Isolation and *in vitro* expansion of disseminated cancer cells from the bone marrow of a transgenic mouse model of breast cancer

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Summary

Metastasis is the major cause of death of cancer patients. Accumulating evidence suggest that metastatic dissemination is an early event. The presence of disseminated cancer cells (DCCs) in patients has been shown to increase the risk of disease recurrence. How the DCCs survive, the underlying factors or events that awaken DCCs are currently discussed and investigated and are fundamental to the understanding of cancer biology. This thesis explores the possibilities to propagate DCCs *in vitro*, and generate cell lines which could later be used for such functional studies.

At first, patient-derived prostate tumor tissue was used to identify *in vitro* conditions for propagating prostate tumor cells as spheres. Later these conditions were used to propagate bone marrow (BM)-derived DCCs from prostate cancer patients. Although the prostate tumor cells grew as spheres BM-DCCs failed to grow under these conditions. A similar observation was made in BALB-neuT mice, a breast cancer model. However, the sphere conditions for culturing mammary epithelial cells were first optimized with cytokines HIL-6 (hyper IL-6) and GRO-α (growth related oncogene α) which were shown to induce proliferation. In addition, DCC density was increased by enriching DCCs with antibodies against EpCAM, Her2 and Sca-1 (EHS). However, these conditions failed to initiate proliferation in DCCs. These results indicated that the conditions optimal for generating mammospheres from MEC were not optimal for DCC culture. Examining the effect of BM cells on primary tumor cells suggested that BM cells exerted a suppressive effect. Reduction in the sphere forming efficiency of tumor cells in medium conditioned by BM of normal or tumor bearing mice suggested a role for BM cell secreted factors in tumor cell growth suppression. On the other hand, primary tumor cells had a growth-promoting effect on BM-DCCs. Tumor cells were able to induce growth of DCC derived spheres in direct co-cultures through intercellular interactions and in transwells through secreted factors. The DCC derived spheres were tumorigenic *in vivo* and the tumor cells isolated from these tumors could further be expanded *in vitro* as cell lines. The tumors and the DCC cell lines expressed CK8/18 and Her2 and displayed an aberrant genomic profile. These cell lines could further be used to elucidate DCC biology.

1. Introduction

1.1. Metastasis and disseminated cancer cells

Metastasis is a leading cause of death in patients suffering from cancer. Cells from the primary tumor that disseminate to distant organs are thought to comprise metastatic founder cells which later develop into manifest metastasis. Studies have shown that the persistence of disseminated cancer cells (DCC) in the bone marrow of breast and prostate cancer patients predicted poor survival and an increased risk of disease relapse (Weckermann, Muller et al. 2001; Braun, Vogl et al. 2005). In a pooled study of patients diagnosed with breast cancer published recently by Janni and colleagues, 676 women diagnosed with early stages of breast cancer were followed for a median of 89 months with bone marrow (BM) aspirations performed 37 months after diagnosis (Janni, Vogl et al. 2011). The authors observed that at follow-up 15.5% (105) of total patients had DCCs and 8% of the total patients had metastasis. In patients with metastasis, 35% of them had DCCs in the BM. An increase of DCC frequency from 15.5% of total to 35% in metastatic patients suggests an enrichment of DCCs in patients with metastasis. This further suggests that DCCs contain a population of cells with a potential to metastasize and emphasize the importance of DCCs in later arising metastasis. Nevertheless, patients with DCCs in BM had a significantly shorter disease free or overall survival. A previous study on cytokeratin positive (CK+) DCCs in BM of breast cancer patients also made similar observations. (Bidard, Vincent-Salomon et al. 2008). Again the presence of DCCs correlated to poorer distant metastasis free and overall survival in the patients. Although DCCs originate from primary tumor there exists a genetic disparity between them. Studies comparing genomic aberrations between DCCs and matched primary tumors showed that DCCs were less aberrant than the primary tumors and they shared very few aberrations with them (Schmidt-Kittler, Ragg et al. 2003). Moreover, studies in both breast cancer mouse models and in human breast cancers have shown that metastatic dissemination of cancer cells happen early during tumor progression (Husemann, Geigl et al. 2008). The process of early dissemination has been suggested in a review through parallel progression model (Klein 2009). This model suggests that cancer cells disseminate early, and at the time of dissemination the DCCs carry few genetic aberrations compared to primary

tumor. The DCCs then accumulate additional aberrations, independent of the genetic evolution of the primary tumor which later forms metastasis (figure 1). This early dissemination model could explain why many patients even after resection of the primary tumor and treatment develop metastasis years after surgery. Although cancer cell dissemination is an early and frequent event, successful metastatic initiation is accomplished only by a few cells at the distant metastatic site. In BM, about 1-10 DCCs per 2 million BM cells can be detected (Klein 2009). Although this number is very low, there could be thousands of DCCs when the whole bone marrow is considered. Still, only a few manifest metastases arise from these cells suggesting that metastatic initiation in most of the cells is inefficient.

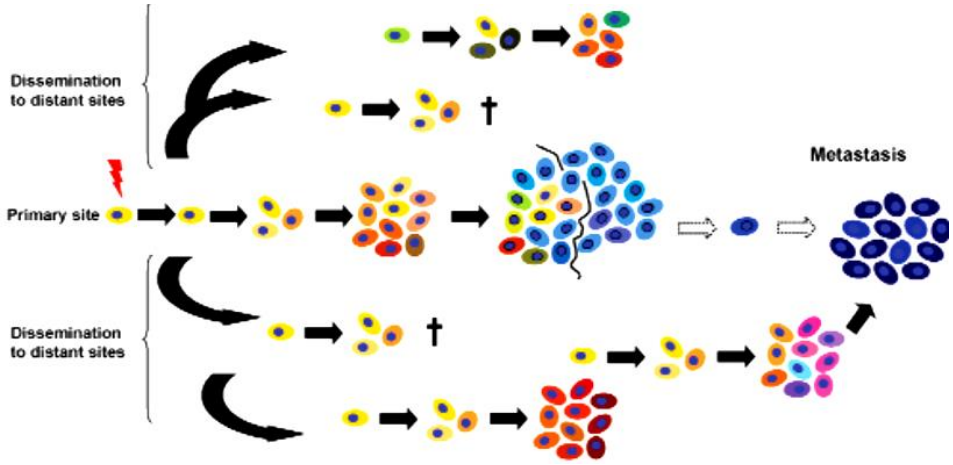


Figure 1: Models explaining cancer cell dissemination.
Parallel progression model states that metastatic dissemination occurs at an early stage during the development of cancer and evolves along with the primary tumor at distant site and later forms overt metastasis even in the absence of primary tumor (Klein 2004).

1.2. Molecular mechanisms involved in metastasis

Metastatic dissemination is a multi-step process. First, the cells in the primary tumor must attain a migratory phenotype followed by invasion of the local vasculature, extravasation at the distant metastatic sites and finally colonize and adapt to the surrounding metastatic niche microenvironment before developing into manifest metastasis (figure 2) (Nguyen, Bos et al. 2009). For prostate and breast cancers the preferred site of dissemination is bone marrow. In mouse models, it has been shown that the prostate cancer cells home to bone marrow niches which are traditionally

occupied by the hematopoietic stem cells (Shiozawa, Pedersen et al. 2011). Although the factors involved in the niche formation are still unknown, chemokine factor CXCL12 (Stromal derived factor-1) which interacts through CXCR4 and CXCR7 receptors, was shown to act as a chemo attractant guiding the DCC to bone marrow metastatic niches in breast cancer (Mukherjee and Zhao 2013). Additionally, vascular endothelial growth factor (VEGF) in the niche microenvironment has been shown to play a role in tumor cell adhesion. (Shiozawa, Pedersen et al. ; Kaplan, Riba et al. 2005; Kaplan, Rafii et al. 2006). In the bone marrow, $VEGFR1^{+}$ hematopoietic progenitor cells and osteoblasts are shown to initiate pre-metastatic niche formation (Kaplan, Riba et al. 2005; Kaplan, Psaila et al. 2007). Although the process of dissemination and the DCC niches in the bone marrow are known, the events that trigger the switch from quiescence to proliferation or the factors responsible for the activation of DCC in the distant metastatic niches years after curative surgery still remain elusive. Over the years, identifying factors responsible for the activation of DCC has become a challenge and the lack of success has hindered our understanding of these cells and events that lead to their activation. Although several chemokines (Viola, Sarukhan et al. 2012) and cytokines (Rao, Dyer et al. 2006; Culig 2011) have been shown to play a role in aiding tumor progression none were shown to have a direct impact on DCC activation.

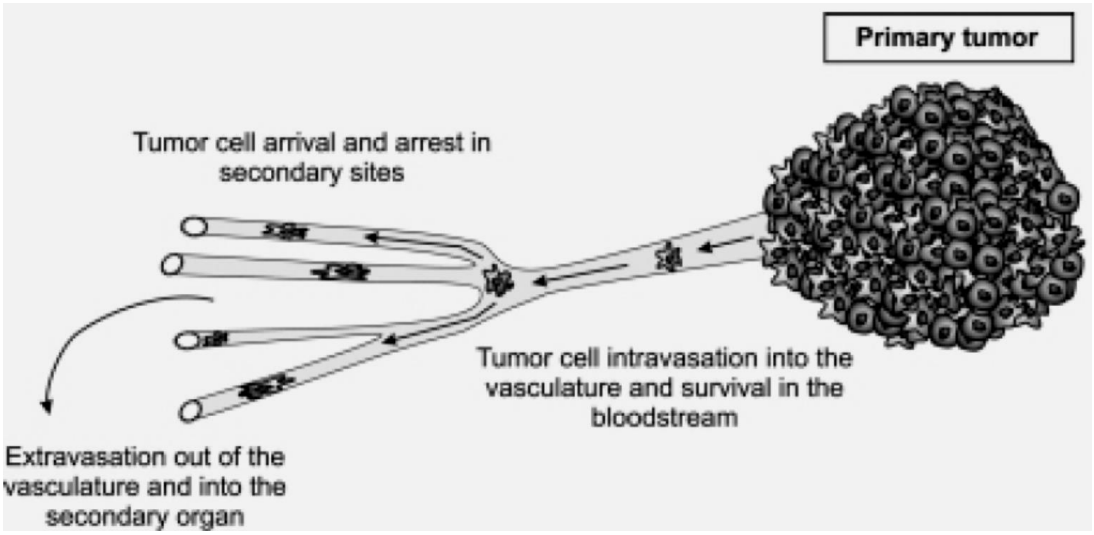


Figure 2. Process of metastatic dissemination of cancer cells.

The cells in the primary tumor first gain a migratory phenotype and later invade the local vasculature. Once they reach a distant metastatic site the cells extravasate out of the

vasculature into the secondary organ, adapt to the surrounding microenvironment of the metastatic niche and later develop into manifest metastasis. (*Figure adapted and modified from* (Allan, Vantyghem et al. 2006-2007))

1.3. Detecting circulating and disseminated cancer cells

Epithelial markers like cytokeratins and epithelial cell adhesion molecule (EpCAM) are routinely used to identify and isolate DCC but they might not be efficient in capturing all the DCCs. The property of cancer cells to disseminate from the primary tumor site to the distant metastatic site is attributed to their ability to undergo epithelial-to-mesenchymal transition (EMT) (Hollier, Evans et al. 2009). Furthermore, recent studies have indicated that during EMT these disseminating cells down regulate EpCAM suggesting that these cells escape detection methods relying on these epithelial markers (Mikolajczyk, Millar et al. 2011; Gorges, Tinhofer et al. 2012). Proliferative potential of CTC in the blood has been used as an alternative method to identify and isolate these cells on collagen adhesion matrix, in prostate cancer patients (Paris, Kobayashi et al. 2009). However, these patients already had metastasis implying that the DCCs were already activated and proliferating. It would be interesting to see if DCC from non metastatic patients also respond similarly. PCR based methods have also been employed in identifying both DCCs and CTCs in peripheral blood and BM of cancer patients. RT-PCR has been used to measure the mRNA for CK19, CK20 and mammoglobin for identifying breast cancer DCCs (Schoenfeld, Kruger et al. 1997; Berois, Varangot et al. 2000; Zhong, Kaul et al. 2000; Bossolasco, Ricci et al. 2002), PSA for prostate cancer DCCs (Halabi, Small et al. 2003) and CEA, MUC1, TIFF in addition to CK20 for gastric cancer DCCs (Dardaei, Shahsavani et al. 2011). The problem with RT-PCR based methods is the availability of DNA or mRNA in low amounts. Genomic and transcriptomic analysis of single DCCs have been established to identify tumor cells based on an aberrant genomic profile (Klein, Schmidt-Kittler et al. 1999; Klein, Seidl et al. 2002). This method overcomes the problem of low mRNA and DNA amounts encountered in the RT-PCR based methods but then, identification of DCCs is still dependant on epithelial markers (cytokeratins and EpCAM). However, another problem with PCR based methods is that the functional properties of the isolated DCCs cannot be evaluated. Since, only a few DCCs are capable of initiating metastasis analyzing their

functional capabilities is also important. Therefore, an *in vitro* model to study DCCs is essential.

1.4. *In vitro* culture of disseminated cancer cells

It has been a challenge over the years to propagate DCCs *in vitro.* This could be due to their presence in low numbers or because the conditions to propagate DCCs *in vitro* are still unknown. However, there have been made attempts to culture DCCs *in vitro*. In the studies published by Pantel and colleagues, cell lines were generated from DCCs isolated from the bone marrow of prostate, breast, colon esophageal and lung cancer patients. However, the possibility of generating DCC cell lines was achieved only after immortalizing the surviving DCCs with SV40 large T antigen (Pantel, Dickmanns et al. 1995; Putz, Witter et al. 1999; Hosch, Kraus et al. 2000). SV40 large T antigen is known to interfere with the general cellular process of a cell. It interacts with several cellular pathways to transform and immortalize the cells. (Ahuja, Saenz-Robles et al. 2005). These cell lines, as the author states, were not assessed for any collateral effects caused by SV40 large T antigen induced transformation. SV40 large T antigen is shown to bind to the tumor suppressor proteins retinoblastoma family of tumor suppressors and p53 to transform the cells (Ahuja, Saenz-Robles et al. 2005). Therefore, the possibility of results from these cell lines in various experiments could be mediated via large T antigen and may be different to normal cells which do not contain the large T antigen. Thus, it is important to expand DCC *in vitro* without any induced transformation and immortalization.

DCCs from prostate cancer were shown to occupy hematopoietic stem cell niches in the bone marrow (Shiozawa, Pedersen et al. 2011). Moreover, recently in breast cancer also it has been shown that DCCs reside in perivascular niches (Ghajar, Peinado et al. 2013) and perivascular niches are shown to maintain hematopoietic stem cells (Ding, Saunders et al. 2012). This could suggest that the DCCs might share some similarities with the stem cells and therefore, may require different conditions to propagate them *in vitro.* In the last decade, *in vitro* culture techniques have been developed to culture stem/progenitor cells. In breast cancer, it has been shown that suspension or non adherent cultures enrich for stem/progenitor cells, grown as spheres called mammospheres (Dontu, Abdallah et al. 2003). Therefore, it

would be worthwhile to test these stem/progenitor enriching *in vitro* culture conditions and see if the bone marrow derived DCCs proliferate in these conditions.

1.5. The BALB-neuT mouse model

Since, patient BM derived DCCs are very rare and variable about 1-10 per 2 million BM cells (Riethdorf, Wikman et al. 2008), we looked at mouse models where DCCs have been reported to be lodging in the BM at a higher frequency. BALB-neuT is a transgenic mouse which develops breast cancers and is a commonly used mouse model to study breast cancer. Integrated into the genome of the mouse the construct consists of a MMTV LTR (Mouse mammary tumor virus, long terminal repeats) region which is transcriptionally active, the neuT oncogene and SV40 polyA recognition sequence (Muller, Sinn et al. 1988). The origin of the oncogene neuT is from the rat and is the corresponding homologue of human Her2. This gene contains a point mutation, at position 664 of the amino acid sequence valine is replaced with glutamine which induces neuroblastoma in the rat. This mutation is in the transmembrane region of the receptor and results in more than 100-fold increase in the receptor kinase activity. Moreover this mutant protein is phosphorylated to a higher degree when compared to the wild type protein (Bargmann and Weinberg 1988). The MMTV promoter responds to estrogen and progesterone and is activated at the onset of puberty in female mice. Disseminated cancer cells can be identified in the bone marrow of BALB-neuT by staining the bone marrow cells with cytokeratin 8/18 (CK8/18), a marker for epithelial cells. The DCCs in the bone marrow of the mice can be identified as early as week 4. The number of DCCs in the bone marrow of this mouse is between 4-20 cells per million bone marrow cells. DCCs were detected in the bone marrow even before tumor became palpable. In the transplanted mouse model, where the recipient wild type mice (BALB/c) received orthotropic transplants from their 3-4 week old transgenic siblings (BALB-neuT), DCCs were also detected. However, the number of DCCs detected in the transplanted mice in comparison to the BALB-neuT was lower. The reason could be due to the presence of 10 mammary glands in the transgenic mouse against 1 gland from the transgenic mouse in the wild type recipient (Husemann, Geigl et al. 2008). Thus, BALB-neuT breast cancer mouse model seemed appropriate for studying DCCs in the current doctoral project.

Aim of the project

Disseminated cancer cells have been difficult to culture *in vitro*. Hence, the knowledge about these cells is limited. Although their persistence in patients even after surgery is associated with high risk of relapse, the reasons for their activation and the factors responsible for their activation is not known. The problems in evaluating DCCs are that they are rare, there are no reliable markers for identifying them and last but certainly not the least there are no *in vitro* models to elucidate them functionally. Therefore, knowing the conditions to expand them *in vitro* and availability of *in vitro* models are critical in further evaluating DCCs.

The aim of this project was to first identify conditions to propagate bone marrow derived DCC *in vitro*, then analyze the functional and molecular characteristics of the activated DCCs and finally expand them *in vitro* to generate cell lines. These cell lines could then serve as models for further understanding DCC biology.

2. Methods

2.1. Mouse experiments

All mouse experiments were performed at the animal facility of the University of Regensburg. The application to work with BALB/c and BALB-neuT mice for the research projects was approved by the Government of Upper Bavaria (Az.209.1/211-2531-108/04).

2.2. Cell culture methods

2.2.1. Processing patient bone marrow

After obtaining informed consent from the patient, bone marrow aspirates were washed with Hanks buffer salt solution (HBSS) and centrifuged at 200xg for 10 min to remove fat and thrombocytes. The supernatant was discarded and cell pellet was resuspended in 9 ml of HBSS and slowly added on top of percoll 60% (6 ml) and centrifuged at 1000xg for 20 min, to remove erythrocytes and granulocytes. The interphase was transferred to a new 50 ml tube. The tube was filled with PBS and centrifuged at 500xg for 10 min. The pellet was resuspended in 2-10 ml PBS depending on the size of the pellet. Erythrocytes and leukocytes in the percoll fraction were counted. The cells were centrifuged at 500xg for 10 min, the pellet was resuspended in MACS buffer (90 µl/10^7 cells) and incubated with anti-CD11b (10 µl/10^7 leukocytes), anti-CD33 (5 µl/10^7 leukocytes) and anti-CD45 (5 µl/10^7 leukocytes) antibody conjugated to anti-APC on a roller for 15 min at 4° C. The cells were washed by adding 10 volumes of MACS buffer and centrifuged at 500xg for 5 min at 4° C. The supernatant was removed and the pellet was resuspended in MACS buffer (60 µl/10^7 cells) and incubated with anti-APC-beads (20 µl/10^7 leukocytes) and anti-Gly A beads (20 µl/10^7 erythrocytes) for 15 min on a roller at 4° C. The cells were washed with 10 volumes of MACS buffer and centrifuged for 5 min at 500xg. The pellet was resuspended in 2 ml of MACS buffer, filtered through 40 µm mesh to remove cell clusters and then loaded onto the magnetic column already equilibrated with 3 ml MACS buffer. The tube was washed with 9 ml MACS buffer which was run

over the column in aliquots of 3 ml. The cells in the flow-through (11 ml) were counted.

2.2.2. Culturing bone marrow DCC of patients

After magnetic sorting, 200000 cells/ml were seeded in sphere medium (see materials, Media for prostate sphere medium) in a 6 cm dish and incubated for 4 weeks at 37° C, 5.5% CO_2 and 7% O_2. The culture dishes were replenished with 1 ml of fresh medium every week until the cultures were terminated. The bone marrow culture dishes were screened every week for spheres.

2.2.3. Mammary tissue digestion and epithelial cell isolation

The mice were killed by asphyxiation with CO_2 or by cervical dislocation. Mammary glands were collected either from BALB/c or BALB-neuT in a 50 ml tube with PBS. The tissue was minced with surgical blades to small pieces and digested in basal medium (DMEM/F12, 100 nM HEPES buffer, 10 mg/ml insulin, 0.5% BSA and 0.5x penicillin/streptomycin) with 200 U/ml collagenase, 10 ng/ml EGF and 200 U/ml hyaluronidase. The tissue was digested for 2-3 hours at 37° C in the incubator mixed every 1 hour. The digested tissue was transferred to a 50 ml tube. After a first centrifugation step of 1 min at 80xg to isolate the organoids the supernatant was collected into a new 50 ml tube and centrifuged at 200xg for 3 min to isolate mammary epithelial cells (MEC). Subsequently, the supernatant was transferred into a new 50 ml tube and centrifuged at 350xg for 4 min to isolate fibroblasts (figure 3). The organoids were either frozen in a freezing medium containing 10% DMSO and 50% FCS in DMEM/F12 or further digested to isolate single cells.

