammary stem/progenitor cell activity (Gyorki et al. , 2009). In our previous studies using the murine chamber model (Chew, Huang, 2013, Chew, Huang, 2012, Chew, Huo, 2014), the inflammatory response from the host was minimal, and only occurred in the peripheral rim of the chamber tissue. Given the increased infiltration of macrophages into chambers seeded with single mammary cells, it is likely that these cells are part of a host response to phagocytosize non-viable cells and clear up debris. However, given the persistence of viable human breast cells in the collagenase digested and FACS biochambers for the 6 weeks of the study, it is also possible that these cells were recruited to promote mammary structure re-formation. Previous studies in these chambers have implicated macrophage recruitment in the neoadipogenesis that occurs in these chambers in response to Matrigel™ and FGF-2 (Lilja, Morrison, 2013, Thomas et al. , 2008). The level of apoptosis as indicated by the percentage of cells positive for cleaved caspase-3 was similarly low (2-3% of whole tissue sections) for all experimental groups, indicating that the majority of the intact tissue, and collagenase digested and FACS sorted single cells were viable and growing. This was also consistent with our proliferation marker results (Ki-67), as mentioned above. In areas within the collagenase group where mixed structures (cells with partial glandular characteristics but lacking complete ductal architecture) were observed, increased level of cells positive for cleaved caspase-3 suggest a higher level of apoptosis compared with other regions in the chamber explants. Unfortunately no ductal or mixed structures could be assessed in the FACS group due to exhaustion of the available tissue.

4.5 Strengths and limitations

Whilst there are now patient-derived tumour xenograft models to study BC growth and metastasis using either human (Dahibawkar et al. , 2015, Fritsche et al. , 2015, Rowan et al. , 2014, Sp et al. , 2015) or mouse-derived cancer cell lines (Singh et al. , 2013, Zou et al. , 2013), there is no mouse model for studying normal breast tissue biology such as that required for examining high MD-associated BC risk. The complex cellular interactions that underpin MD and BC cannot be easily created in an *in vitro* setting, thus our pilot study demonstrated the value of our murine biochamber as a model to assess altered cellular function in HMD and LMD human mammary tissues. After mechanical and enzymatic digestion, single cells from human tissue reformed mammary structures. As the biochambers were incubated for only 6 weeks, the structures observed could be preliminary. Faraldo and team transplanted mouse mammary epithelium fragments and sorted cell populations into cleared mammary fat pads in an attempt to study epithelial differentiation and stem cell activity *in vivo;* they found that while outgrowths were detected 4-5 weeks after the transplantation of epithelial fragments, complete outgrowths from sorted mammary basal cells may take up to 12 weeks (Faraldo et al. , 2015). Hence, increasing implant duration from 6 weeks is likely to allow continued development and organisation of formed epithelial structures. This system could also be amenable to adding extracellular matrix elements such as collagen that may facilitate epithelial-stromal cell interactions and promote colonisation and growth of the mammary structures. Assessment of the activation state and orientation of infiltrating macrophages in future studies could also allow further definition of the role that murine macrophages play in xenograft outgrowth

5. Conclusion

Our study showed single cells isolated from collagenase digestion with or without flow cytometry sorting from HMD and LMD human breast tissue reformed into epithelial structures after being maintained in murine bioengineering chambers for 6 weeks, without the need for prior humanizing of the input site. The value of this murine biochamber model lies in it being a foundation for further analyses in single

cell subtypes that may be responsible for increased breast cancer risk in an *in vivo* setting, although more work is needed to test the ideal conditions needed for glandular reconstitution. As intact chambers recapitulated the input breast tissue (Chew, Huang, 2012), future work with collagenase and FACS-isolated samples will focus on extending the incubation period and adding ECM elements in an attempt to develop the ideal experimental condition for isolated single cells to recapitulate breast tissue features of high and low MD origin in biochambers.

Competing interests

The authors declare that they have no competing interests.

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Ethical approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of St Vincent's Hospital and Peter MacCallum Cancer Centre and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in this study involving animals were in accordance with the ethical standards of St Vincent's Hospital at which the studies were conducted.

References

Anbazhagan R, Osin PP, Bartkova J, Nathan B, Lane EB, Gusterson BA. The development of epithelial phenotypes in the human fetal and infant breast. The Journal of pathology. 1998;184:197-206.

Bocker W, Moll R, Poremba C, Holland R, Van Diest PJ, Dervan P, et al. Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept. Lab Invest. 2002;82:737-46.

Boyd NF, Byng JW, Jong RA, Fishell EK, Little LE, Miller AB, et al. Quantitative classification of mammographic densities and breast cancer risk: results from the Canadian National Breast Screening Study. Journal of the National Cancer Institute. 1995;87:670-5.