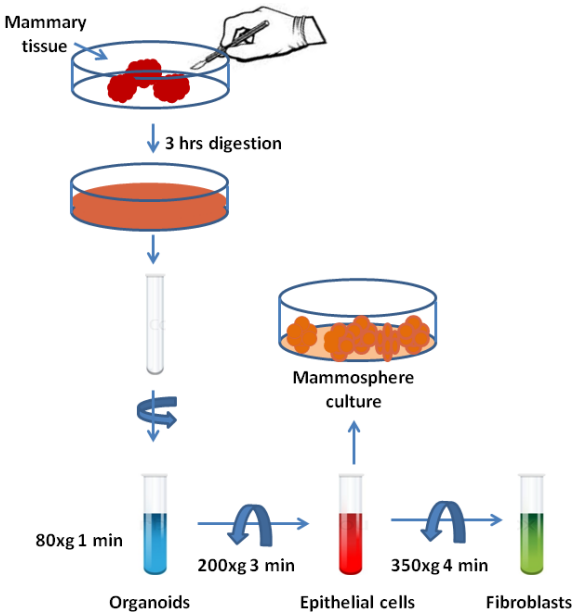


Figure 3: Schematic representation of epithelial cell isolation from murine mammary glands.

The mammary tissue is minced with a scalpel to small pieces, enzymatically digested and afterwards epithelial cells are isolated by differential centrifugation at 200xg for 3 min. The isolated epithelial cells were later cultured in non-adherent serum-free conditions to generate mammospheres.

2.2.4. Preparation of single cells from organoids

Freshly isolated or frozen organoids were used for isolation of epithelial cells. If frozen, the organoids were first thawed and transferred into 15 ml tube with 4 ml of pre-warmed basal medium. The tube was centrifuged at 40xg for 2 min at room temperature (RT) and the supernatant was discarded. Fresh or thawed organoids were resuspended in 3 ml of trypsin-EDTA and incubated for 1 min at RT. The suspension was carefully and slowly mixed for 2 min with a 5 ml pipette along the tube wall avoiding formation of bubbles. Then, 5 ml of trypsin neutralizing solution (TNS) were added, incubated for 1 min and centrifuged at 300xg for 3 min at RT. The supernatant was discarded and the pellet was resuspended in 3 ml of freshly prepared dispase (5 mg/ml) and 200 µl of 1 mg/ml DNase solution. The suspension was mixed using a 5 ml pipette along the tube wall for 2 min. Additional 200 µl of DNase solution was added, if the solution was thick suggesting release of DNA, and mixed for another 1 min. The suspension was filtered through a 40 µm mesh to remove any large cell clusters. The flow-through was centrifuged at 300xg for 3 min

at RT. After washing in 5 ml of 1x PBS the pellet was resuspended in 2-5 ml of PBS (depending on the pellet size) and viable cells were counted on a Neubar chamber.

2.2.5. Isolating cells from murine bone marrow

Tibias and femurs were collected either from BALB/c or BALB-neuT in PBS or basal medium. The soft tissue surrounding the bones was removed. One of the bone ends was cut with scissors to expose the bone marrow. Using a 26G needle and 1 ml syringe the bone marrow was flushed out into a 50 ml tube. Alternatively, for some experiments the flushed bones were put into a mortar to break the bones using mortar and pestle. The broken bones were washed with 1x PBS to collect the remaining bone marrow cells after flushing. Afterwards, the cells were pooled and centrifuged at 200xg for 10 min. The supernatant was discarded and the pellet was resuspended in 9 ml of 1x PBS. The cell suspension was slowly and carefully overlaid onto 6ml of 65% percoll in a 15 ml tube to form a layer and centrifuged for 20 min at 1000xg to remove erythrocytes. The interphase was then carefully collected using a 5 ml pipette and transferred into a new 50 ml tube. The tube was filled up with 1x PBS and centrifuged for 10 min at 500xg for washing the cells. The cells were resuspended in 5 ml of 1x PBS and counted.

2.2.6. Enrichment of bone marrow DCC

The bone marrow cells were enriched for EpCAM, Her2 and Sca-1 (EHS) after density centrifugation. After counting, the cells were centrifuged for 10 min at 400xg. Supernatant was discarded and the cell pellet was resuspended in MACS buffer (100 µl / 10^7 cells). Primary antibodies anti-EpCAM-bio (10 µg/ml), anti-Her2 (10 µg/ml) and anti-Sca-1-FITC (10 µl/10^7 cells) were added and incubated for 15 min at 4° C on a roller. After incubation, 10 volumes of MACS buffer were added and centrifuged for 10 min at 400xg. Supernatant was discarded and the pellet was resuspended in 4 volumes of MACS buffer containing anti-biotin, anti-IgG2a+b and anti-FITC magnetic microbeads and incubated for 15 min at 4° C on a roller. After incubation 10 volumes of MACS buffer was added and centrifuged for 10 min at 400xg. The procedure for sorting the cells on the columns is as explained in 2.2.1. All cells bound by magnetic

microbeads stick to the magnetic column and the rest flow though the column. After carefully transferring the column with positive fraction to a 15 ml tube, 5 ml of MACS buffer were added to the column and cells were flushed out into the tube. The negative fraction was again run through the column and the positive fraction collected in the tube with the 1st positive fraction.

2.2.7. Sphere forming assay (SFA)

Epithelial cells isolated from digested murine mammary glands or tumors were seeded under non adherent conditions in poly-HEMA-coated plates to generate spheres. The cells were seeded at a density of 50000 cells/ml for co-cultures in sphere medium (SM), with 10 ng/ml EGF, 10 ng/ml bFGF, 4 µg/ml heparin, 20 ng/ml hyper-IL-6, 5 ng/ml GRO-α and 1x B27 in basal medium. In experiments involving optimization or for studying the effect of conditioned medium on cells, the cells were seeded at a density of 10000 cells/ml. For control experiments only EGF, bFGF and heparin were added to the basal medium. The culture plates were incubated in a cell culture incubator at 37° C in atmosphere containing 5.5% CO_2 and 7% O_2.

2.2.8. Preparation of poly-HEMA and low attachment plates

To prepare coating solution, 2.4 g of poly-hydroxy-ethyl-methyl-acrylate (Poly-HEMA) were added to 20 ml of 95% ethanol and put on a shaker at 65° C to dissolve for ~8 hours. To the above dissolved poly-HEMA, 80 ml of 95% ethanol was added and mixed. The prepared solution could be stored indefinitely at 4° C. Poly-HEMA solution was added to the cell culture dishes at a final density of 0.8 mg/cm (w/v). The plates were dried overnight in a sterile hood with lids slightly opened. The poly-HEMA-coated plates can be stored indefinitely until use at +4° C.

2.2.9. Methylcellulose preparation

Medium with methylcellulose (MC) was prepared to culture bone marrow cells *in vitro*. Methocel was used as a thickening agent in sphere cultures to limit the lateral

movement and thereby aggregation of cells. A stock solution of 1% MC was prepared and diluted to 0.2% in sphere medium for culturing bone marrow cells. Briefly, 1 g of MC was added to 20 ml of boiling basal medium and mixed on a magnetic stirrer for 2 hours at 95° C. Eighty ml of cold basal medium was added and allowed to dissolve overnight at 4° C on a magnetic stirrer. The medium was collected into an ultracentrifuge tube and centrifuged for 30 min at 7500xg to remove any undissolved MC. The medium was transferred into a 50 ml tube leaving the pellet at stored at 4° C. The medium with MC is stable for 4 weeks.

2.2.10. General cell membrane labeling with PKH26 and CellVue®

PKH26 (PKH) and CellVue (CV) are general cell membrane labeling dyes. PKH26 is visible under Cy3 and CV under Cy5 filter. Cells prepared from BM, MEC or cell lines were centrifuged at 400xg for 5 min in a 50 ml tube. The cells were washed once in 1x PBS or serum free medium to remove any traces of serum. The supernatant was discarded and the cell pellet was resuspended in 1 ml Diluent C. Immediately, before staining 1 µl of PKH or 2 µl of CV were added to 999 µl or 998 µl of Diluent C solution respectively. The dye solution was added to the cell suspension, mixed gently and incubated for 5 min. The staining reaction was stopped by adding 2 ml of 20% BSA for 1 min and centrifuged at 400xg for 10 min. After discarding the supernatant, the pellet was resuspended in medium containing BSA, transferred to a new 50 ml tube and centrifuged for 10 min at 400xg. The cells were washed 3 times with BSA containing medium to remove unbound dye and counted on a Neubauer chamber.

2.2.11. Co-culture

Mammary epithelial cells (MEC) isolated from BALB/c or BALB-neuT were cultured with BALB-neuT bone marrow cells enriched for EHS (see 2.2.6). The mammary epithelial cells were stained with CellVue and EHS-fraction with PKH26 (see 2.2.10). The cells were seeded in a poly-HEMA-coated 6 cm dish in 5 ml sphere medium. MECs were seeded at a density of 50000 cells/ml together with 200000 cells/ml of EHS and incubated at 37° C, 5.5% CO_2 and 7% O_2. The cultures were replenished with fresh medium every week until they were terminated.

2.2.12. Transwell assay

Transwell plates are cell culture dishes with a transwell insert. The insert has a microporous (0.4 µm) membrane which separates the dish in to upper (UC) and lower compartments (LC). The microporous membrane allows only soluble factors to pass through between the compartments (figure 4). In the UC 50000 cells/ml of MECs or 10000 cells/ml of cell lines were seeded. The cells in the UC were stained with PKH26. In the lower chamber 100000 cells/ml EHS cells were seeded in sphere medium containing 0.2% methylcellulose. The medium was changed once every week for the first two weeks and after the second week the medium was changed every third day. For screening or changing medium, the upper chamber was carefully removed and placed in a new petri dish with 5 ml basal medium. After screening or changing medium the upper chamber was carefully placed back in the transwell dish. This was done to avoid any spillage of cells in the upper chamber while transporting the plates to the microscope or while changing the medium in the upper chambers.

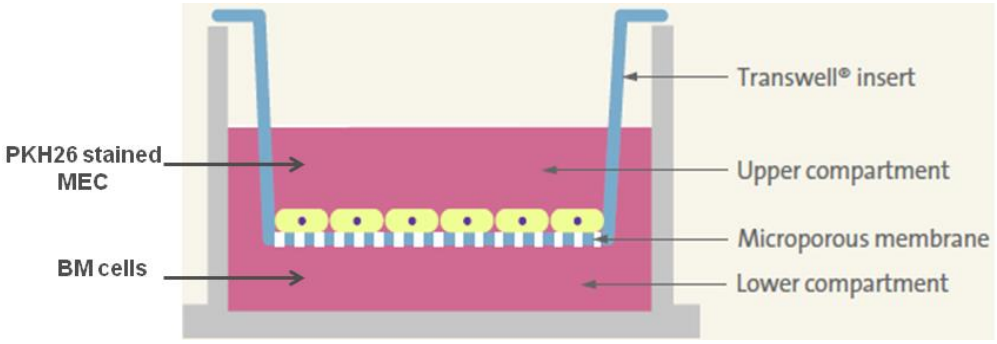


Figure 4: Transwell assay experimental setup

The transwell assay is divided into an upper and a lower compartment by a microporous membrane. Transwells can be used to study the effect of two different cell types on each other. The pore size of the membrane used for the experiments in this study is 0.4 µm which allows only soluble factors to pass through the membrane. The upper chamber contains primary mammary cells or cell lines stained with PKH26 and the lower chamber contains BM cells enriched for EHS. MEC: Mammary epithelial cells.

2.2.13. Conditioned medium preparation

After density centrifugation, 200000 cells/ml of bone marrow cells from BALB/c, BALB-neuT or NSG mice were seeded in basal medium supplemented with EGF (5 ng/ml), bFGF (5 ng/ml), heparin (4 µg/ml) and B27. After 3 days the cultures were

collected in a 50 ml tube and centrifuged for 10 min at 1000xg to pellet the cells. The supernatant was sterile filtered through a 0.22 µm filter to remove any cells in the conditioned medium. The medium was used immediately or stored at -80° C until further use.

2.3. Molecular methods

2.3.1. Immunocytochemical staining: Her2 staining on adhesion slides

Bone marrow (BM) cells on the adhesion slides were taken out of -20° C and thawed for 20 min. Blocking solution (5% mouse serum in 1x TBS) was added to the slides to rehydrate the cells and to block unspecific binding of antibodies to the cells. After 20 min the blocking solution was discarded and primary antibody against Her2 (table 1) was added and incubated for 60 min. The primary antibody was discarded and the slides were washed 3 times for 3 min in 1x TBS. The slides were incubated with the secondary antibody (table 1) for 25 min and washed 3 times for 3 min each in 1x TBS followed by incubation with avidin-biotin complex (ABC) coupled with alkaline phosphatase (AB/AP system) for 25 min. Finally, the development system of the BCIP/NBT for alkaline phosphatase enzymatic substrate was added for 10 min. The slides were washed 3 times for 3 min and screened for Her2 positive cells. The positive cells were typically violet-to-black in color. TUBO, a tumor cell line derived from a murine primary mammary tumor of BALB-neuT which expresses Her2, was used as a positive control. For antibodies and concentrations used, please refer to table 1.

2.3.2. Cytokeratin 8/18 staining on adhesion slides

The procedure for CK8/18 staining of cells on adhesion slides is as explained in 2.3.1, except for the antibodies (table 1). TUBO cells express CK8/18 and were used as a positive control.

Table 1: List of antibodies used for immunocyto- and immunohisto chemical staining against CK8/18 and Her2.

Antigen	Against	Conjugate	Source	Isotype	Conc.	Product code
c-erbB-2 (Her2-neu)	Mouse,Rat, Human		Rabbit	IgG	5 µg/ml	29D8
IgG	Rabbit	Biotin	Goat	IgG	5.5 µg/ml	D-111-065-144
Cytokeratin 8/18	Mouse,Rat, Human		Guinea pig	IgG	1:200	GP11
IgG	Guinea pig	Biotin	Goat	IgG	1.6 µg/ml	D-106-065-003

2.3.3. Haematoxylin and eosin staining of paraffin embedded tumor tissue

Paraffin embedded tumor tissue was cut into 5 µm sections onto a slide. The sections were deparfinized in Xylol for 10 min twice and rehydrated first in 100% ethanol for 3 min and then in 80% ethanol for 3 min. The sections were washed in 1x PBS for 1 min thrice and incubated with hematoxylin for 45 sec. The slides were rinsed in tap water for a short duration and washed in tap water for 30 min in a glass cuvette. Eosin (0.1%)was added to the tissue sections and after 2 min they were washed with ddH_2O for 1 min. The stained sections were dehydrated in 70% ethanol for 2 min, 100% ethanol for 2 min and finally in xylol for 15 min. Mounting gel was added to the tissue sections and a cover slip was placed carefully on the gel avoiding bubbles and were left to dry overnight.

2.3.4. Immunohistochemical staining of paraffin embedded tumor tissue

Paraffin tissue sections were stained for CK 8/18 or Her2. Briefly, the paraffin slides were rehydrated as explained in 2.3.3. Antigen retrieval was performed by boiling the slides in microwave oven for 2 min and cooling for 2-3 min at RT and was repeated 5 times. After the slides were washed 3 times in 1x PBS for 1 min, they were incubated in 3% H_2O_2 and methanol for 30 min at RT to neutralize endogenous peroxidase activity. After washing thrice in 1x PBS for 1 min, tissue sections were blocked with 5% goat serum for 30 min. The sections were incubated with primary antibody (table

1) for 60 min and washed with 1x PBS 3 times for 1 min. After incubating with secondary Ab for 30 min, the slides were washed in 1x PBS once for 3 min and incubated with ABC-HRP for 30 min. The ABC-HRP was washed with 1x PBS 3 times for 3 min, DAB development system was added and incubated for 5 min. The slides were first washed 3 times in PBS, once in ddH_2O for 2 min and counter-stained with haematoxylin for 3 mins. Haematoxylin was discarded and washed for 2 min first with ddH_2O, then with tap water and again with ddH_2O. The slides were dehydrated gradually in 80% and 100% ethanol for 3 min each and in xylol for 10 mins. Mounting gel was added to the slides and was left overnight to dry.

2.3.5. Isolation and amplification of mRNA from single spheres

The mammospheres from NMEC and TMEC cultures were picked with a 2 µl pipette tip in 1 µl volume of the medium using a microscope. For co-cultures, single PKH positive spheres were picked. The isolated spheres were added to a 4 µl lysis buffer with 0.4 µl tRNA and stored at -20° C.

Isolation and amplification of mRNA was performed as published. (Hartmann and Klein 2006).

2.3.6. Preparation of genomic DNA along with mRNA

Using *Ampl1*™ kit from Silicon Biosystems, genomic DNA was prepared from the supernatants collected during mRNA isolation from single cells.

2.3.7. PCR

To identify samples carrying the rat Her2 transgene, PCRs were performed on the genomic DNA isolated from spheres, tumor cells or cell lines. Transgene specific primers were added to the PCR reaction mixture along with the DNA to amplify the rat Her2 transgene.

The contents of the PCR reaction were as follows:

Reagents	Volume
10 x PCR-Puffer	1 µl
Primer 5' (100 mM)	0,5 µl
Primer 3' (100 mM)	0,5 µl
BSA (20mg/ml)	0,25 µl
Taq-Polymerase (5 U/µl)	0,1 µl
H2O	7,25 µl
DNA	0,5 µl

Primers used for amplification of Her2 transgene were:

Gene	5'-Primer	3'-Primer	PCR Band	Annealing temp
Transgen (MMTVHer2/neu)	GTA ACA CAG GCA GAT GTA GG	ATC GGT GAT GTC GGC GAT AT	220 bp	58º C
Her2 (Her2 GG)	GAG ACG CTC AAC CTC AA	TTG GGC ACT TGT TGG TCT GTA G	180 bp	58º C

2.3.8. Array CGH data analysis

The array-CGH (aCGH) was performed on Agilent microarrays and the data was analyzed using Agilent Workbench 6.5®. The aberration algorithm used was ADM-2.

2.3.8.1. Labeling of sample DNA (preparation for array CGH procedure)

Test and reference DNA were labeled with SureTag DNA Labeling Kit (Agilent) according to the manufacturer's instructions. Briefly, 1.5 - 2.0 µg of purified input DNA (WGA product of unamplified genomic DNA) was supplemented with 5 µl of Random Primer Mix and filled up with H_2O to 31 µl. Samples were denatured at 95°C for 10 or 3 min in the case of unamplified DNA and WGA products, respectively. Sample tubes were transferred to ice and incubated for 5 min. The labeling reaction with exo-Klenow fragment consisted of: 31 µl of denatured template DNA, 10 µl of 5x

reaction buffer, 5.0 µl of 10x dNTP mix, 3.0 µl of Cy5-dUTP (test) or Cy3-dUTP (reference) and 1.0 µl of Exo(-) Klenow fragment. For all the experiments gender-matched test and reference samples were used. The labeling reaction was run at 37° C for two hours followed by inactivation at 65°C for 10 min.

Labeled DNA was purified using Ultra 0.5 purification system (30 kDa – cut-off). Briefly, DNA samples were supplemented with water to a total volume of 480 µl and applied on the purification column. Samples were centrifuged for 10 min at 14000xg. Following removal of the flow-through 480 µl of H_2O was applied on the column and samples were centrifuged again (10 min, 14000xg). Spin columns were removed from the spin column assembly, inverted and placed in fresh collection tube. DNA was collected by centrifugation for 1 min at 1000xg at room temperature. The quantity of purified DNA was assessed using Nano Drop ND-1000 instrument.

2.3.8.2. Array comparative genomic hybridization

Array CGH was performed on oligonucleotide-based SurePrint G3 Mouse CGH 4x180K microarray slides (design code: 027411) according to the protocol provided by the manufacturer (Agilent Technologies) with slight modifications. Hybridization mix was comprised of 5.0 µg of Mouse Cot1-DNA (Invitrogen), 11 µl of 10x Blocking Reagent (Agilent Technologies) and 55 µl of 2x Hi RPM hybridization buffer. The hybridization mix was mixed with 19 µl of test and reference DNA and applied on the array. Hybridization was carried out at 65° C for 24 h after which, they were washed twice for 2:30 min in Oligo aCGH/ChIP-on-Chip wash buffer 1 at room temperature, twice for 30 sec in Oligo aCGH/ChIP-on-Chip Wash Buffer 2 at 37° C. Washed slides were immersed in acetonitrile to remove all remaining traces of the wash buffers and were later scanned using an Agilent Microarray Scanner Type C.

2.3.8.3. Processing and analysis of the aCGH data

Microarray TIFF files were processed and quantified with the Agilent Genomic Feature Extraction Software (version 10.7). The resulting SHP files were imported and analyzed with Agilent Genomic Workbench Software (version 6.5 Lite or 7.0).

Aberrant regions were recognized using ADM-2 algorithm with a threshold set to 6.5. To avoid detection of false positive aberration calls, aberration filters were applied to define the minimum log2 ratio (0.2 and 0.3 for unamplified DNA and WGA products, respectively) and the minimum number of probes in the aberrant interval (5 and 10 for unamplified DNA and WGA products, respectively).

2.3.8.4. Cluster analysis

Cluster analysis was performed using Euclidean distance based on the aCGH data of the cell lines. This clustering feature was provided in the Workbench 6.5® by Agilent.

2.3.9. Genomic DNA preparation

The genomic DNA was isolated using Qiagen QIAmp DNA mini kit according to the manufacturer's instructions.