Chew GL, Huang D, Huo CW, Blick T, Hill P, Cawson J, et al. Dynamic changes in high and low mammographic density human breast tissues maintained in murine tissue engineering chambers during various murine peripartum states and over time. Breast Cancer Res Treat. 2013;140:285-97.

Chew GL, Huang D, Lin SJ, Huo C, Blick T, Henderson MA, et al. High and low mammographic density human breast tissues maintain histological differential in murine tissue engineering chambers. Breast Cancer Res Treat. 2012;135:177-87.

Chew GL, Huo CW, Huang D, Blick T, Hill P, Cawson J, et al. Effects of Tamoxifen and oestrogen on histology and radiographic density in high and low mammographic density human breast tissues maintained in murine tissue engineering chambers. Breast Cancer Res Treat. 2014;148:303-14.

Chua AC, Hodson LJ, Moldenhauer LM, Robertson SA, Ingman WV. Dual roles for macrophages in ovarian cycle-associated development and remodelling of the mammary gland epithelium. Development. 2010;137:4229-38.

Couto E, Qureshi SA, Hofvind S, Hilsen M, Aase H, Skaane P, et al. Hormone therapy use and mammographic density in postmenopausal Norwegian women. Breast Cancer Res Treat. 2012;132:297-305.
Dahibawkar M, Forsberg MA, Gupta A, Jaffe S, Dulin K, Eisenbrey JR, et al. High and low frequency subharmonic imaging of angiogenesis in a murine breast cancer model. Ultrasonics. 2015.
Daniel CW, Young LJ, Medina D, DeOme KB. The influence of mammogenic hormones on serially transplanted mouse mammary gland. Experimental gerontology. 1971;6:95-101.
Debels H. Advances in tissue engineering ; a novel technology making use of an in vivo vascularized chamber. Acta Chir Belg. 2015;115:104-10.
Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes & development. 2003;17:1253-70.
Duss S, Brinkhaus H, Britschgi A, Cabuy E, Frey DM, Schaefer DJ, et al. Mesenchymal precursor cells maintain the differentiation and proliferation potentials of breast epithelial cells. Breast Cancer Res. 2014;16:R60.
Eirew P, Stingl J, Eaves CJ. Quantitation of human mammary epithelial stem cells with in vivo regenerative properties using a subrenal capsule xenotransplantation assay. Nat Protoc. 2010;5:1945-56.
Faraldo MM, Glukhova MA, Deugnier MA. The transplantation of mouse mammary epithelial cells into cleared mammary fat pads. Methods Mol Biol. 2015;1293:161-72.
Fritsche H, Heilmann T, Tower RJ, Hauser C, von Au A, El-Sheikh D, et al. TRAIL-R2 promotes skeletal metastasis in a breast cancer xenograft mouse model. Oncotarget. 2015;6:9502-16.
Gyorki DE, Asselin-Labat ML, van Rooijen N, Lindeman GJ, Visvader JE. Resident macrophages influence stem cell activity in the mammary gland. Breast Cancer Res. 2009;11:R62.
Kim ND, Oberley TD, Yasukawa-Barnes J, Clifton KH. Stem cell characteristics of transplanted rat mammary clonogens. Experimental cell research. 2000;260:146-59.
Lilja HE, Morrison WA, Han XL, Palmer J, Taylor C, Tee R, et al. An adipoinductive role of inflammation in adipose tissue engineering: key factors in the early development of engineered soft tissues. Stem cells and development. 2013;22:1602-13.
Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, et al. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nature medicine. 2009;15:907-13.
Lin SJ, Cawson J, Hill P, Haviv I, Jenkins M, Hopper JL, et al. Image-guided sampling reveals increased stroma and lower glandular complexity in mammographically dense breast tissue. Breast Cancer Res Treat. 2011;128:505-16.
Linton L, Martin LJ, Li Q, Huszti E, Minkin S, John EM, et al. Mammographic density and breast cancer: a comparison of related and unrelated controls in the Breast Cancer Family Registry. Breast Cancer Res. 2013;15:R43.
Mikaelian I, Hovick M, Silva KA, Burzenski LM, Shultz LD, Ackert-Bicknell CL, et al. Expression of terminal differentiation proteins defines stages of mouse mammary gland development. Veterinary pathology. 2006;43:36-49.
Morisson W. The O'Brien Institute of Microsurgery. 2015.
Organisation WH. Breast Cancer: prevention and control. 2015.