Materials:

Antibodies

Reagents	Manufacturer
29D8 (anti-Neu/-HER-2)	Cell signaling
BA-9500 (anti-goat-IgG; Biotin)	Vector Laboratories
D-106-065-003 (anti-goat-IgG; Biotin)	Dianova
D-106-165-003 (anti-goat-IgG; Cy3)	Dianova
D-111-065-144 (anti-rabbit-IgG; Biotin)	Dianova
DLN-12082 (anti-c-erbB-2/-HER-2/-neu)	Dianova
G8.8-Bio (anti-EpCAM; Biotin)	Biolegend
GP11 (anti-cytokeratin 8/18)	Progen
Microbeads (anti-IgG2a+b)	Miltenyi Biotech
Microbeads (anti-biotin)	Miltenyi Biotech
Microbeads (anti-FITC)	Miltenyi Biotech
Sca-1- (anti-mouse Sca-1:FITC)	Miltenyi Biotech

Buffers and solutions

Solutions	Composition
20x SSC (Saline–Sodium Citrate)	0,3 M sodium citrate
	3 M NaCl
	70 % ethanol
	70 ml 100% ethanol
	30 ml water
	85 % ethanol l
	85 ml 100% ethanol
	15 ml Water
Hematoxylin	2 g haematoxylin
	0,4 g sodium iodate
	100 g aluminium potassium sulfate
	100 g chloralhydrate
	2 g citric acid
Differentiative epitthelial medium	RPMI medium without L-glutamine
	10 % FCS
	200 U/mL penicillin
	200 U/mL streptomycin
	2 mM L-glutamine
PBS (Phosphate Buffer saline)	8,5 mM Na_2HPO_4
	2 mM KH_2PO_4 NaCl
	150 mM NaCl
	pH 7,4
PCR-buffer + dNTPs	100 mM Tris-HCl
	500 mM KCl
	10 mM $MgCl_2$
	1 mM of each nucleotide
TBE-buffer, pH 8,3	89 mM TRIS
	89 mM boric acid
	2 mM $EDTANa_2$
TE-Puffer, pH 7,4	10 mM TRIS-HCl
	1 mM EDTA

Cell culture media

Solutions	Composition	Final conc
Basal medium	500 ml DMEM/F12	
	5 ml HEPES buffer	100 nM
	5 mlPen/Strep	1x
	BSA	0.5%
	500 µl Insulin	5 µg/ml
Sphere medium (primary culture)	48.8 ml basal medium	
	1 ml B27	1x
	50 µl EGF	10 ng/ml
	50 µl heparin	4 µg/ml
	20 µl bFGF	10 ng/ml
	25 µl GRO-α	5 ng/ml
	10 µl HIL-6	20 ng/ml
Sphere medium (BM transwell)	38.8 ml basal medium	
	10 ml 1% metthylcellulose	0.2%
	1 ml B27	1x
	50 µl EGF	10 ng/ml
	50 µl heparin	4 µg/ml
	20 µl bFGF	10 ng/ml
	25 µl GRO-α	5 ng/ml
	10 µl HIL-6	20 ng/ml
Sphere medium (cell lines)	48.8 ml basal medium	
	1 ml B27	1x
	50 µl EGF	10 ng/ml
	50 µl heparin	4 µg/ml
	20 µl bFGF	10 ng/ml
TUBO medium	to 500 ml DMEM	
	100 ml FCS	20%
	5 ml 2mM L-glutamine	1x
C3HT10$^{1/2}$ medium	to 500 ml DMEM	
	25 ml FCS	5%
	5 ml 2mM L-glutamine	1x
DCC cell line medium	500 ml RPMI 1640	

	50 ml FCS	10%
	5 ml 2mM L-glutamine	1x
	5 ml Pen/Strep	1x

Cell lines

Material	Origin
TUBO	Cell line from BALB-neuT primary tumor.
C3HT10$^{1/2}$	mouse embryonic fibroblasts (received from Prof. John Stingl, Cambridge University, Cambridge, UK)
Immortalised fibroblast	mouse fibroblasts (received from Prof. Robert Weinberg, Whitehead Institute, MA, USA)
NIH3T3	mouse embryonic fibroblasts

Consumables

Product	Manufacturer	Catalog#
Adhesion slides	Roth	H8701
Filter (0.22 µm)	Roth	P666.1
Syringe needles (26G)	Braun	C7181
Cell culture plates	Schubert and Weiss, OMNILAB	FALC351007
Cell culture flasks	Sarsted	831.810.302
Glass slides	Langenbrinc	03-0001
6 well plates	Greine	657160
Reaction tubes 1,5 ml	CLN Gmb	CLN-BÖT1.5
Reaction tubes 0,2 ml	Abgen	AB-0337
Transwell plates (10 cm)	Cornin	3419

Enzyme, growth factors and Development system

Product	Manufacturer	Catalog #
2-Log DNA-Ladder 1kb	NEB	N32005
AB/AP-System	DAKO	AK-5000
AB/HRP-System	DAKO	PK-4000

AB-Serum	Bio-Rad	805315
Agarose	Sigma-Aldrich	A3038
B27	Invitrogen	17504-44
BCIP/NBT	BioRad	1706432
bFGF	Sigma-Aldrich	F0291
BSA	Roche	1071145001
CellVue®	Sigma-Aldrich,	MINICLATER-1KT
Collagenase Type I	CellSystems biotech	LS4196
DAB	DAKO	K3468
DEPC-H_2O	Invitrogen	750023
Diluent C	Sigma-Aldrich	CGDIL-6x10 ml
Dispase	Invitrogen	D4818
DMEM	Pan-Biotech	P04-03500
DMEM/F12	Pan-Biotech	P04-41500
EGF	Sigma-Aldrich	E9644
Eosin	Sigma-Aldrich	54802
Ethanol absolute	J.T. Baker	8006
Ethidium bromide (1 %)	Sigma-Aldrich	E8751
Eukitt (Mounting gel)	Sigma-Aldrich	03989-100ML
Expand Long Template Enzyme Mix	Roche	11 759 060 001
FCS	PAN Biotech	P30-3702
Goat serum	DAKO	X0907
GRO-α	RnDsystems	275GR
Hematoxylin	Sigma-Aldrich	MHS16
Heparin	Sigma-Aldrich	H3149
Hyaluronidase Type IV-S	Sigma-Aldrich	H4272
Igepal	Sigma-Aldrich	I3021-50ML
Insulin	Sigma-Aldrich	I9278
Isopropanol	Fluka	59300-2.5ML
Levamasol	Sigma-Aldrich	L9756
L-Glutamin (200 mM)	Pan-Biotech	P04-80100
Matrigel	BD Biosciences	356231
Methanol	VW	1.06009.2500

Methyl cellulose	Sigma-Aldrich	M0512-100G
Mouse serum	DAKO	X0910
Mse I 50 U/µl	New England Biolabs	R0525M
OPA Plus	Amersham Biosciences,	27-0901-02
Panscript Taq-Polymerase	PAN Biotec	MB-30010250
Paraformaldehyde	VW	104005
Penicillin/Streptomycin (10 U/µL)	Pan-Biotech	P1-010
Percolll	Amersham Biosciences	17089102
PKH26	Sigma-Aldrich	MINI26-1KT
Polyhema	Sigma-Aldrich	P3932
Proteinase K	Roche	03115828001
RPMI 1640	Pan-Biotech	P05-17500
Sodium citrate	Sigma-Aldrich	73894-100ML
T4 DNA Ligase	Roche	10799009001
Tween 20	Sigma-Aldrich	P9416
Xylol	Roth	9713.3

Equipment

Instrument	**Manufacturer**
Cell culture incubator	Heraeus
Flourescence microscope	Leica
Heat block thermo mixer 5436,	Eppendorf
Inverted microscope	Leica
Refrigerated centrifuge	Eppendorf
Micromanipulator Microinjector 5242,	Eppendorf
MJResearch Peltier Thermal Cycler PTC-200	Bio-Rad
Voltmeter EPS 200,	Pharmacia Biotech
UV-screen	INTAS
Video camera	INTAS

Water bath	Memmert
Centrifuge	Eppendorf
Cytospin centrifuge	Hettich
Dissection tools for mouse	Heiland

Kits

Kits used	**Manufacturer**
Ampli 1™	Silicon Biosystems
PKH26 general cell membrane labeling	Sigma-Aldrich
CelVue general cell membrane labeling	Sigma-Aldrich
QIAmp DNA mini kit	Qiagen
SureTag DNA Labeling Kit	Agilent technologies
SurePrint G3 Mouse CGH 4x180K microarray slides	Agilent technologies

Oligonucleotides and Primers

All oligonucleotides and primers were manufactured by Metabion, Munich.

Primer	**Sequence**
ddMse11	TAA CTG ACA G-ddC
Her2 AT 5’	CAG ATT GTC TCA CCA GGT G
Her2 AT 3’	CTC ATT GCT ATT CCA AAT GCC
Her2 GG 5’	GAG ACG CTC AAC CTC AA TTG
Her2 GG 3’	GGC ACT TGT TGG TCT GTA G
LIB1	AGT GGG ATT CCT GCT GTC AGT
Transgene 5’ (MTV)	GTA ACA CAG GCA GAT GTA GG
Transgene 3’ (BRL)	ATC GGT GAT GTC GGC GAT AT

Software

Product	Manufacturer
Axiovision Ver.4.8	Carl-Zeiss GmbH
Graphpad®	Graphpad software Inc.
Agilent Workbench 6.5	Agilent technologies

List of Abbreviations

In this work, internationally accepted chemical symbols and abbreviations of SI units (Système International d`Unités) were used.

Ab	Anti body
bp	Base pair
BCIP	5-Bromo-4-chloroindol-3-ol dihydrogen phosphate ester mono-p-toluidinium salt
BM	Bone marrow
BM-Tr	Bone marrow of transplanted mouse
BSA	Bovine serum albumin
bFGF	Basic fibroblast growth factor
cDNA	Complementary deoxyribonucleic acid
aCGH	Array comparative genomic hybridization
CM	Conditioned medium
CK	Cytokeratin
CSC	Cancer stem cell
DCC	Disseminated cancer cell
ddH2O	Deionized distilled water
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
EGF	Epidermal growth factor
EpCAM	Epithelial cell adhesion molecule
EHS	EpCAM, Her2 and Sca-1 enriched
FCS	Fetal calf serum

FITC	Fluorescein isothiocyanate
GRO-α	Growth related oncogene-alpha
HE	Haematoxylin-Eosin
ICC	Immunocytochemistry
IgG	Immunoglobulin G
IHC	Immunohistochemistry
HIL-6	Hyper-interleukin-6
MEC	Mammary epithelial cells
MMTV	Mouse-mammary tumor virus
MRD	Minimal residual disease
NBM	Normal bone marrow
NMEC	Normal mammary epithelial cells
NSG	NOD scid IL2 receptor gamma chain knockout mouse
NSG BM	NSG mouse bone marrow
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Para-formaldehyde
RKI	Robert Koch Institute
RT	Room temperature
Sca-1	Stem cell antigen-1
SDF-1	Stromal derived factor-1
SM	Sphere medium
StM	Standard medium
SV40	Simian Virus 40
TBM	Tumor bone marrow
TBS	Tris buffer saline
TMEC	Tumor mammary epithelial cells
TW	Transwell
UV	Ultra violet
WT, wt	Wild type

3. Results

The main aim of this study was to establish 1) *in vitro* culture conditions to propagate and expand bone marrow derived disseminated cancer cells (DCC) and 2) to establish *in vitro* DCC cell line model to study DCCs.

3.1. Prostate bone marrow DCC and tumor cell culture

The first aim of my doctoral thesis was to identify and establish conditions to culture DCCs *in vitro*. I first tested the standard conditions, used in sphere culture to generate prostatospheres from tumor cells of prostate cancer patients. Single cells were isolated from the tumor tissues and seeded in basal medium (see materials buffers and solutions) supplemented with EGF, bFGF and B27 at a density of 10000 cells/ml. Cultures were observed for spheres after 7 days and spheres with a diameter of 50 µm and above were counted (figure 5). An average of 60 prostatospheres was counted in the sphere cultures. These conditions were then used to culture DCCs from the bone marrow (BM) of prostate cancer patients. DCCs from the BM of prostate cancer patients cultured in sphere conditions did not generate any spheres. We then cultured the patient BM in adherent conditions to see whether attachment could initiate growth in the DCCs. The cultures were screened for adherent colonies but this condition also failed to initiate growth in DCCs (table 2). Although, in bone marrow 3D cultures there were some sphere-like structures they never grew beyond 20 µm even after 4 weeks in culture.

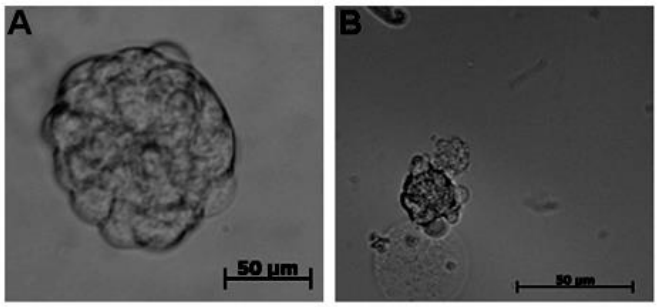


Figure 5: Bone marrow and prostate tissue of patients in culture.
A) Prostatosphere from single cells isolated from prostate tumor tissue of prostate cancer patients post surgery. 10000 cells/ml were seeded and cultured for 7 days and spheres $\geq$ 50 µm were counted and B) sphere-like structure in the prostate cancer patient bone marrow cultures prostate cancer patients. The BM cells were cultured for 4 weeks at a density of 200000 cells/ml. Although sphere-like structures appeared in the BM cultures they never exceeded 20 µm.

Table 2: Bone marrow and tumor tissue of prostate cancer patients in culture.

Sample type	No. of samples	Condition	Spheres/Colonies
Prostate tumor tissue	2	3D	60 (55+65)
Prostate BM	5	3D	0
Prostate BM	9	2D	0

3D cultures were screened for spheres and 2D cultures were screened for adherent colonies. 2D: adherent and 3D: non adherent or suspension cultures.

When the patient bone marrow was cultured the positivity of the sample for the DCCs or the number of DCCs in the positive samples was not known. So, in order to know the number of DCCs that went in to culture, patient BM was stained with EpCAM (epithelial cell adhesion molecule) or CK8/18 (cytokeratin 8 and 18) epithelial markers to identify the DCCs (table 3).

Table 3: EpCAM and CK8/18 status of patient BM samples.

Sample Id	Condition	EpCAM (per 10^6)	CK 8/18 (per 10^6)
PC-272	2D	n.a	Negative
PC-277	2D	Negative	n.a
PC-280	2D	n.a	Negative
PC-284	2D	n.a	Negative
PC-298	2D	n.a	1
PC-316	2D	n.a	3
PC-324	2D	n.a	Negative
PC-325	2D	n.a	Negative
PC-326	2D	Negative	Negative
PC-816	3D	2	1
PC-826	3D	5	Negative
PC-831	3D	2	Negative
PC-838	3D	4	Negative
PC-901	3D	6	n.a

n.a=not assessed. 2D: adherent and 3D: non adherent or suspension cultures.

3.2. Identifying *in vitro* DCC culture conditions using BALB-neuT breast cancer mouse model

Patient bone marrow samples were highly variable with respect to the number of samples positive for DCCs and the number of DCCs per positive sample (table 3).

Moreover, there is variability between prostate cancer patients also . Thus, we used the BALB-neuT breast cancer mouse model. In BALB-neuT mice DCCs can be detected in bone marrow (BM) by cytokeratin 8/18 (CK8/18) staining from week 4 at a frequency of 1-10 per $5x10^5$ BM cells. The Workflow of the experiments involving BALB-neuT BM DCC culture is shown below in figure 6.

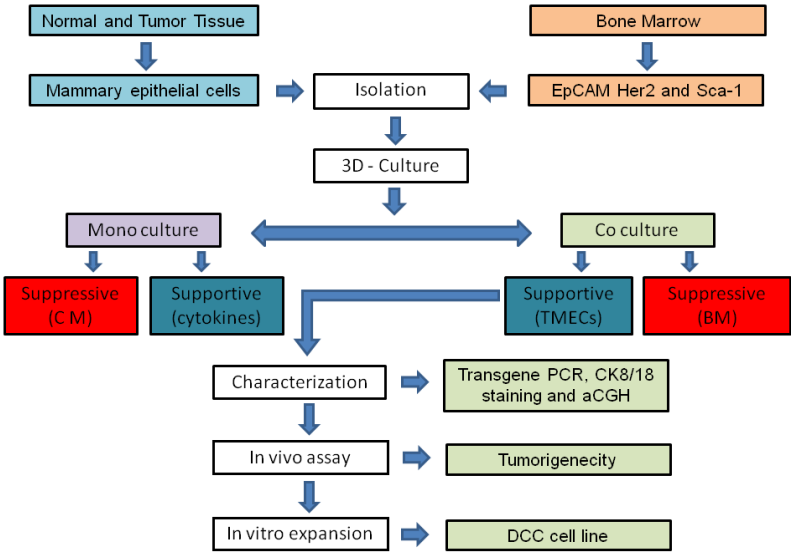


Figure 6: Workflow of the project.
Mammosphere assay was first established using mammary epithelial cells isolated from the mammary glands of normal or tumor tissue and was later optimized. The optimized conditions were then applied to the BM cells enriched for EpCAM, Her2 and Sca-1 from tumor bearing mice to culture DCCs *in vitro*. Since the DCCs failed to grow in mammosphere conditions the possible suppressive effect of BM cells on mammary cells was tested in coculture or monoculture. Eventually, the supportive effect of mammary epithelial cells was also tested in both mono- and coculture experiments. Spheres formed in coculture experiments were later characterized molecularly and functionally and then expanded *in vitro* to generate cell lines.

3.2.1. Establishing protocol for generating mammospheres

Formation of spheres from mammary epithelial cells (MEC) isolated from mammary glands of both normal (BALB/c, wild type) and transgenic (BALB-neuT) mice was first tested to establish a working protocol for generating mammospheres. Epithelial cells isolated from mammary glands of either BALB/c or BALB-neuT were cultured under non adherent conditions to generate spheres as mentioned in materials and methods (2.2.3). The cells were seeded standard sphere medium (StM) (see Materials for buffers and solutions) supplemented with growth factors EGF, bFGF, which are the

standard growth factors used for sphere culture, along with B27 supplement, a serum replacement used for the survival of cells under non adherent conditions (Liao, Zhang et al. 2007). The cells were cultured at a density of 10000 per ml for 7-10 days and subsequently spheres with a diameter of 50 µm and above were counted (figure 7). These basic conditions were subsequently modified.

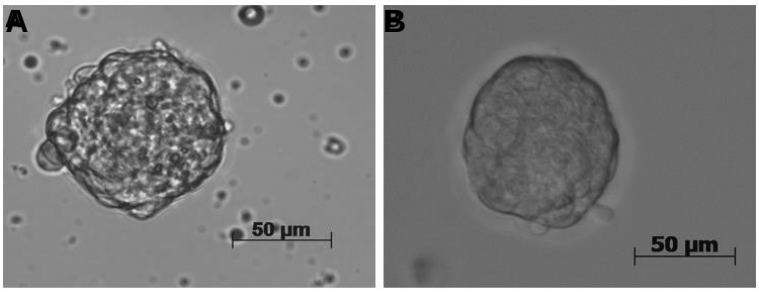


Figure 7: Sphere forming assay.
Mammospheres from mammary epithelial cells isolated from A. BALB/c and B. BALB-neuT. Spheres 50 µm in diameter or above were counted. Number of cells seeded for mammosphere generation was 10000 cells/ml in standard growth factors; EGF, bFGF along with B27 supplement, a serum replacement used in suspension cultures.

3.2.2. Increase in the cell density increases sphere number but not the frequency of sphere forming cells

To examine if increase in cell density increases the frequency of sphere forming epithelial cells, normal or tumor MEC was seeded at different cell densities. Cells were seeded at 10000, 25000, 50000 and 100000 cells/ml in 5 ml of StM, and spheres were counted after one week. The frequency of sphere forming cells per 1000 cells was higher at 25000 cells/ml for TMEC and 10000 cells/ml for NMEC (figure 8 A). The frequency of sphere forming cells decreased with the increase in cell density for both NMEC and TMEC. The reduction in the frequency of sphere forming cells was mainly due to the aggregation of cells at higher densities. As seen in figure 8B, with the increase in density the number of clusters also increased especially in TMEC sphere cultures. The cell clusters were more prominent at 100000 cells/ml (figure 8C). The number of clusters was highest at a density of 100000 cells/ml which may have resulted in the decrease of both the frequency of sphere forming cells and the number of spheres. The TMEC compared to NMEC produced more than twice the number of spheres in culture. These results indicate that cell density in cultures

does affect the frequency of sphere forming cells. For experiments optimizing different conditions, cells at a density of 10000 cells/ml was used for both NMEC and TMEC.

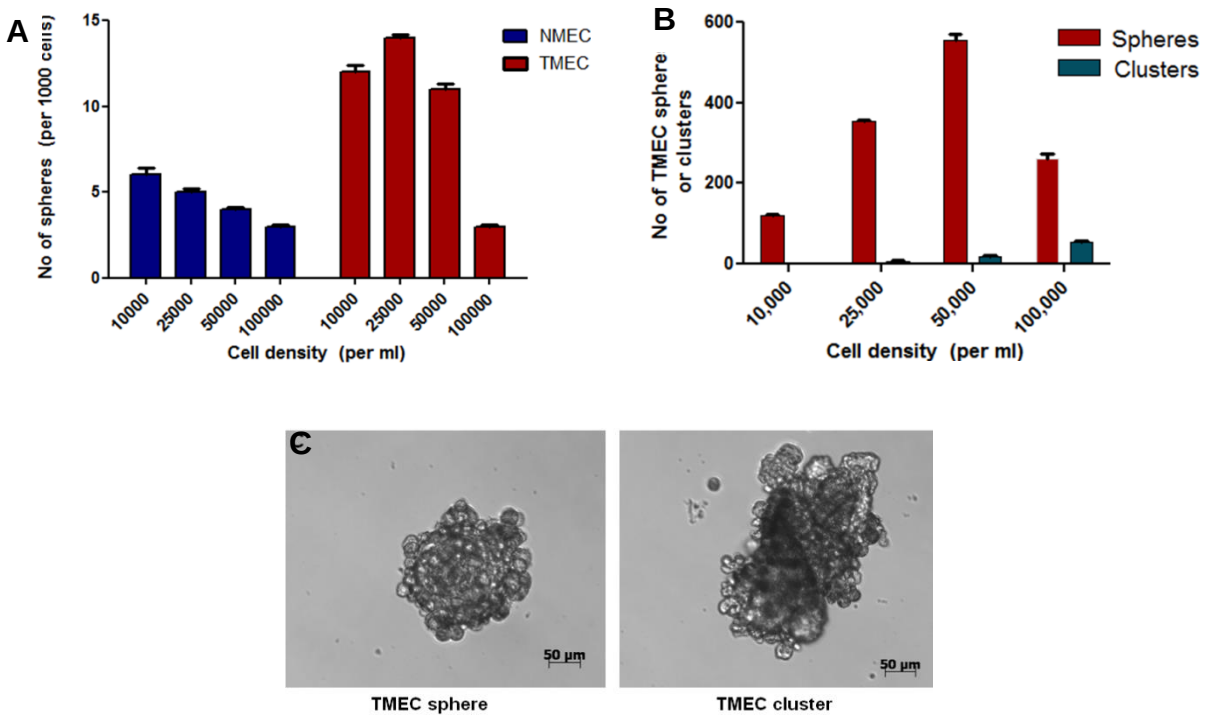


Figure 8: Optimizing cell density for sphere culture.
NMEC and TMEC cells were seeded in different densities to identify the optimal condition for sphere generation with the standard growth factors EGF and bFGF, (A) Sphere forming efficiency of NMEC and TMEC at different cell densities, (B) Spheres and clusters in TMEC cultures at different cell densities and (C) sphere and cluster in TMEC cultures at a density of 100000 cells/ml. NMEC: normal mammary epithelial cells, TMEC; tumor mammary epithelial cells.

3.2.3. Effect of hyper Interleukin-6 (HIL-6) on mammosphere formation

The standard sphere culture conditions containing growth factors EGF and bFGF did not yield any success in generating DCC spheres even in prostate bone marrow where DCCs were detected (3.1). Therefore, cytokines known to have a mitogenic effect on cells were tested in addition to EGF and bFGF. Two different cytokines were first tested on MECs to optimize conditions for sphere culture assuming that these conditions would trigger growth in BM DCCs.

Interleukin-6 (IL-6) is a pro-inflammatory cytokine known to play a role in cell proliferation. IL-6 signals through its membrane bound specific receptor, IL-6r, and

two surface bound gp130 molecules. Hyper IL-6 (HIL-6) is a fusion protein, where IL-6 is fused to its soluble receptor (sIL-6r). This molecule does not require its specific membrane bound IL-6 receptor and signals through gp130 alone. Since all cells express gp130 it is advantageous to use HIL-6 as even non IL-6 responsive cells (cells lacking IL-6r) respond to HIL-6. To assess the effects on mammosphere formation different concentrations of HIL-6 were tested, from 1 ng to 50 ng. The number of spheres increased with the increase in HIL-6 concentration from 1 ng to 20 ng but decreased at 50 ng (figure 9 A). However, the decrease in the sphere number at 50 ng was not due to fusion of spheres. Although the average sphere diameter increased from 1 to 50 ng (figure 9 B) the highest number of spheres could be observed at a concentration of 20 ng. For further mammosphere experiments, we therefore used HIL-6 at an end concentration of 20 ng/ml.

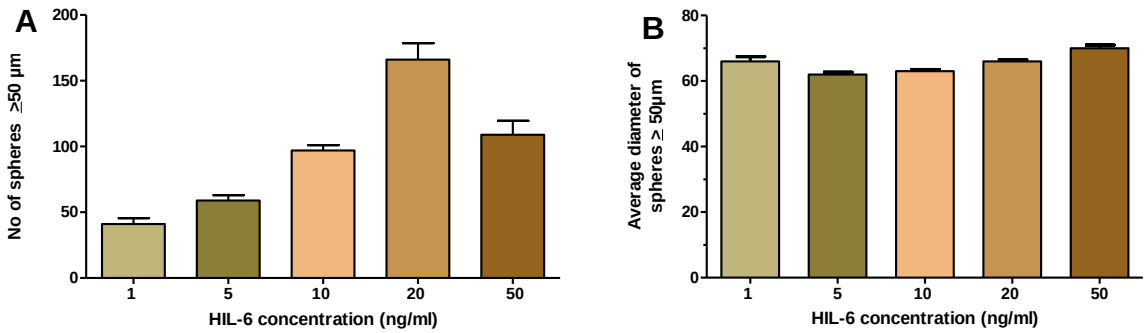


Figure 9: Effect of HIL-6 at different concentrations on mammosphere generation.

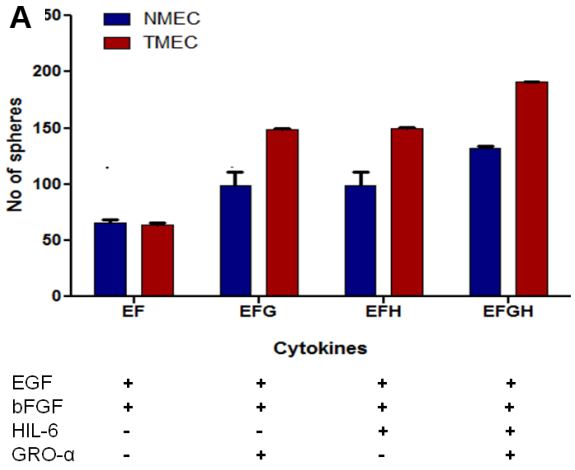
10000 cells/ml were seeded in a 6 well plate in 5 ml. After seven days in culture pictures of every sphere was taken using Axiovison software under the microscope and spheres ≥50 µm were counted after measuring the diameter of every single sphere. (A) total number of spheres was counted and (B) average diameter of spheres was also calculated by measuring the diameter of every sphere in culture. The experiment was performed in triplicates.

3.2.4. GRO-α and HIL-6 on mammospheres generation

Another cytokine GRO-α, growth related oncogene α, also known as melanoma growth stimulatory activity (MGSA) alpha belongs to CXCL1 family of chemokines. GRO-α is known to have a potent mitogenic effect on the neighboring cells especially on premalignant epithelial cells (Coppe, Patil et al.) Since DCCs are epithelial derived cells I found it apt to test GRO-α first on mammary epithelial cells. To observe the effects of GRO-α alone or in combination with HIL-6, GRO-α was added to normal

and tumor mammary epithelial cells in sphere medium supplemented with and without HIL-6. 10000 cells/ml were seeded and cultured for 7-10 days thereafter the number of spheres was counted. As shown in figure 10, the number of spheres in cultures treated with either HIL-6 or GRO-α increased compared to the control medium containing only EGF and bFGF. Among the different conditions tested (figure 10 A), the number of spheres in cultures with either HIL-6 or GRO-α was similar indicating that GRO-α has an equal potential ability to induce proliferation in cells as HIL-6 (figure 10 B). In conditions where both GRO-α and HIL-6 were present the number of spheres increased. Although the number of spheres generated by the tumor cells was higher the effect of both cytokines on both the normal and tumor (figure 10) mammospheres was similar. Based on these results the sphere medium with HIL-6 (20 ng/ml) and GRO-α (5 ng/ml) in addition to EGF and bFGF was found optimal and was used for future sphere cultures. This medium hereafter will be referred to as sphere medium (SM) and the medium with EGF and bFGF only will be called standard medium (StM)

A



B

NMEC				
	EF	**EFG**	**EFH**	**EFGH**
EF	*n.a*	*p<0.01*	*p<0.01*	*p<0.01*
EFG	*p<0.01*	*n.a*	*p=1.0000*	*p<0.01*
EFH	*p<0.01*	*p=1.0000*	*n.a*	*p<0.01*
EFGH	*p<0.01*	*p<0.01*	*p<0.01*	*n.a*
TMEC				
	EF	**EFG**	**EFH**	**EFGH**
EF	*n.a*	*p<0.0001*	*p<0.0001*	*p<0.0001*
EFG	*p<0.0001*	*n.a*	*p>0.1143*	*p<0.0001*
EFH	*p<0.0001*	*p>0.1143*	*n.a*	*p<0.0001*
EFGH	*p<0.0001*	*p<0.0001*	*p<0.0001*	*n.a*

Figure 10: Effect of cytokines on the mammosphere forming efficiency of MEC.

(A) Sphere forming efficiency of normal (NMEC) and tumor mammary epithelial cells (TMEC) isolated from murine mammary glands. Different cytokines were added to the sphere medium and their effect on the mammosphere generation was observed and (B) overview of *p values* from t-test performed comparing different conditions. E: EGF, F: bFGF, H: HIL-6, G: GRO-α. The cells were seeded at a density of 10000 cells/ml 5 ml of medium. Spheres $\geq$50 µm were counted after 7 days in culture. The experiment was performed in triplicates. **n.a* = *not applicable.*

3.3. Mammary epithelial cells but not the freshly isolated fibroblasts generate spheres

The epithelial cell isolation from mammary glands is based on the principle of differential centrifugation where the epithelial cells are isolated centrifuging at 200xg for 3 min and fibroblasts at 350xg for 4 min. Although these two cell types are isolated at different centrifugal speeds the possibility of fibroblast contamination in the epithelial fraction could not be ruled out. Therefore, the sphere forming ability of the fibroblasts under non adherent conditions was tested.

In the first experiment we checked the sphere forming ability of established murine fibroblast cell lines (NIH3T3, C3HT10$^{1/2}$ and Immortalized fibroblasts) in SM. All tested cell lines were able to form spheres under mammosphere conditions, figure 11 A and B. The C3HT10$^{1/2}$ spheres although spherical were not compact as the epithelial cell derived mammospheres. On the other hand, the immortalized fibroblast cells formed compact spheres similar in appearance to mammospheres. NIH3T3 cells did grow in suspension but looked more like cell aggregates, loosely bound, not compact and without a clear outer periphery typical for epithelial cell derived mammospheres. Surprisingly, the fibroblast cell lines generated more spheres compared to NMEC (~8 fold) or TMEC (~5 fold) (figure 11) at the same cell density (10000 cells/ml).

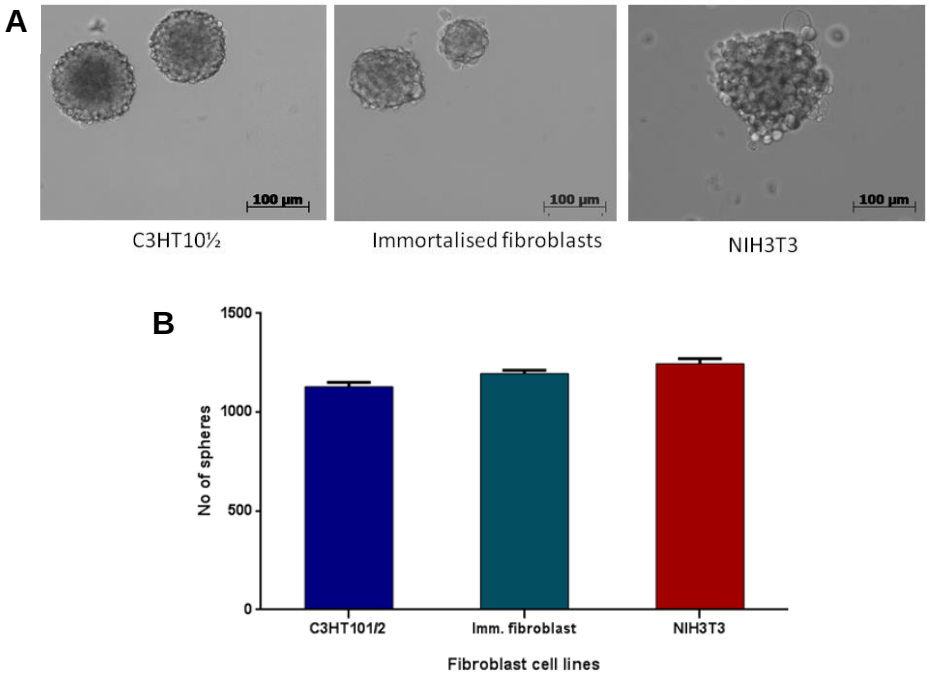


Figure 11: Sphere assay of fibroblast cell lines.
(A) Fibroblast cell line spheres under mammosphere conditions with EGF, bFGF, GRO-α, HIL-6 and (B) number of spheres generated by each fibroblast cell line with a seeding density of 10000 cells/ml. The experiment was performed in triplicates and repeated twice.

Since these cell lines have been in culture for long periods of time they could have acquired growth capabilities differing from primary cells. Therefore, we asked, if freshly isolated fibroblasts from murine mammary tissue also possess the ability to survive in suspension and form spheres.

Mammary epithelial and fibroblast cells were isolated by differential centrifugation from both BALB/c and BALB-neuT mammary tissue. Since primary fibroblasts were never tested for their ability to form spheres, they were seeded at 50000 cells/ml to exclude low cell density as one of the factors leading to the failure of fibroblasts to form spheres in SM. After 7 days the fibroblast cultures were screened for spheres and compared to spheres from epithelial cultures. The mammary epithelial cells formed spheres in non adherent conditions in SM, but fibroblasts neither from BALB/c nor BALB-neuT could form spheres under identical conditions (figure 12 A and B). These results indicate that albeit the fibroblast cell lines have sphere forming ability, freshly isolated mammary fibroblasts lack the capability to survive in suspension cultures and form spheres.

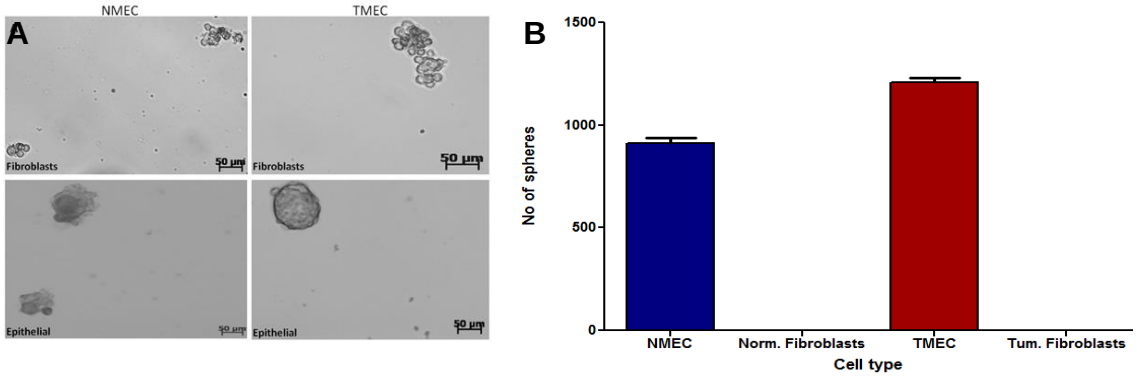


Figure 12: Comparison of sphere forming ability of mammary epithelial and fibroblast cells.
(A) freshly isolated mammary fibroblasts and epithelial cells from murine mammary glands were cultured in sphere medium; (B) spheres ≥ 50 µm and above were counted after 7 days in culture for NMEC, TMEC and their respective fibroblasts. All experiments were done in triplicates and repeated twice. Seeding density was 50000 cells/ml.

3.4. *In vitro* propagation of murine bone marrow derived DCC

To test if SM can induce proliferation in DCCs, BM DCCs were cultured in the SM. Bone marrow from BALB-neuT (TBM) and normal BALB/c (NBM) were collected and mono nucleated cells were isolated by 60% percoll gradient (P60) centrifugation. BM cells were either cultured directly or further enriched for EpCAM, Her2 (EH) or EpCAM, Her2 and Sca-1 (EHS) and cultured in sphere medium. As shown in table 4, none of the bone marrow samples cultured, P60, EH or EHS could form spheres.

Table 4: BM cells cultured in different conditions to propagate DCC *in vitro*.

Bone marrow	Enrichment	Depletion	Medium	Spheres	Colonies
BALB/c	No	No	SM	No	-
BALB-neuT	No	No	SM	No	-
BALB/c	EH	No	SM	No	-
BALB-neuT	EH	No	SM	No	-
BALB/c	EHS	No	SM	No	-
BALB-neuT	EHS	No	SM	No	-
BALB/c	EHS	No	RPMI+FCS	-	No
BALB-neuT	EHS	No	RPMI+FCS	-	No
BALB/c	-	EHS	RPMI+FCS	-	No
BALB-neuT	-	EHS	RPMI+FCS	-	No

EH: EpCAM, Her2; EHS: EpCAM, Her2, Sca-1; DCCD: DCC density.

The BM samples, enriched and non-enriched were stained with CK8/18 to confirm the presence of DCCs. Although, both non-enriched and EH or EHS enriched BM were positive for CK8/18 (figure 13) they did not grow in sphere conditions. Furthermore, the DCC density (DCCD) in the BM when stained with CK8/18 increased after enrichment (table 6) with 125 CK+ cells in EHS, 60 CK+ in EH and 7 in non enriched BM. There were a few small sphere-like structures in cultures with EHS enriched cells but these never grew in size and were below 20 µm in diameter. There were many clusters with loosely bound cells in non-enriched BM cultures but nothing that could be defined as spheres. Interestingly, these loosely bound cell clusters reduced with EHS enrichment.

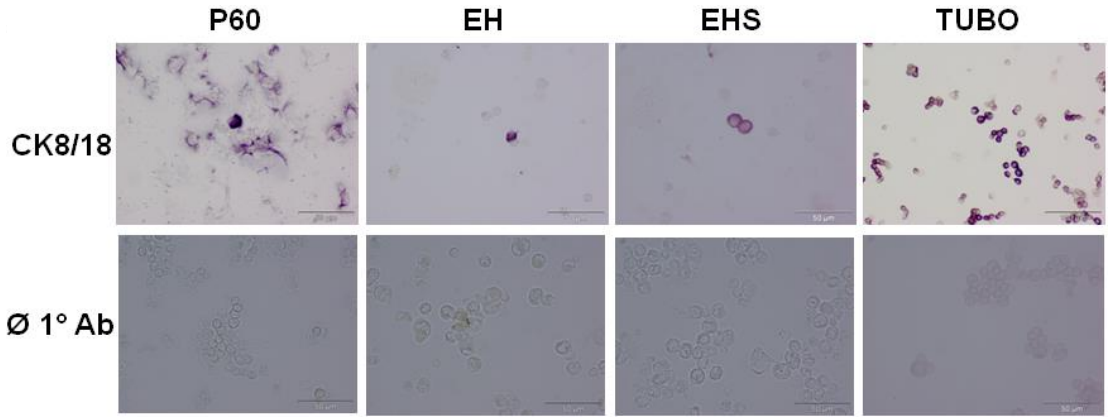


Figure 13. CK8/18 staining of bone marrow before and after enrichment.

BM of BALB-neuT was stained for CK8/18 after percoll 60 (P60), EpCAM and Her2 enrichment (EH) and EpCAM, Her2 and Sca-1 (EHS) enrichment for the presence for disseminated cancer cells on adhesion slides. TUBO expresses CK8/18 and was used as the positive control. Ø 1° Ab: without primary antibody.

Fetal calf serum (FCS) contains many factors which support the growth of cells *in vitro* and is routinely used in cell culture. To see if serum could trigger growth in DCCs in normal cell culture conditions, EHS cells were isolated from 3 mice each for BALB/c and BALB-neuT were cultured separately on 3 regular cell culture dishes in RPMI with 10% FCS and observed for the formation of any adherent colonies. As indicated in table 5, addition of FCS failed to initiate DCC growth. These results imply that the mammosphere condition was not efficient in triggering growth of the bone marrow DCC nor was the normal cell culture. Nevertheless, the density of DCCs (DCCD) after enrichment with EpCAM, Her2 and Sca-1 increased (table 5). Therefore for further cultures EHS enriched BM was used.

Table 5: DCCD in enriched and un-enriched BM.

Bone marrow	Enrichment	DCCD
BALB/c	No	0
BALB/c	EH	0
BALB/c	EHS	0
BALB-neuT	No	7
BALB-neuT	EH	60
BALB-neuT	EHS	125

EH: EpCAM, Her2; EHS: EpCAM, Her2, Sca-1; DCCD: DCC density.

3.5. Effect of bone marrow cells on the sphere forming efficiency of epithelial cells

3.5.1 Coculture of bone marrow cells with TMECs

We hypothesized that the bone marrow only supports for the survival of DCC but exerts a growth suppressive effect. To test this hypothesis TMECs were co-cultured with bone marrow cells of transgenic mice (TBM). Additionally, to see if in the absence of DCCs the effect of BM cells on TMECs would be any different from TBM with DCCs, TBM was depleted of EpCAM, Her2 and Sca-1 (TBM w/o EHS) and co-cultured with TMECs. NSG mice are deficient in mature T cells, B cells and natural killer cells (NK) and are defective in several cytokine pathways (Shultz, Schweitzer et al. 1995; Shultz, Lyons et al. 2005). To observe if NSG also has a suppressive effect TMECs were cocultured with NSG bone marrow cells (NSG-BM). TMECs were stained with PKH26, a cell membrane labeling dye, to differentiate from the BM cells in co-cultures. After seven days in culture the number of PKH26 stained spheres were counted as shown in figure 14 A. In the presence of TBM cells the number of mammospheres decreased compared to the number of mammospheres in TMEC monoculture, figure 14 B. TMEC mammospheres further decreased when cultured with EHS depleted TBM cells. The reduction of TMEC spheres in NSG-BM was significant (*t-test p=0.0257*) suggesting some effect of NSG-BM cells on TMEC. However, compared to NSG-BM the effect of TBM on TMEC *(t-test p<0.001*) was highly significant and the effect of TBM depleted of EpCAM, Her2 and Sca-1 on TMEC (t-test *p<0.0001*) was even more significant. There were no spheres in the BM monoculture controls. These results suggest that the BM cells could exert a suppressive effect on tumor cells.