Pece S, Tosoni D, Confalonieri S, Mazzarol G, Vecchi M, Ronzoni S, et al. Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell. 2010;140:62-73.
Prater MD, Petit V, Alasdair Russell I, Giraddi RR, Shehata M, Menon S, et al. Mammary stem cells have myoepithelial cell properties. Nature cell biology. 2014;16:942-50, 1-7.
Proia DA, Kuperwasser C. Reconstruction of human mammary tissues in a mouse model. Nat Protoc. 2006;1:206-14.
Purkis PE, Steel JB, Mackenzie IC, Nathrath WB, Leigh IM, Lane EB. Antibody markers of basal cells in complex epithelia. Journal of cell science. 1990;97 (Pt 1):39-50.
Quandt Z, Flom JD, Tehranifar P, Reynolds D, Terry MB, McDonald JA. The association of alcohol consumption with mammographic density in a multiethnic urban population. BMC Cancer. 2015;15:1094.
Rowan BG, Gimble JM, Sheng M, Anbalagan M, Jones RK, Frazier TP, et al. Human adipose tissue-derived stromal/stem cells promote migration and early metastasis of triple negative breast cancer xenografts. PLoS One. 2014;9:e89595.
Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, et al. Generation of a functional mammary gland from a single stem cell. Nature. 2006;439:84-8.
Singh M, Ramos I, Asafu-Adjei D, Quispe-Tintaya W, Chandra D, Jahangir A, et al. Curcumin improves the therapeutic efficacy of Listeria(at)-Mage-b vaccine in correlation with improved T-cell responses in blood of a triple-negative breast cancer model 4T1. Cancer Med. 2013;2:571-82.
Soule HD, McGrath CM. A simplified method for passage and long-term growth of human mammary epithelial cells. In vitro cellular & developmental biology : journal of the Tissue Culture Association. 1986;22:6-12.
Sp N, Darvin P, Yoo YB, Joung YH, Kang DY, Kim DN, et al. The combination of methylsulfonylmethane and tamoxifen inhibits the Jak2/STAT5b pathway and synergistically inhibits tumor growth and metastasis in ER-positive breast cancer xenografts. BMC Cancer. 2015;15:474.
Spike BT, Engle DD, Lin JC, Cheung SK, La J, Wahl GM. A mammary stem cell population identified and characterized in late embryogenesis reveals similarities to human breast cancer. Cell stem cell. 2012;10:183-97.
Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, et al. Purification and unique properties of mammary epithelial stem cells. Nature. 2006;439:993-7.
Stomper PC, D'Souza DJ, DiNitto PA, Arredondo MA. Analysis of parenchymal density on mammograms in 1353 women 25-79 years old. AJR Am J Roentgenol. 1996;167:1261-5.
Sun X, Robertson SA, Ingman WV. Regulation of epithelial cell turnover and macrophage phenotype by epithelial cell-derived transforming growth factor beta1 in the mammary gland. Cytokine. 2013;61:377-88.
Thomas GP, Hemmrich K, Abberton KM, McCombe D, Penington AJ, Thompson EW, et al. Zymosan-induced inflammation stimulates neo-adipogenesis. Int J Obes (Lond). 2008;32:239-48.
Yi Q, Ying Y, Xiaoli W, Fu Q, Wang W, Wu J, et al. Impact of cell dissociation on identification of breast cancer stem cells. Cancer Biomarkers. 2013;12:125-33.
Zou Z, Bellenger S, Massey KA, Nicolaou A, Geissler A, Bidu C, et al. Inhibition of the HER2 pathway by n-3 polyunsaturated fatty acids prevents breast cancer in fat-1 transgenic mice. Journal of lipid research. 2013;54:3453-63.

Table 1 Demographic characteristic of study participants (n=7)

Selected characteristics	Number, N, or Mean
Age at surgery date	Mean 41 years (Range 36-49 years)
Birads Category	
4	2
3	3
2	2
1	0
Risk Factors	
BRCA-, strong Fhx	2
BRCA1+	3
BRCA 2+	2
Past history of BC or DCIS	4
Menopausal status	
Pre	6
Peri	0
Post	1
Parity	
Parous	6
Nulliparous	1

BI-RADS score 1: Predominantly fat, 2: Scattered fibroglandular densities, 3: Heterogeneously dense, 4: Extremely dense. BC = BC. FHx = Family history. IDC = Invasive ductal carcinoma. DCIS = ductal carcinoma *in situ*. Some participants have >1 risk factors.

Table 2 Summary of experimental results (n=3-4 mice/women, 7 women)

Participant ID	Histology of original human breast tissue	Histology of chamber intact breast tissue	Collagenase only chamber results	Collagenase & FACS chamber results
1	1A	1A	√	N/A
2	1A	1A	X	X
3	1A	1A	N/A	√
4	0A	0A	√	√
5	0A	0A	√	N/A
6	1A	1A	N/A	√
7	1A	1A	√	√

1A: abundant epithelium observed on H&E stained slides; 0A: scarce epithelium observed on H&E stained slides; √: formed glandular organoids of human tissue origin observed on histology; X: no formed glandular organoids of human tissue origin observed on histology. N/A: not available due to inadequate numbers of cells isolated for chamber implantation.