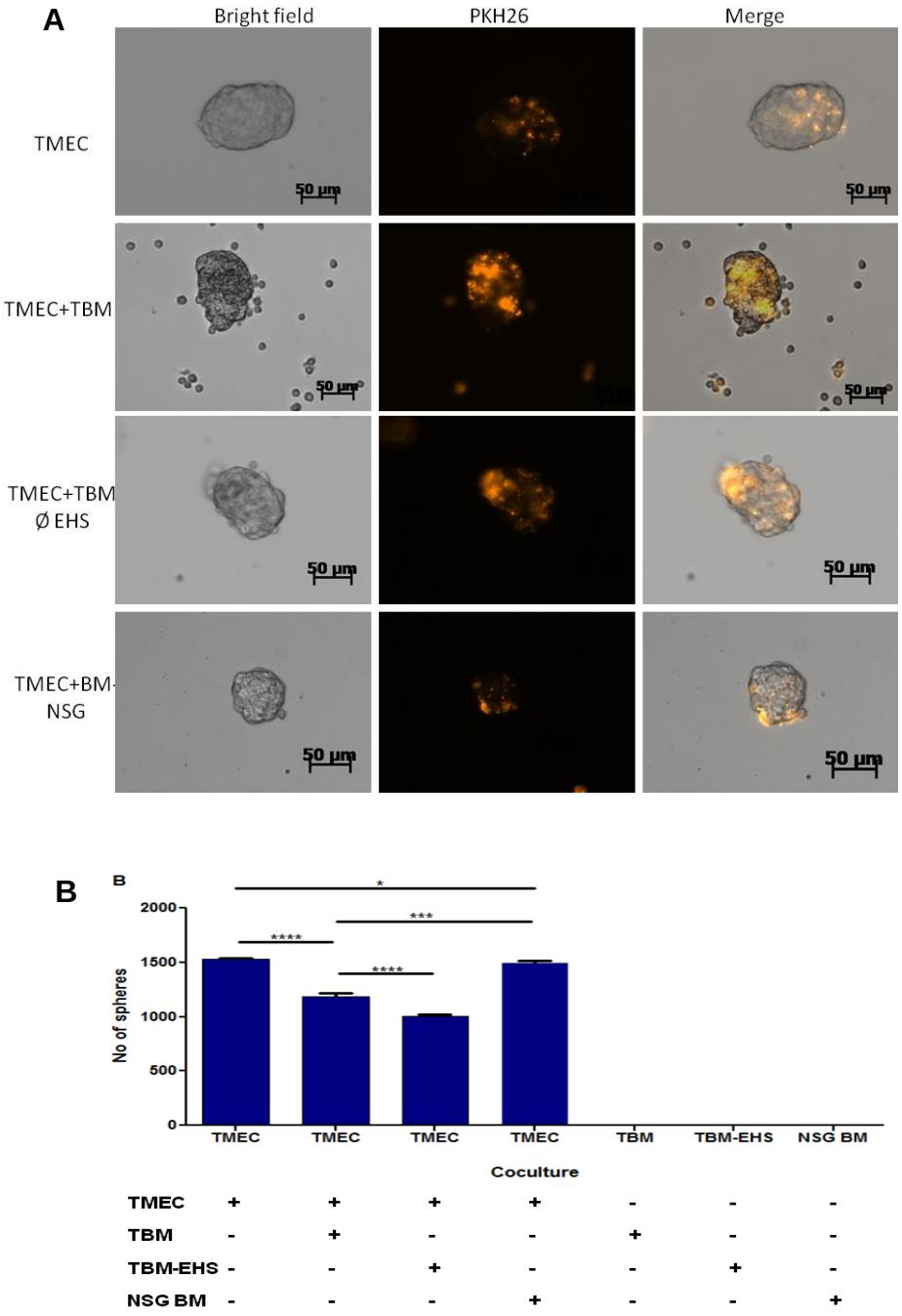


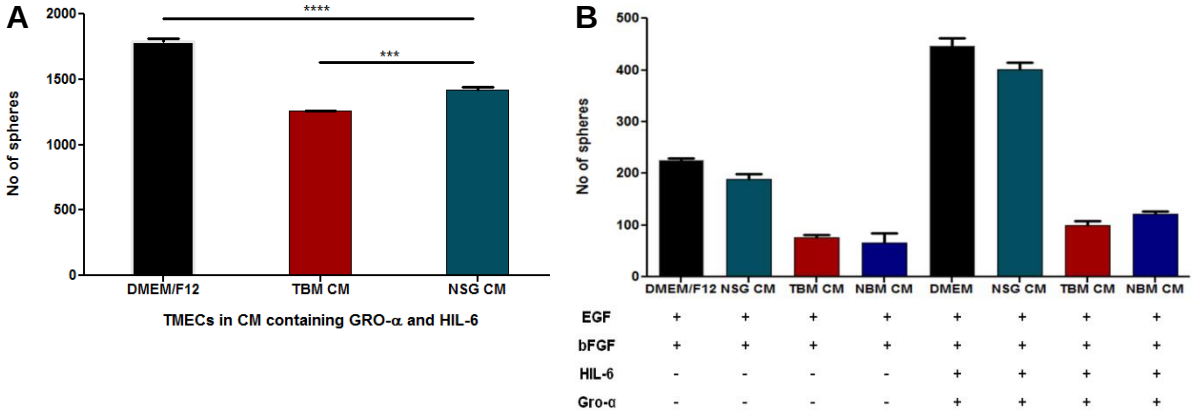
Figure 14: The effect of BM cells on TMEC and their ability to form spheres.
Spheres stained with PKH26 and with a diameter of 50 µm or above were counted. 50000 cells/ml of TMEC and 200000 cells/ml of BM cells were seeded in cocultures. (A) Spheres from TMEC cultured in different culture conditions, (B) number of spheres from TMEC in different culture conditions were counted and culturing for 7 days. Statistical analysis was performed using unpaired t-test; *p>0.05; ***p<0.001; ****p<0.0001. TBM Ø EHS/TBM-EHS: EpCAM, Her2 and Sca-1 depleted. Experiment was repeated thrice in triplicates.

3.5.2 Effect of BM conditioned medium on sphere forming ability of TMECs

We hypothesized that factors secreted by bone marrow cells may play a role in suppressing DCC growth in the bone marrow. Therefore, conditioned media (CM) from bone marrow of tumor mice (TBM) and bone marrow of NSG mice (NSG-BM) were prepared by culturing the cells in non adherent conditions for 3 days with EGF and bFGF. The medium was collected and filtered through a 0.22 µm filter to remove cells. Later TMECs were seeded at a density of 50000 cells/ml and cultured in their respective CM supplemented with EGF, bFGF, GRO-α and HIL-6. Mammospheres formed in both the CM with reduced frequency (figure 15 A). The reduction in NSG-BM compared to SM (t-test *p<0.0001*) and TBM compared to NSG-BM CM (t-test *p<0.001*) were significant. To test the effect of BM conditioned medium in the absence of GRO-α and HIL-6 and later if GRO-α and HIL-6 can overcome the effect of CM, TMECs were seeded in CM without GRO-α and HIL-6 and compared to CM supplemented with GRO-α and HIL-6. In addition, the epithelial cell density was decreased to 10000 cells/ml for ease of counting spheres and to exclude high cell density as one of the factors for high sphere numbers as seen in the previous experiment (figure 15 A). As controls TMECs were seeded in standard sphere medium with or without GRO-α and HIL-6. The medium was always supplemented with EGF and bFGF for all conditions. Spheres $\geq$ 50 µm were counted after one week in culture. The number of spheres in the NSG-BM CM without GRO-α and HIL-6 compared to normal medium (DMEM/F12) without GRO-α and HIL-6, reduced significantly (figure 15 B and C). The effect of conditioned medium from TBM on TMECs was even more significant when compared to NSG-BM CM with a further decrease in sphere numbers. Addition of GRO-α and HIL-6 did not increase the sphere forming efficiency of TMEC cells in TBM CM. The number of TMEC spheres in TBM CM decreased with respect to NSG-BM CM with GRO-α and HIL-6. This effect was also highly significant (figure 15 C).These results indicate that BM conditioned medium of BALB-neuT does have a suppressive effect on the tumor cells

Is this suppression an innate property of the BM or is it due to the presence of primary tumor or DCCs in the bone marrow? To test if the suppressive effect of the BM is a consequence of the primary tumor or DCCs present in the BM, TMECs were grown in NBM CM to check if NBM CM also suppressed the growth of TMEC. Interestingly, TMEC in NBM CM also generated less spheres either in the presence

or absence of GRO-α and HIL-6 compared to TMEC spheres in their respective NSG-BM conditioned media cultures. This reduction in sphere numbers was also highly significant (figure 15 B and C). Based on these results it can be concluded that the BM exerts a suppressive effect on TMECs and supplementing the conditioned media with GRO-α and HIL-6 could not abrogate this effect.



C

CM supplemented with EGF and bFGF				
	DMEM/F12	NSG CM	TBM CM	NBM CM
DMEM/F12	*n.a*	*p<0.01*	*p<0.0001*	*p<0.0001*
NSG CM	*p<0.01*	*n.a*	*p<0.0001*	*p<0.0001*
TBM CM	*p<0.0001*	*p<0.0001*	*n.a*	*p=0.4014*
NBM CM	*p<0.0001*	*p<0.0001*	*p=0.4014*	*n.a*
CM supplemented with EGF, bFGF, GRO-α and HIL-6				
	DMEM/F12	NSG CM	TBM CM	NBM CM
DMEM/F12	*n.a*	*p<0.01*	*p<0.0001*	*p<0.0001*
NSG CM	*p<0.01*	*n.a*	*p<0.0001*	*p<0.0001*
TBM CM	*p<0.0001*	*p<0.0001*	*n.a*	*p=0.0156*
NBM CM	*p<0.0001*	*p<0.0001*	*p=0.0156*	*n.a*

Figure 15. Suppressive effect of BM CM on TMEC.
(A) number of spheres in the BM conditioned media from BALB-neuT (TBM) NSG (NSG-BM) mice, cell density 50000 per ml; (B) comparison of spheres between TBM ,NSG-BM and BALB/c BM (NBM) conditioned medium to with and without GRO-α and HIL-6 and (C) overview of p-values from unpaired t-test performed to examine the effect of different CM on TMECs. Cell were seeded at 10000 cells/ml. Experiment was performed thrice in triplicates and spheres $\geq$ 50 µm were counted. EGF and bFGF are standard growth factors in the sphere medium and GRO-α and HIL-6 are added in addition these factors. CM: conditioned medium, NBM: BALB/c bone marrow, NSG-BM: NSG mouse bone marrow, TBM: BALB-neuT bone marrow and n.a: not applicable.

3.6. Effect of mammary epithelial cells on bone marrow DCCs

3.6.1. Mammary epithelial cells induce proliferation in bone marrow DCCs

Tumor cells secrete many factors and some of these have a growth promoting effect on their neighboring cells (Celis, Gromov et al. 2004). We sought to see if these tumor cells or their secreted factors have any effect on the DCCs in the bone marrow. Bone marrow from the BALB-neuT was isolated, enriched for EHS and cocultured with TMECs to observe for their effect on BM DCCs. To differentiate between the two cell populations the BM cells were stained with PKH26, a general cell membrane labeling dye. After 1 week spheres $\geq$ 50 µm stained with PKH26 were counted in the cocultures with TMECs (figure 16 A and B) indicating that the TMECs do induce growth in the DCCs in the bone marrow. EHS cells from BALB-neuT were also cultured with NMECs to see if the growth inducing effect on DCCs was an exclusive property of the TMECs. In the presence of NMECs the DCCs were also able to form spheres suggesting that the growth inducing effect was not a property confined to TMECs (figure 16 A and C). As a control, NMEC was cocultured with NBM to examine if NMECs can activate cells in the normal BM other than DCCs. The number of PKH26+ spheres in the co-cultures was high and was comparable to their respective MEC controls. We assume that many of the spheres contained a mixture of DCCs and MECs making it difficult to distinguish between DCC only and DCC+MEC derived spheres. As controls, NBM and NMECs from three different BALB/c mice were isolated and co-cultured in SM. The NBM cells were stained with PKH26 to distinguish them from NMEC. NMEC did not have any effect on the NBM cells confirming that the growth inducing effect is selectively on the DCCs.

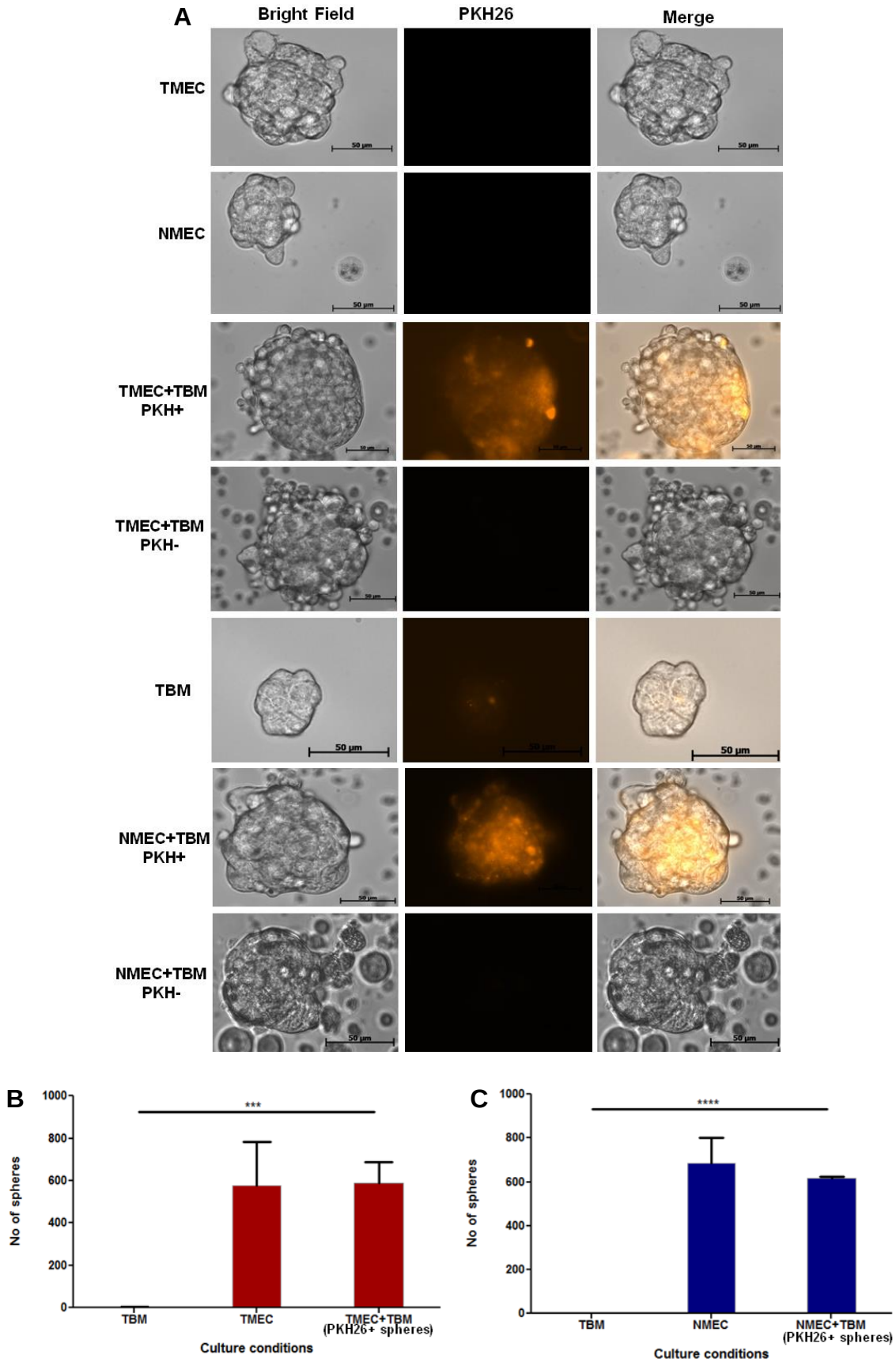


Figure 16: Propagation of DCCs through coculture of TBM with TMECs and NMECs.

(A) Spheres from coculture experiment, NMEC and TMEC spheres, DCC spheres in TBM stained with PKH26 visible under Cy3 channel and unstained spheres originating either from

NMEC or TMEC in the coculture and TBM spheres in monoculture and (B) graph showing TBM DCC spheres with tumor mammary epithelial cells (TMEC) as cocultures and (C) graph showing TBM DCC spheres cocultured with normal mammary epithelial cells (NMEC). PKH26 stained spheres ≥ 50 µm were counted. The results are from 3 independent experiments of TMEC cocultures and 2 independent experiments of NMEC cocultures. ****p<0.001*, *****p<0.0001.*

3.6.2. PCR to confirm the identity of TBM spheres

To confirm the identity of the BM spheres in the cocultures, PKH26+ spheres from the cocultures were picked and checked for the presence of Her2 transgene through PCR. As controls spheres from NMEC and TMEC were picked and a PCR was performed for the transgene. TMECs carry the rat Her2 oncogene but not NMECs (figure 17 A). The resultant PCR product for rat Her2 oncogene is 220 bp. As expected all the spheres picked from TBM+TMEC co-cultures carried the transgene serving as a positive control (figure 17 B). Importantly, the PKH26+ spheres picked from NMEC+TBM co-cultures carried the Her2 transgene confirming the origin of the spheres.

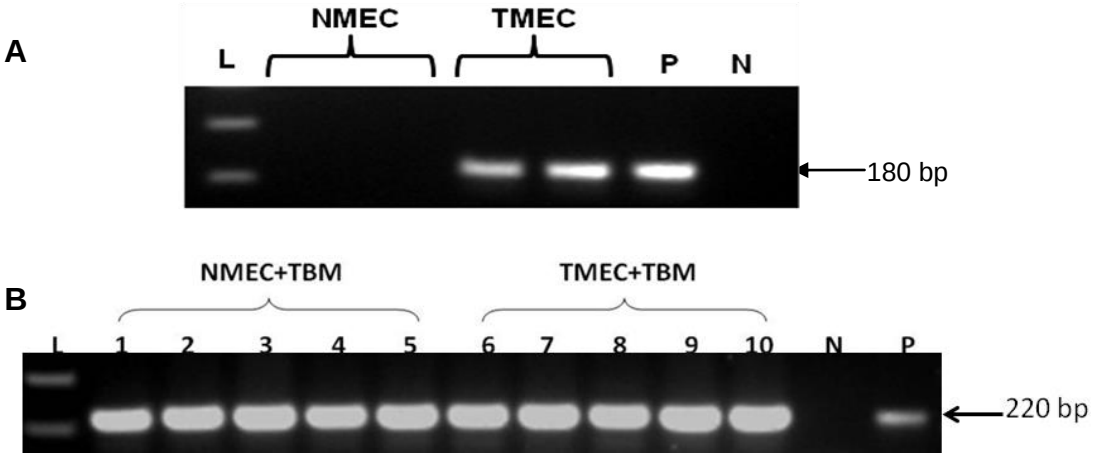


Figure 17: Transgene PCR to confirm the identity of PKH26+ spheres.

(A) PCR for Her2 transgene on PKH26 positive (PKH+) spheres picked from NMEC and TMEC cocultures and (B) PCR for Her2 transgene on spheres picked from NMEC and TMEC monoculture controls. NMEC do not carry the transgene hence NMEC mammospheres are negative for the rat Her2 PCR. PCR band for Her2 transgene is 220 bp and for wild type is 180 bp.

The PKH26+ spheres could expand *in vitro* as cell lines and were tumorigenic when injected sub cutaneously into NSG mice (table 6). It was surprising to note that the

cell lines lacked the Her2 transgene. Furthermore, neither the tumor cells nor the cell lines expressed CK8/18 or Her2. Therefore, the identity of the cell lines or the tumors could not be determined as they were neither epithelial nor DCC derived.

Table 6: Characterization of the cell lines and tumors derived from PKH26+ spheres.

Experiment	Spheres	Cell lines	Tumor	Transgene in cell lines	CK8/18 and Her2 staining
TMEC + TBM	PKH26+	yes	2/2	absent	Negative
TMEC + TBM	PKH26-	yes	2/2	absent	Negative

3.7. Effect of tumor cell secreted factors on TMECs

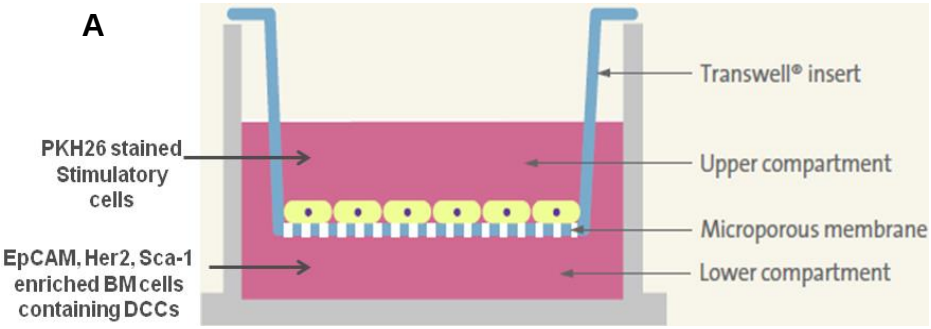
Do cell secreted factors also play a role in activating DCCs? To investigate the role of secreted factors in eliciting DCC growth *in vitro*, a transwell assay was setup. In addition, the transwell separated the two cell populations thereby preventing contamination by either population.

For this assay, a metastatic mouse mode, a modification of the BALB-neuT model was used (Husemann, Geigl et al. 2008). Briefly, pre-malignant mammary gland of a 4 week old BALB-neuT mouse is transplanted in a 4 week old BALB/c mouse. The cells from the transplanted tissue after malignant transformation disseminate to the bone marrow of the recipient BALB/c mouse. The advantage of this mouse model is that only the DCC in the BM carry the transgene while in BALB-neuT all the cells carry the rat Her2 transgene. Hence, this mouse model provided a better and cleaner background in identifying DCCs.

3.7.1 Transwell assay to test the effect of secreted factors on BM DCCs

Bone marrow from 5 transplanted mice were pooled and later enriched for EHS. 4 mice of the 6 pooled had metastasis. The EHS enriched BM of transplanted mice (EHS-BMTr) was seeded in the lower chamber and NMEC or TMEC were seeded in the upper chamber. The EHS-BMTr was also cocultured with TUBO, an established primary tumor cell line from BALB-neuT, and C3HT10$^{1/2}$, a murine embryonic

fibroblast cell line, to see if these cell lines would also influence DCC proliferation. As an additional measure to identify contamination in the BM compartment of the transwell plates, the MECs and cell lines were stained with PKH26 (figure 18 A). As a control one transwell plate only with PKH26+ cells in the upper chamber was added and treated similarly as the rest of the transwell plates. The cells were cultured for 4 weeks with medium change at regular intervals (2.2.11). After four weeks spheres negative for PKH26 and $\geq$ 50 µm could be observed in the BM compartment, figure 18 B. Spheres from BM formed only in transwells where TMEC and TUBO cells were the stimulators. Neither NMEC nor C3HT10$^{1/2}$ cell line could stimulate the BM DCCs to proliferate. Although, NMECs induced growth in DCCs in the coculture assay they failed to induce any growth in DCC in the transwell assay (figure 18 B). After quantifying the BM spheres transwell cultures, the upper chamber with TMEC or TUBO was removed and the BM was further cultured for 4 weeks. This was done to examine if the activated DCCs required continuous stimulation for being in an active state. Interestingly, by week 8 the number of spheres in the BM cultures even after the removal of the stimulators increased (figure 18 C). As controls the normal bone marrow was cultured in the presence of NMEC and TMEC, which were earlier shown to have a positive effect on the DCC proliferation in TBM, in the coculture assay (3.5.1). NBM cells did not form any spheres. Neither TMEC nor NMEC had any influence on normal BM cells and neither of them could induce sphere formation in NBM. The results indicate that tumor cell secreted factors play a role in activating the BM DCCs.



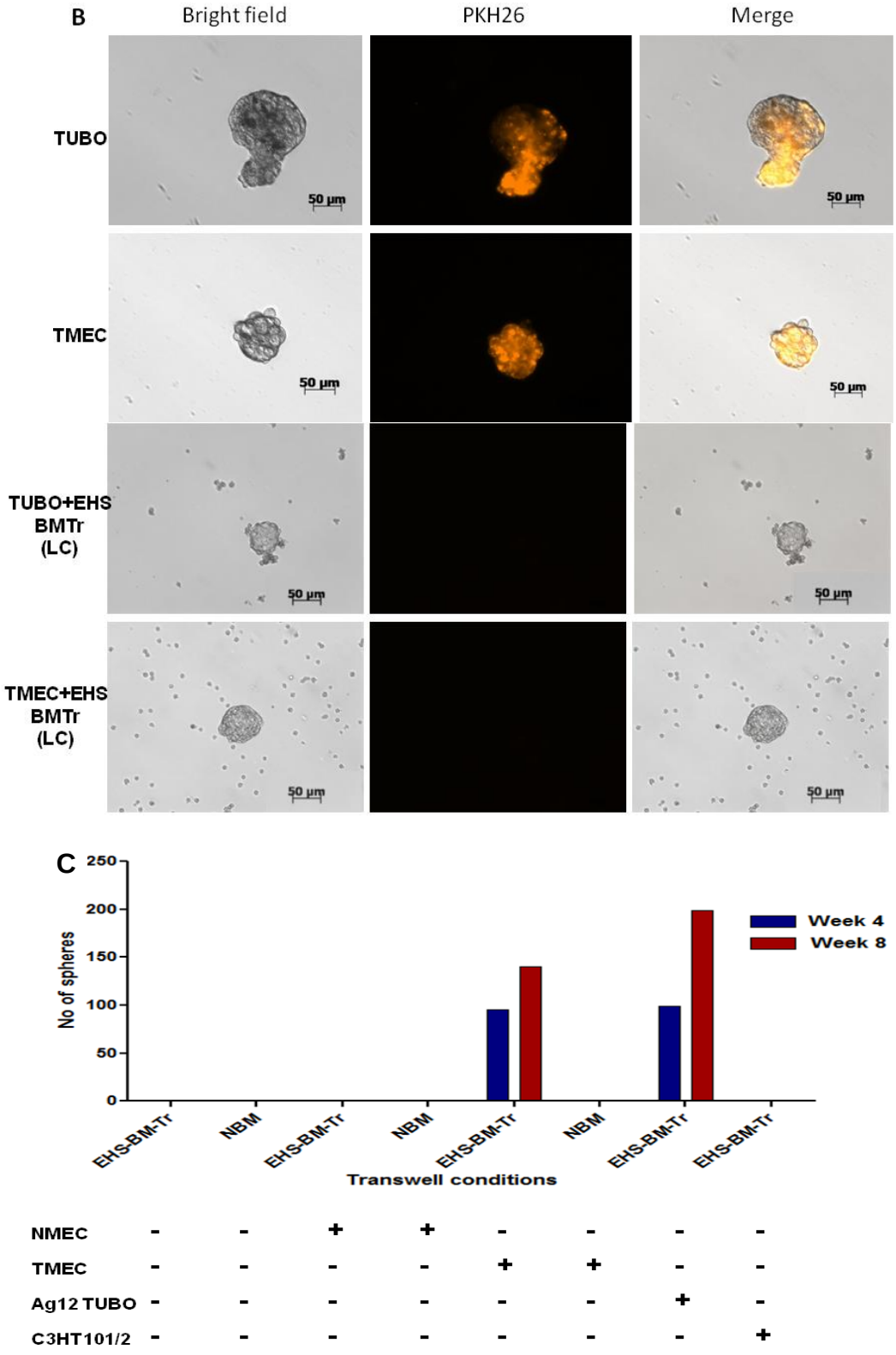


Figure 18: Transwell assay to propagate DCC from the bone marrow of transplanted mice.

Cell lines and MECs were stained with PKH26 and seeded in the upper chamber. The MECs were seeded at 50000 cells/ml and cell lines at 10000 cells/ml. BM from 6 transplanted mice were pooled, enriched for EHS and seeded at 100000 cells/ml in the lower chamber of the transwell plate.(A) Spheres from different transwell conditions tested. TMEC and TUBO are stained with PKH26 to identify any contamination in the BM chamber. (B) number of spheres

formed in different culture conditions with the upper chamber until week 4 and thereafter without the upper chamber until week 8. LC: lower chamber

3.7.2 Cytokeratin 8 and 18 staining of the transplanted bone marrow

To determine the number of DCCs present in the BM of transplanted mice (BM-Tr), adhesion slides of the BM-Tr were prepared to stain for cytokeratin 8 and 18 (CK8/18). The CK 8/18 staining was positive for the transplanted bone marrow, figure 19. About 175 cells per $1*10^6$ BM cells were stained positive for CK8/18 (table 6).

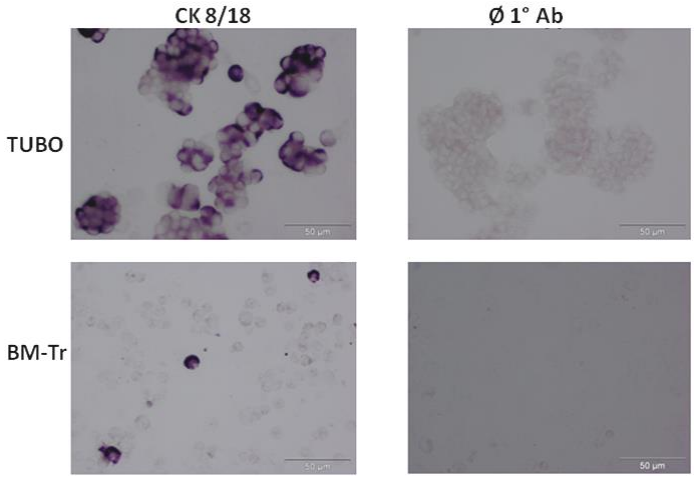


Figure 19: Cytokeratin 8/18 staining of BM-Tr.
Adhesion slides prepared from the BM of transplanted mice were stained for CK 8/18. TUBO cell line was used as a positive control for CK staining along. CK positive cells are dark purple colored cells.

3.7.3. EpCAM, Her2 and Sca-1 cells from the bone marrow have the ability to form spheres in a transwell assay

So far, the BM was enriched for all the three markers, EpCAM, Her2 and Sca-1, making it difficult to determine the population of cells that contained sphere forming DCCs. To identify the cell population with sphere forming DCCs, the bone marrow of eight transplanted mice were pooled, split into three parts and enriched for EpCAM, Her2 or Sca-1 as shown in figure 20 A. Two of the 8 pooled mice had metastasis. Since, TUBO was shown to induce growth in DCC; the enriched cells were cultured in transwells in the presence of TUBO. After 3 weeks, spheres appeared all the three cell populations (EpCAM, Her2 and Sca-1) in transwells cultured with TUBO. EpCAM

had the highest number of spheres and Sca-1 the least. TUBO failed to induce any growth in the EpCAM, Her2 or Sca-1 enriched cells of NBM in transwells (figure 20 B). After counting the spheres, the upper chamber was removed and the BM cells were cultured further. The spheres in all three cell fractions increased in number by week 6. There was a three-fold increase of spheres in Sca-1 and around two-fold for EpCAM. In Her2, the increase in sphere number was around 1.4 fold. These results indicate that both EpCAM and Sca-1 had a higher frequency of proliferating DCCs than Her2.

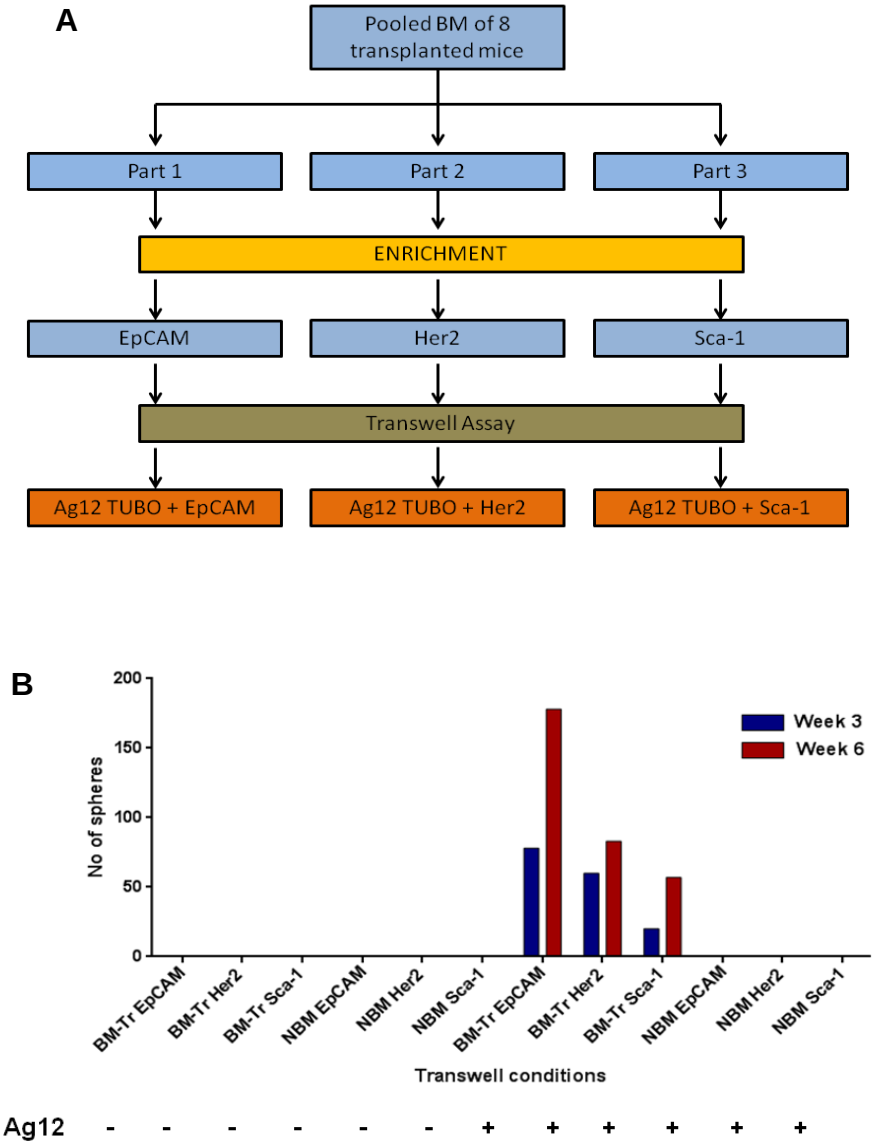


Figure 20: Sample preparation and transwell assay.
(A) Schematic representation of BM enrichment from transplanted mice and culture and, (B) number of spheres from the BM in the lower chamber of the transwell in week three and in week six. After week three the upper chamber was removed and the lower chamber with BM was cultured further until week six. Spheres with a diameter of 50 µm and above were counted.

3.7.4. CK 8/18 staining of the BM-Tr of EpCAM, Her2 and Sca-1 fractions

To determine the number of DCCs in the BM enriched for EpCAM, Her2 or Sca-1, adhesion slides from the enriched fractions prepared before transwell culture were stained for CK 8/18 (figure 21). The number of CK 8/18 positive cells in EpCAM and Her2 BM fractions was similar while in Sca-1 the number of CK positive cells was far less (table 6). Surprisingly, the number of spheres in the Her2 fraction did not increase much though cells positive for CK in Her2 was high (table 7).

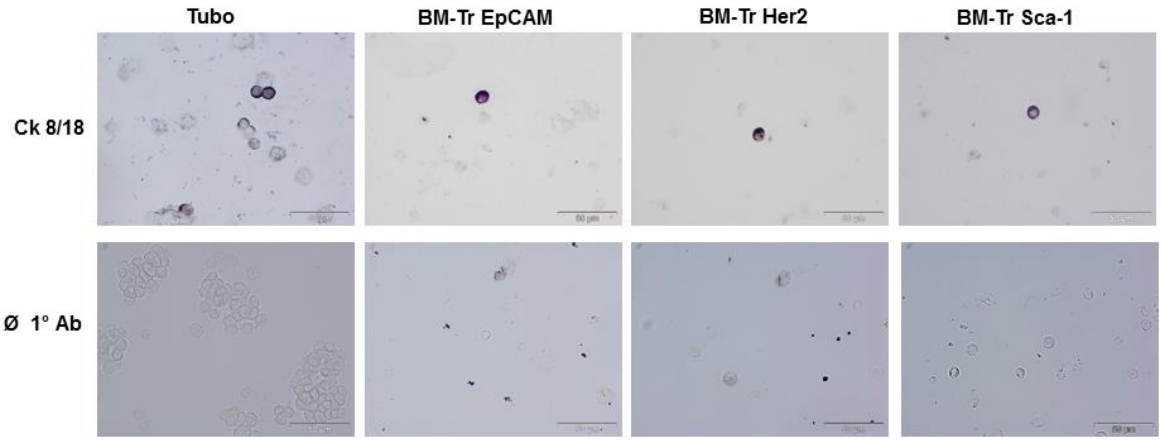


Figure 21: CK 8/18 staining of EpCAM, Her2 and Sca-1 fractions of BM-Tr.
Adhesion slides or cytospins from the bone marrow of EpCAM or Her2 or Sca-1 enriched fraction were stained for Her2 or CK to indentify the number of DCCs in their respective fractions. CK positive cells are stained dark purple in color. TUBO was used as control. Ø 1° Ab; without primary antibody, BM-Tr: bone marrow of transplanted mice.

Table 7: Number of CK positive cells counted for different BMTr fractions per 10^6 bone marrow cells on adhesion slides.

				No. of spheres/CK8/18 cells	
BM sample	**DCCD**	**Spheres Week 3**	**Spheres week 6**	**Week 3**	**Week 6**
BM-Tr (EHS)	175	99	199	0,57	1,14
BM-Tr EpCAM	260	70	178	0,27	0,68
BM-Tr Her2	267	60	83	0,22	0,31
BM-Tr Sca-1	80	20	57	0,25	0,71

3.7.5. PCR to confirm the identity of spheres from the BM of transplanted mice

To confirm the presence of transgene and thereby the origin of sphere forming cells, genomic DNA of BM spheres picked from 3.6.1 and 3.6.3 were examined for the presence of Her2 transgene. As shown in the figure 22, all the spheres picked transwell experiments carried the transgene. These results imply that the spheres from BM-Tr were derived from activated DCC and the factors secreted by TMEC and TUBO can selectively activate bone marrow DCCs.

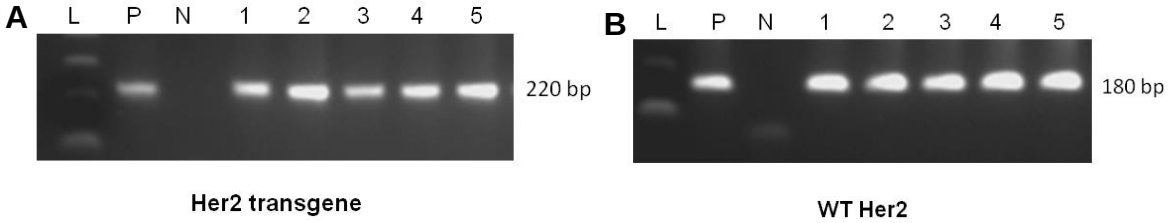


Figure 22: Transgene PCR on the isolated DNA of spheres from different transwell conditions.
Gel (A) shows the presence of rat Her2 transgene in the spheres picked at 220 bp and the gel (B) shows PCR bands for the WT Her2 (control) at 180 bp. 1: BM-Tr Her2, 2: BM-Tr EpCAM, 3: BM-Tr Sca-1, from 3.6.2 transwell experiment and, 4: TMEC+BM-Tr (EHS) and 5: TUBO+BM-Tr (EHS) from 3.6.1 transwell experiment. P: Positive, N: Negative and L: Ladder.

3.8. *In vivo* tumorigenicity of spheres generated from the transwell assays

The ability to proliferate *in vitro* does not imply that the DCCs posses the capability to initiate tumors. Therefore, the tumorigenicity of DCC spheres from the transwells was tested *in vivo*. BM spheres from the transwells (table 8) were picked and transplanted orthotopically into BALB/c or sub-cutaneously into NSG mice.

Table 8: *In vivo* transplantation of spheres generated in different transwell conditions.

Mouse id	Condition		No of DCC spheres	Injection site	Tumor
	Feeder Cells	Test Cells			
3940 (BALB/c)	TUBO	EHS	50	Mammary fat pad	Yes
3941 (BALB/c)	TUBO	EpCAM	50	Mammary fat pad	Yes
3942 (BALB/c)	TUBO	Her2	50	Mammary fat pad	Yes
3943 (BALB/c)	TUBO	Sca-1	32	Mammary fat pad	Yes
4072 (BALB/c)	TMEC	EHS	40	Mammary fat pad	Yes
90-17 (NSG)	TMEC	EHS	50	Sub cutaneous	Yes

All the mice transplanted with DCC spheres developed tumors implying that some cells within the spheres displayed tumor initiating potential. The tumors were removed and a small part of the tumor was cryo-preserved and another part was paraffin embedded. Rest of the tumor was digested to prepare single cells (see 3.8.2). The paraffin embedded tumor tissue sections were stained with haematoxylin and eosin and all tumors appeared to be epithelial in origin (figure 23).

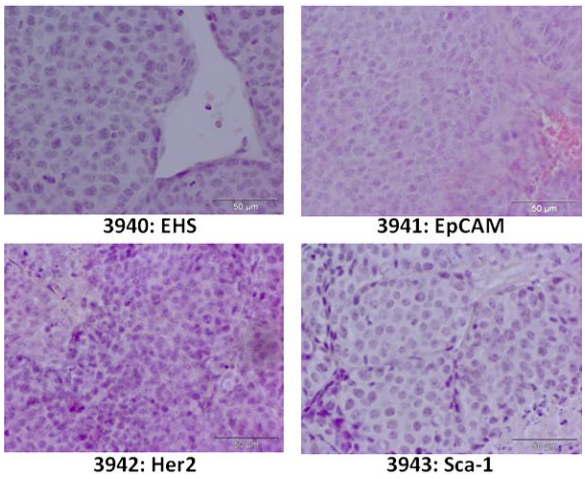


Figure 23: H & E staining of paraffin embedded sections.
Paraffin sections of tumors derived from transwell sphere transplants in the mammary fat pad of BALB/c mice. 3940, 3941, 3942 and 3943 are the mouse numbers of the respective transwell sphere transplants.

3.9. Generation of DCC cell lines

The second aim of my project was to generate cell line from DCCs which can later be used to study these cells. Primary cell culture is very challenging especially in

culturing and generating cell lines from a particular cell type (Epithelial or fibroblast). Fibroblasts could sometimes grow faster and outgrow epithelial cells when cultured together or when epithelial cultures are contaminated with fibroblasts. Tumor tissue consists of both epithelial and fibroblast cells. Thus, to generate epithelial cell cultures isolated from tumor tissue it is important to eliminate fibroblast contamination from these cultures.