Figure Legends:

Fig.1 The biochamber model

The schematic flow chart describes the source of the biochamber at time of implantation, the silicone material that the chamber is composed of and the vascular supply from the inferior epigastric pedicle in the groin of the SCID mice. The chamber was placed in a dissected pocket in the mouse groin. The bottom end was sealed with bone wax first, and the superior end was closed also with bone wax after insertion of chamber content. 1: abdominal aorta; 2: right external iliac artery; 3: right inferior epigastric artery and vein, mobilised as a pedicle for chamber content (in grey); 4: harvested chamber tissue in formalin showing its vascular supply.

Fig. 2. The method of reconstituting digested single cells of human breast tissue using the xenograft model.

Breast tissue accrued from prophylactic mastectomy procedures were partitioned into the following 4 groups: original human tissue (in formalin), control (intact tissue), collagenase digested and FACS isolated cells. The number of mice utilised again depended on the final total number of live cells isolated, as we aimed to implant 1.5-2 million live cells into each chamber. FACS: fluorescence activated cell sorting; SCID: Severe combined immunodeficiency; CK: cytokeratin, H&E: haematoxylin and eosin.

Fig.3. Representative histological sections of original human breast tissue and intact breast tissue maintained in murine chambers (intact tissue chamber).

Original human breast tissue at 2.5x and 40x magnification, respectively (a and c). Human breast tissue maintained in murine chambers for 6 weeks, at 2.5x and 40x magnification, respectively (b & d). n= 3-6 mice/women; 5 women. Scale bar for a & b = 100µm; scale bar for c and d = 10 µm.

Fig. 4 Histological sections of intact, collagenase digested and FACS sorted breast tissue maintained in murine chambers for 6 weeks.

Intact human mammary tissue (a1&2), human mammary tissue that underwent collagenase digestion to a single cells (b1&2), and human mammary tissue that underwent collagenase digestion and then FACS (c1&2). Arrows indicate H&E stained glandular structures in a1&2, and the only gland-resembling structure from the entire biochamber explant material in b1&2 and c1&2.

Fig. 5 Representative photomicrographs of human-specific pan-CK and -14 double immunofluorescent staining.

Intact human mammary tissue (a, b, c, d), human mammary tissue that underwent collagenase digestion overnight down to a single cell level (e, f, g, h), and human mammary tissue that underwent collagenase digestion overnight followed by FACS (I, j, k, m). Blue: DAPI (a, e, i); green: CK-14 (b, g, j); red: panCK (c, g, k); merged images (d, g, m). For photomicrographs (e—m) glandular structures shown here are the only ones found from representative biochamber explant materials. Scale bar = 10 µm.

Fig. 6 Representative photomicrographs of human-specific CK-14 and CK-19 immunofluorescent staining.

CK-14 and CK-19 staining in human mammary tissue that underwent collagenase digestion overnight down to a single cell level and was grown in biochambers. A large ductal outgrowth (a-g) and small (e-h) ductal outgrowth are shown. Blue: DAPI; green: CK-14; red: CK-19; merged images (d &h). Scale bar = 10 µm.

Fig. 7 Representative photomicrographs of Ki-67 nuclear staining.

Intact human mammary tissue (a), human mammary tissue that underwent collagenase digestion overnight down to a single cell level (b) human mammary tissue that underwent collagenase digestion overnight and then flow cytometry sorting (c). Arrows indicate positive Ki-67 nuclear staining in (a), and the only gland-resembling structure from the entire biochamber explant material in (b) and (c). Scale bar = 10 µm.

Fig. 8 Representative photomicrographs of mouse macrophage (F4/80) staining.

Intact human mammary tissue extracted from murine biochambers (a), human mammary single cells recombined in murine biochambers (b), human mammary single cells sorted by flow cytometry and recombined in murine biochambers (c). Arrows indicate positive mouse macrophage staining. Scale bar = 10 µm.

Fig. 9 Analysis of Cleaved Caspase-3 staining.

Cleaved caspase staining in biochamber outgrowths. (a) Representative image from ductal structure; (b) Representative image of a mixed structure; (c) Average

percentage of caspase positive cells in each group; (d) percentage of caspase positive cells according to type of outgrowths. Data are mean +/- SEM. Short arrow: mammary duct; long arrow: a mixed structure as defined in Methods; ns: not significant; ND: no data.