3.9.1. Establishing a method to generate cell lines *in vitro* from mammary epithelial cells of BALB-neuT mouse

Although the freshly isolated fibroblasts did not survive in non-adherent, serum-free conditions, contaminating fibroblasts could survive in the presence of serum. The possibility of the fibroblasts outgrowing epithelial cells also could not be completely ignored. Therefore, a method was first established to eliminate or at least minimize fibroblast contamination and generate an epithelial cell line from BALB-neuT primary tumors. Therefore, two procedures 1) low centrifugation speed (200xg) for isolating epithelial cells from digested mammary tissue and 2) serum free medium, in which fibroblasts cannot survive were used. MECs were isolated from an 18 week old BALB-neuT mouse (BnT18) and 10000 cells per cm^2 were seeded in 2 different conditions, DMEM+20%FCS which is used to culture TUBO cell line and RPMI+10%FCS commonly used to culture epithelial cell lines. The cells were seeded in a T-25 flask and incubated at 37° C, 5.5% CO_2 and 7% O_2. After 24 hours of plating the cells, the medium was removed and replaced with StM (basal medium+EGF+bFGF+heparin+B27) and incubated for 24 hours. The cells were later trypsinized and centrifuged at 200xg for 3 min to pellet epithelial cells and re-plated in their respective serum containing medium. This was repeated 3 times and later the cells were checked morphologically under the microscope for any stromal cell contamination in the culture flasks (figure 24 A). The cells seeded in DMEM+20% FCS were very few and did not survive to generate a cell line however; the cells in RPMI+10% FCS were able to grow into a cell line (figure 24 B) and looked epithelial in morphology as short compact colonies of cells. The cells were also stained with epithelial markers CK8/18 and Her2 and compared to TUBO cell line used as a positive control to confirm the epithelial identity of the cell line (figure 24 C). Based on

these results RPMI+10%FCS was used to generate cell lines from tumors generated by transwell spheres *in vivo.*

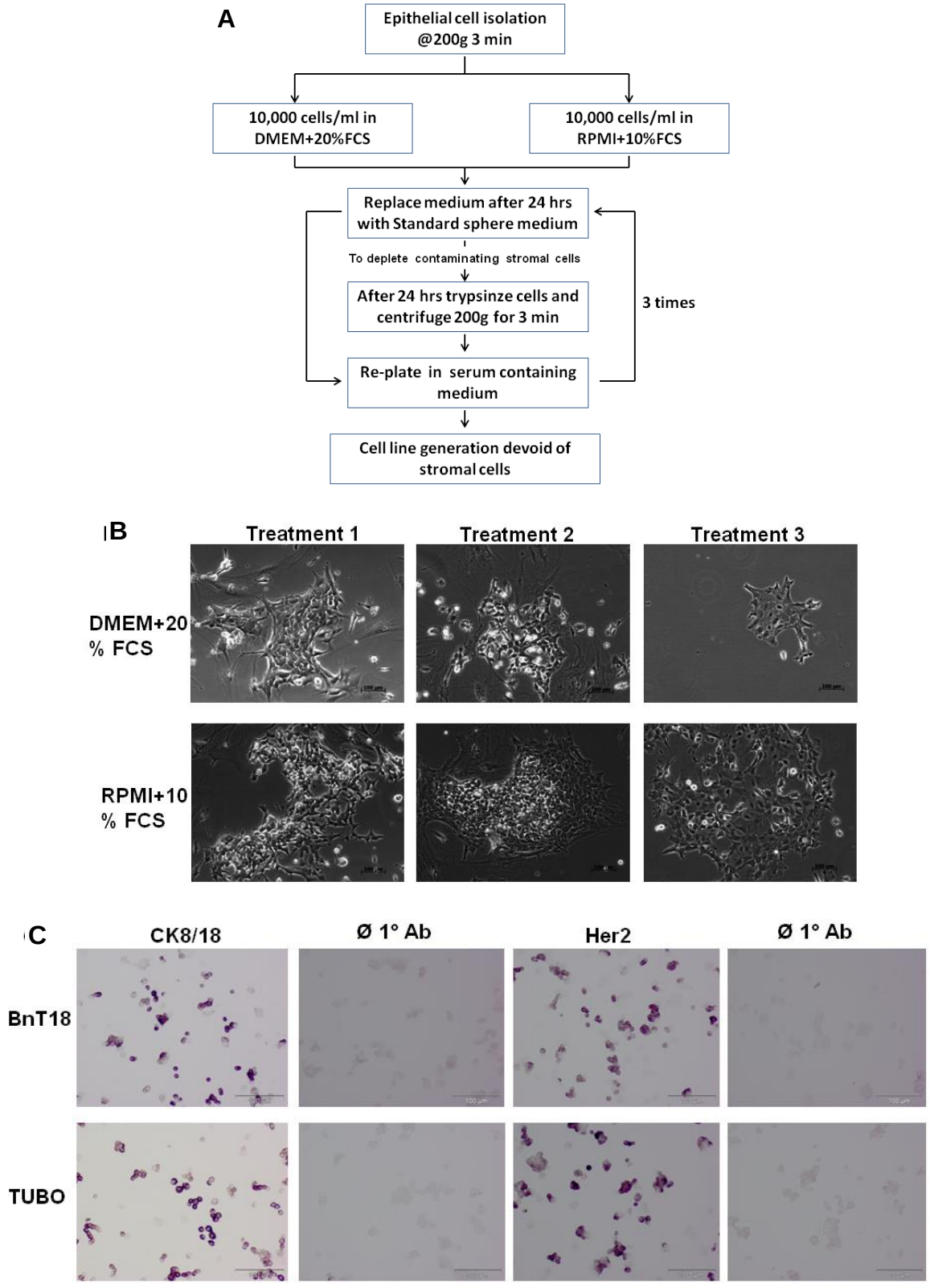


Figure 24: Generating cell line from MEC of BALB-neuT mouse *in vitro*.
(A) Workflow of the procedure for *in vitro* cell line generation. (B) phase contrast pictures of MECs seeded in DMEM+20% FCS or RPMI+10% FCS after each treatment, shown as a box

in the workflow. (C) Adhesion slides prepared from BnT18 cell line cultured in RPMI+10% FCS with 500000 cells per slide were stained for CK8/18 and Her2. TUBO cell line used as a positive control for CK8/18 dn Her2. BnT18; cell line generated from MECS of an 18 week old BALB-neuT mouse, Ø 1° Ab: without primary antibody.

3.9.2. Generation of cell lines from tumors derived from transplanted DCC spheres

To answer the question if the cells from DCC initiated tumors could expand in normal cell culture conditions, single cells prepared from the tumors were seeded in RPMI medium supplemented with 10% FCS in a normal cell culture flask at a cell density of 10, 000 cell/cm^2. The medium was changed the next day to remove non adherent or dead cells as explained in the methods section. The culture flasks were treated as explained in the previous section. Tumor cells isolated from all the tumors seeded to generate cell lines were able to grow and expand *in vitro* in a week. The primary cell lines could be maintained and propagated *in vitro* for several passages (figure 25). The cells in the cell culture flasks looked epithelial growing together as small colonies representing cobblestone morphology.

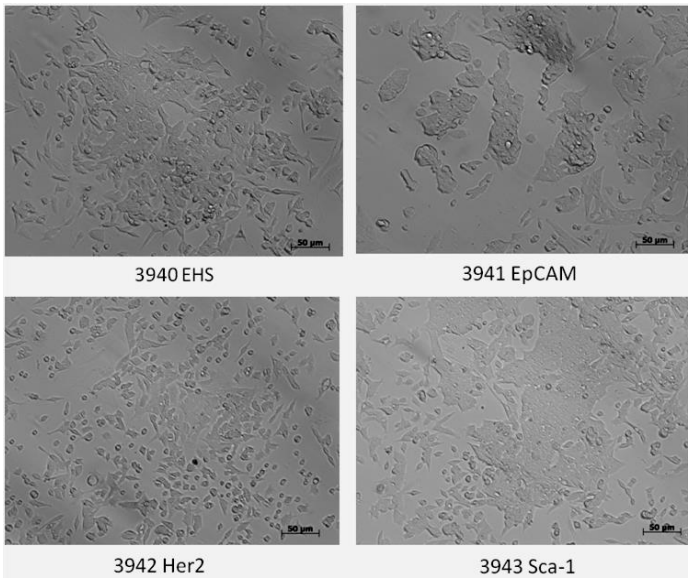


Figure 25: *In vitro* expansion of activated DCC.
Cell lines generated from the tumor cells of tumors originated from transwell spheres. The single cells were generated from tumors grown in BALB/c mice transplanted with transwell spheres. 10000 cells/cm^2 were seeded in RPMI + 10% FCS in a cell culture flask and treated as explained in 3.8.1.

3.10. Staining tumors and cells derived from *in vivo* transplanted spheres

Paraffin embedded tumor tissues from transwell spheres transplanted into BALB/c mice and cells isolated from these tumors were stained with CK8/18 and Her2 to confirm that they were of epithelial and DCC origin.

3.10.1. Immunohistochemical staining of paraffin embedded tumors with CK8/18 and Her2

From paraffin embedded tumors (section 3.7) 5 µm thick sections were prepared. The tumor sections were stained for CK 8/18 and Her2 to confirm if the tumors were epithelial and originated from the DCC derived spheres. As a control, primary tumor from BALB-neuT was also stained for CK 8/18 and Her2. All the tumors from the transwell spheres expressed CK8/18 and Her2 (figure 26).

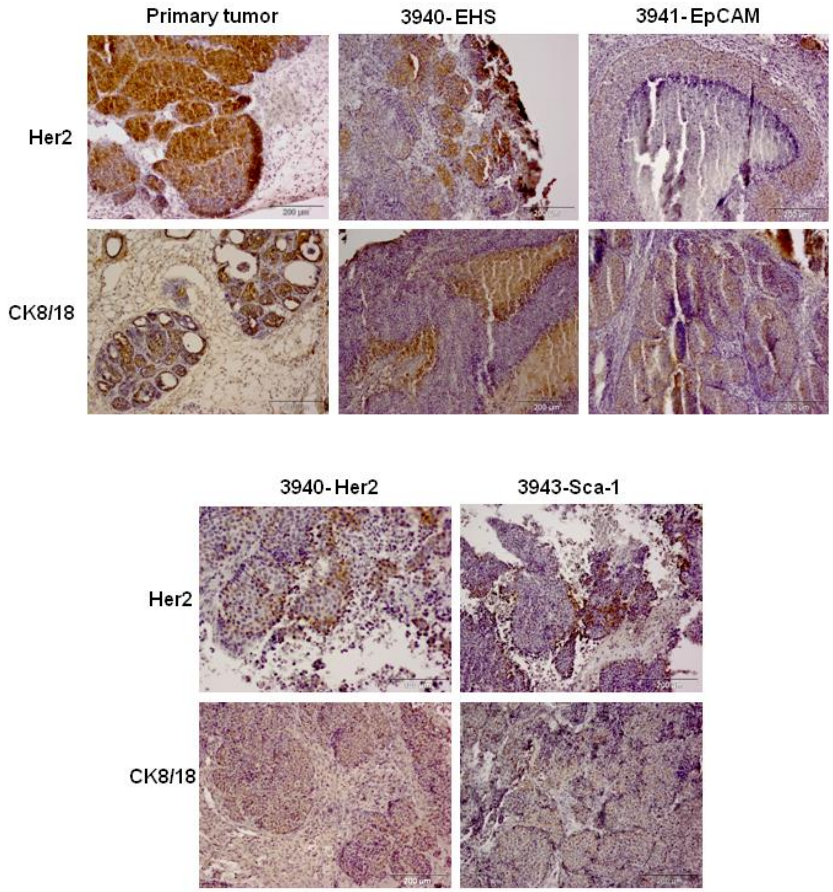


Figure 26: Immunohisto-chemical staining with CK8/18 and Her2.
Paraffin embedded tumor tissue sections were prepared from tumors formed by transplanting spheres from different transwell conditions in the mammary fat pad of BALB/c mice. The

tumor tissues were stained for CK8/18 and Her2. Primary tumor of BALB-neuT was used as a control.

3.10.2. CK8/18 and Her2 staining of tumor cells and cell lines on adhesion slides

Adhesion slides were prepared from tumor cells and cell lines established from these tumor cells and stained for CK8/18 and Her2 to see if the cells expressed cytokeratin and Her2 and confirm their epithelial origin. Cells from all the tumors (figure 27 A) and cell lines (figure 27 B) were positive for CK8/18 and Her2. TUBO cells were used as control for both the staining.

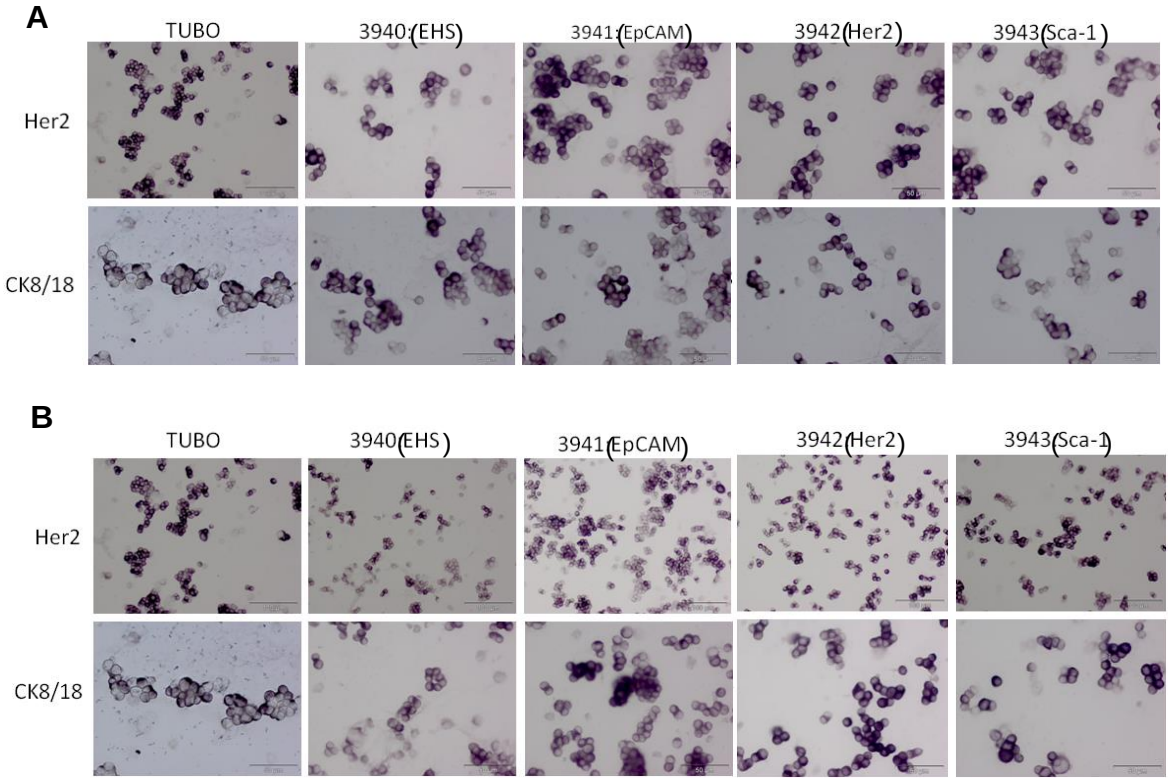


Figure 27: Immunocytochemical staining of tumor cell lines.
Adhesion slides with 500000 cells per slide were prepared from tumor cells isolated from DCC induced tumor and cell lines established from the isolated tumor cells. The slides were stained for CK8/18 and Her2. (A) Cytokeratin 8/18 and Her2 staining of tumor cells and (B) CK8/18 and Her2 staining of tumor cell lines. TUBO was used as a positive control for CK8/18 and Her2. EHS: EpCAM, Her2 and Sca-1.

3.11. Her2 transgene PCR of tumor cell and DCC cell lines

Additionally, a PCR was performed on the DCC derived tumor cells and DCC cell lines generated from these tumor cells to look for the presence of the rat Her2 transgene and confirm their DCC origin. All the cells from tumors and the cell lines carried the transgene (figure 28 A) again confirming their DCC origin. A PCR for the WT Her2 was also performed as a control (figure 28 B), lower panel. Together the results from CK8/18, Her2 staining along with Her2 transgene PCR suggests the tumors and the cell lines are DCC derived.

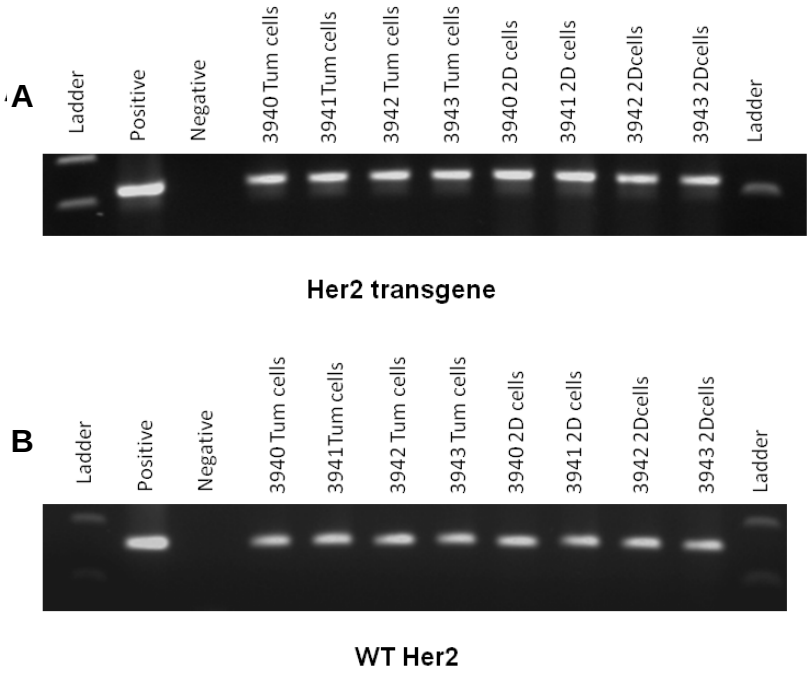


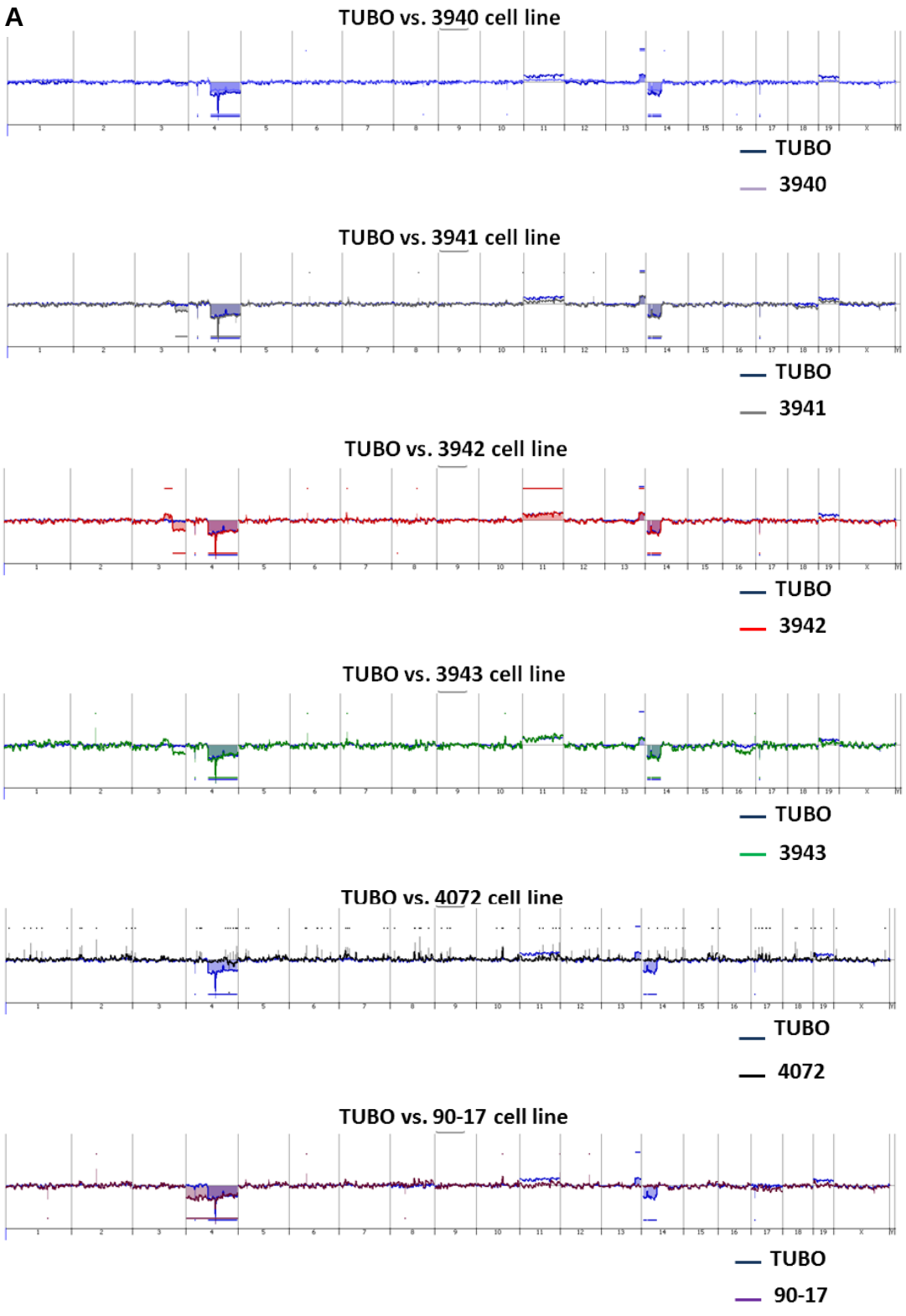
Figure 28: Transgene PCR on genomic DNA isolated from tumor cells and the cells from the cell lines generated from the tumors.

Genomic DNA was isolated from tumor cells and cell lines established from transwell spheres and checked for the presence of Her2 transgene. PCR was performed for rat Her2 oncogene and the corresponding PCR bands were observed on 1.5% agarose gel after gel electrophoresis. Upper gel (A) shows Her2 transgene at 220 bp while the lower gel (B) shows bands for WT Her2 at 180 bp. 2D = cell lines.

3.12. Genomic aberration of cell lines by array comparative genomic hybridization

Tumor cells are known to exhibit an aberrant genomic profile with deletions and amplifications across their genome. An aCGH was performed on cell lines generated from the tumors of transplanted transwell DCC spheres along with TUBO to see if they were genomically aberrant and compared to TUBO. TUBO carried least number

of aberrations, mostly deletions, and 4072 was highly aberrant with aberrations across all chromosomes and mostly amplifications (figure 29 A and B). However, deletion on chromome 4 was shared by all the cell lines while deletion on chromosome 14 was shared only between TUBO, 3940, 3941, 3942 and 3943.



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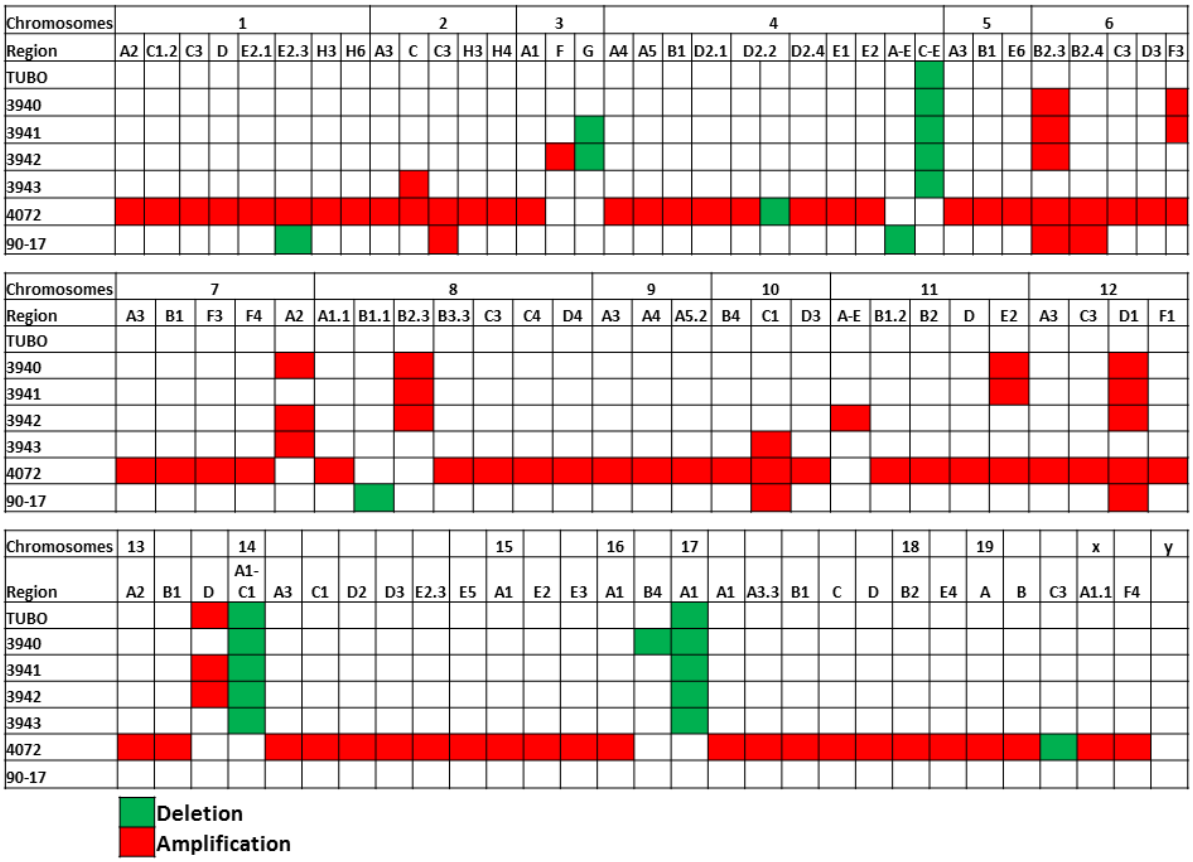


Fig 29: aCGH profile of DCC cell lines.

Tumor from the mice generated by the DCC sphere transplants were digested and isolated cell single cells were grown as adherent cultures to generate DCC cell lines. gDNA was isolated from the DCC lines and an aCGH was performed to obtain a genomic profile of the cell lines. (A) An aCGH profile of TUBO compared to DCC cell lines. Cell lines 3940, 3941, 3942 and 3943 were derived from tumors generated by spheres grown in the presence of TUBO. DCC cell lines 4072 and 90-17 were derived from tumors generated by spheres grown in the presence of TMECs and (B) an overview of genomic aberrations of all the cell lines. Red: Amplification, Green: Deletion

3.13. Cluster analysis of all the cell lines based on the aCGH data

The DCC cell lines were heterogeneous in their genomic profile and shared a few aberrations with TUBO. To see how similar the cell lines are to each other and to TUBO, a cluster analysis was performed applying Euclidean distance using the aCGH data of all the cell lines. As shown in figure 30, cell lines 4072 and 90-17 from the same experiment but were shown to be similar to each other. Although the spheres were injected in different mice and at different sites (table 6) they were clustered together. On the other hand 3940 and 3943 were similar to each other and

were also placed closer to TUBO suggesting these two cell lines (3940 and 3943) are more similar to TUBO than rest. 3941 and 3942 were placed away from the rest of the cell lines in two separate clusters implying that were different from the other cell lines. The DCC cell lines, 90-17 and 4072, derived from spheres grown in the presence of TMECs were closer to each other and also to TUBO. DCC cell lines 3941 and 3942 were clustered separately from each other and also from TUBO.

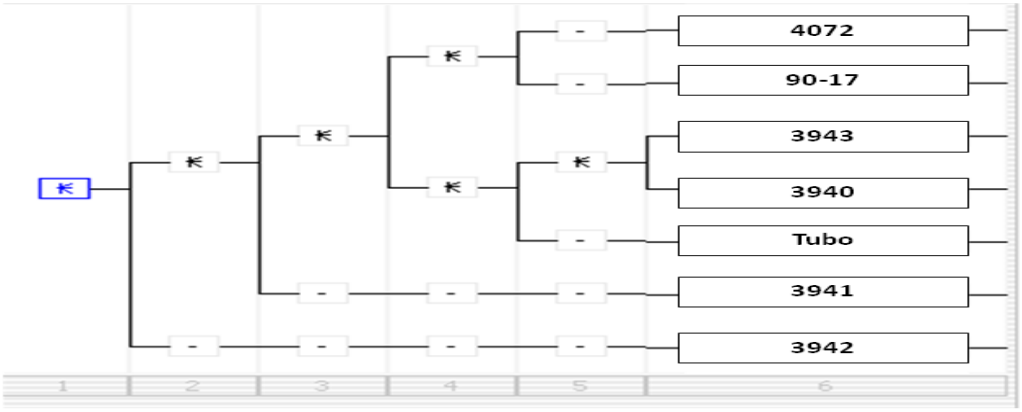


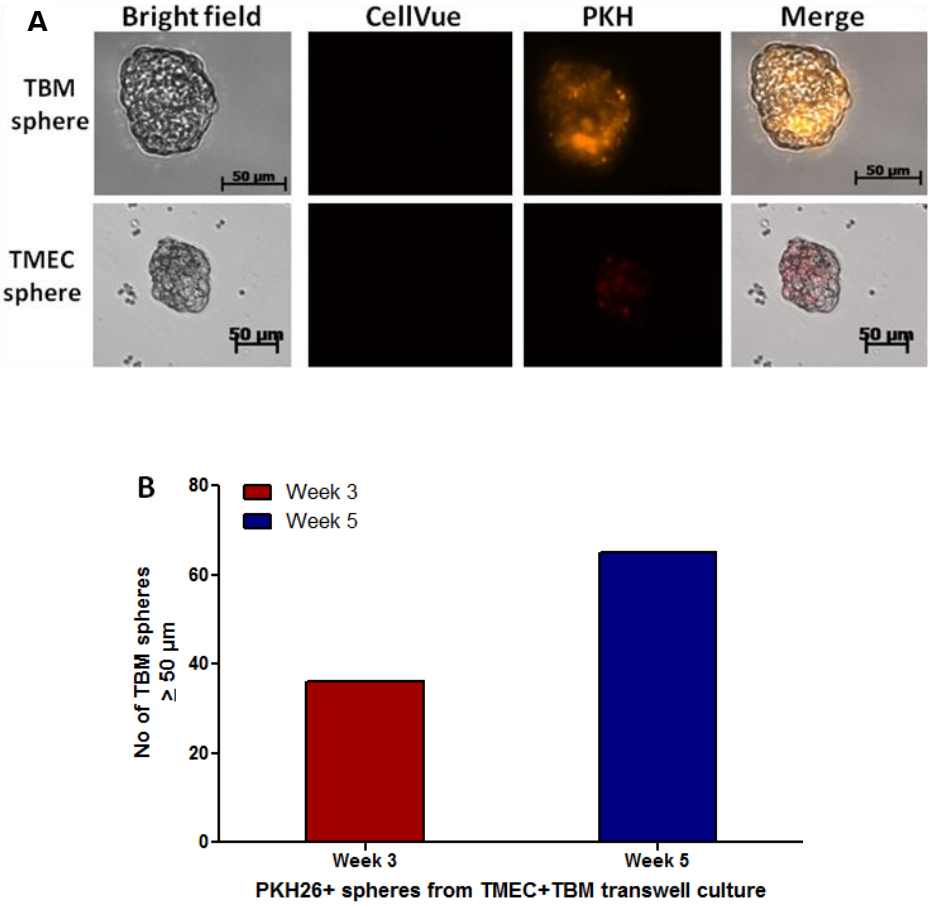
Figure 30: Euclidean distance or Manhattan clustering of the cell lines using the aCGH data.

The cell lines were clustered using the aCGH data of the cell lines using Euclidean distance. The distance between two clusters is calculated as the arithmetic mean of distances between all possible pairs from the two clusters. The clustering has two major groups, one with 3942 and the second with the rest of the cell lines. The difference between the groups is equal to length of the line joining them at the nearest node.

3.14. DCCs from BALB-neuT also form spheres in the presence of TMECs in a transwell setup

The DCCs cultured so far in a transwell setup were isolated from the BM of transplanted mice. Some of these mice in the both the transwell experiments had metastasis representing an M1 state of breast cancer. To examine if DCCs from BALB-neuT BM representing M0 stage disease can also propagate, BM of an 8 week old BALB-neuT mouse isolated enriched for EHS and cultured in the presence of TMECs isolated from the same mouse. The BM cells were stained with PKH26 and TMECs with CellVue, another unspecific cell membrane labeling dye which is visible under cy5 filter. The BM cells were seeded in the lower chamber and TMECs in the upper chamber (materials and methods 2.2.11). The transwell was observed every week for spheres. After 3 weeks spheres appeared in the BM chamber and spheres

≥50 µm in diameter were counted (figure 31 A and B) and later the transwell with TMEC was removed and further cultured for another 3 weeks. The number of spheres from week 3 to week 5 an almost increase by 2 folds, similar to the observations made in earlier transwell experiments. The spheres were picked and whole transcriptome amplification and genome DNA isolation was performed. The genomic DNA carried the rat Her2 oncogene (figure 31 C) and expressed epithelial markers CK8/18 and EpCAM (figure 31 D) confirming their epithelial origin. The spheres were injected in to mice to test their tumorigenic ability, but unfortunately the mouse died of lymphoma and *in vivo* tumorigenicity of these in vitro activated DCCs could not be evaluated. However, an aCGH performed on one of the spheres showed an aberrant genomic profile but completely different to TUBO and the DCC cell lines (figure 31 E). Nevertheless, the aberrant genomic profile of the sphere may suggest that the sphere contained tumor cells.



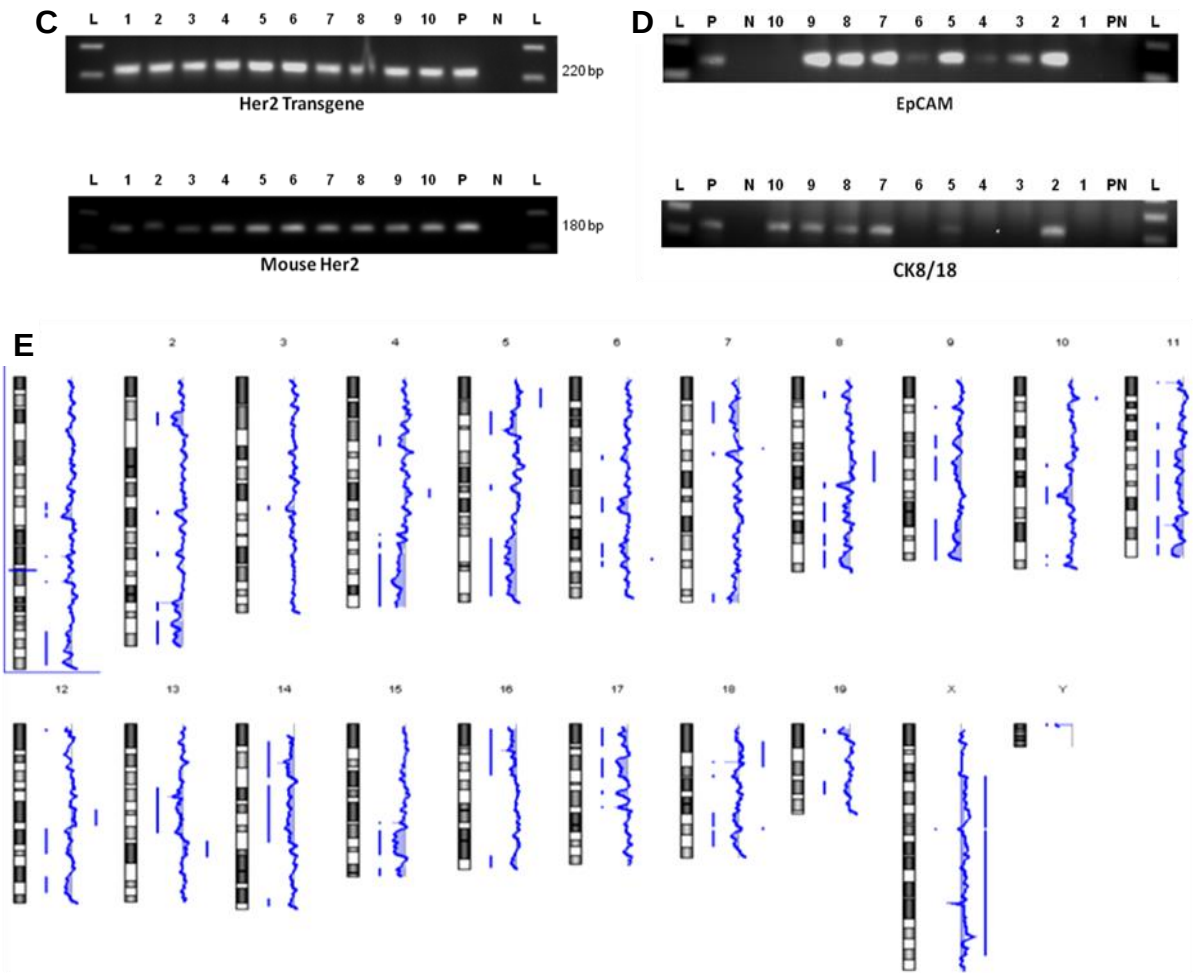


Figure 31. Transwell coculture of TBM of 8 week old mouse with TMEC.
Bone marrow from an 8 week old mouse was isolated, enriched for EHS and cocultured with TMEC isolated from the same mouse in a transwell. The EHS cells were stained with PHK26 and the TMECs with CV to differentiate both the cell types. Spheres with a diameter of 50 µm or above were counted. (A) BM spheres stained with PKH26 (PKH) and TMEC spheres stained with CellVue, (B) number of spheres counted on week 3 in the presence of TMECs and on week 5 after removing upper chamber containing TMECs (C) Her2 transgene in the genomic DNA (gDNA) of all the DCC spheres checked, (D) EpCAM and CK on cDNA libraries prepared from the DCC spheres. cDNA libraries and gDNA were prepared from the same spheres. (E) Array CGH profile of the DCC sphere 7 (from the PCR gel bands).

4. Discussion.

The main aim of this study was to 1) identify conditions suitable to propagate disseminated cancer cells (DCC) *in vitro* and 2) later expand them and generate cell lines. Limitations in studying DCC are that they are rare and there are no suitable markers to identify them. A major shortcoming in the progress of understanding these cells is the absence of a suitable *in vitro* model, a DCC cell line. It has been a challenge over the years to propagate these cells *in vitro*.

Prostate BM samples cultured in both adherent and non adherent conditions failed to proliferate although prostate tumor epithelial cells did generate spheres in similar conditions. Identifying optimal conditions for propagating patient derived bone marrow DCC was always challenging. Various factors such as amount of bone marrow available, sample positivity for DCC and number of DCCs present per sample including patient variability (M0 or M1 stage or gleason score) could influence the outcome of the results. For example, if the BM in culture does not produce any spheres, it could be because of 1) the absence of DCCs in the BM sample in culture, 2) the low number of DCCs in the sample in culture or 3) the culture conditions applied to propagate DCCs were not optimal. There were too many variables to consider. Thus, we examined DCCs in BALB-neuT, an established mouse model for breast cancer, to identify conditions suitable to propagate them *in vitro*. Studies in our group have shown that these mice contain DCCs in the bone marrow and can be detected by CK8/18 staining of the bone marrow cells from week 4 (Husemann, Geigl et al. 2008). Therefore, we found this mouse model suitable for isolating and establishing conditions for propagating DCCs and later expanding them *in vitro*.

4.1. Mammosphere assay and bone marrow culture

4.1.1. Mammosphere assay

DCCs are rare in the bone marrow, 1-10 per million BM cells (Husemann, Geigl et al. 2008 (Husemann, Geigl et al. 2008). Low DCC numbers per mouse limited us from testing different culture conditions on these cells initially. Furthermore, conditions to grow DCCs *in vitro* are not known. Thus, we first tried to optimize culture conditions

on murine mammary epithelial cells as spheres and later applied these conditions to culture DCCs *in vitro*. Studies have shown that non adherent serum-free cultures enrich for stem/progenitor cells (Dontu, Abdallah et al. 2003) and importantly it has been shown that fibroblasts cannot grow in serum-free non-adherent conditions (Kulkarni and McCulloch 1994). Furthermore, recent studies have shown that both DCCs and CTCs contain a population of cells expressing markers thought to be associated with stem/progenitor cells like CD44, ALDH in CTCs (Theodoropoulos, Polioudaki et al. 2010) and CD44, CD326 (EpCAM) and ALDH in DCCs (Reuben, Lee et al. 2011). Hence, the BM cells were grown in serum-free non-adherent conditions as spheres to prevent differentiation induced by serum and adherent conditions (Bertolero, Kaighn et al. 1986; Masui, Wakefield et al. 1986) and preserve the stem/progenitor cells. Mammary tissue digestion, epithelial cell isolation and mammosphere culture conditions methods were adapted and modified from previously published work (Liao, Zhang et al. 2007; Eirew, Stingl et al. 2008). While both the groups (Liao et al. and Eirew et al.) used FCS in their cultures, serum was omitted to avoid differentiation of DCCs. Conditions were first optimized to culture mammospheres from both BALB/c and BALB-neuT mammary epithelial cells *in vitro*. The optimized sphere medium contained cytokines HIL-6 (hyper interleukin-6) and GRO-α (growth related oncogene-α) in addition to EGF and bFGF. Addition of these two cytokines increased the sphere forming efficiency of mammary epithelial cells. IL-6 is a pro-inflammatory pleiotrophic cytokine which acts through its IL-6 receptor and two gp130 molecules activating JAK/STAT pathway and thereby promotes proliferation. Very few cells express IL-6r but all cells express gp130. HIL-6 is a fusion protein where IL-6 is fused to its soluble IL-6 receptor forming an IL-6/sIL-6r complex. This fusion protein can bind to gp130 and initiate gp130 mediated signaling cascade, called trans-signaling. Therefore, non responsive cells which lack IL-6 receptor also respond to HIL-6 as they express gp130 (Fischer, Goldschmitt et al. 1997; Rose-John, Waetzig et al. 2007). IL-6 was shown to play a role in inducing proliferation and is also involved in several inflammatory diseases and oncogenesis in breast cancers (Sansone, Storci et al. 2007), colon (Becker, Fantini et al. 2005), and prostate, reviewed in (Barton 2005). HIL-6 increased the number of mammospheres when added to the sphere medium. The number of spheres increased with the increase in the concentration of HIL-6 until 20 ng/ml. The number mammospheres reduced at 50 ng/ml. It could be assumed that the reduction of

spheres at 50 ng/ml was due to the fusion of spheres close to each other. However, this was not the case. The reduction in sphere numbers may have been due to the high concentration of HIL-6 which reduced the sphere forming efficiency of MECs. We therefore found 20 ng/ml HIL-6 to be optimal for sphere culture. GRO-α on the other hand is a CXC chemokine belongs to IL-8 family of cytokines and signals through its chemokine receptor CXCR2 activating MAPK through PI3K-Ras-Raf pathway. GRO-α is secreted by different cell types including melanoma cells, epithelial cells, neutrophils and macrophages. This chemokine is also involved in inflammation and wound healing (Luan, Shattuck-Brandt et al. 1997; Wang, Hendricks et al. 2006) and was also shown to play a role in melanoma and esophageal tumor progression (Luan, Shattuck-Brandt et al. 1997; Wang, Hendricks et al. 2006). GRO-α has a mitogenic effect on the neighboring premalignant epithelial cells and is a known potent epithelial cell growth factor (Coppe, Patil et al. 2010). Adding GRO-α to MECs in culture increased the number of mammospheres. The effect of GRO-α was as significant as HIL-6 had on MECs (figure 10). Both cytokines play a role in proliferation albeit acting through different growth promoting pathways. Therefore, addition of these two factors to the mammosphere culture resulted in an increased number of spheres when compared to either factor added alone. Further, when tested for their ability to induce sphere formation on fibroblasts, GRO-α and HIL-6 did not induce any sphere formation from freshly isolated mammary fibroblasts but the fibroblast cell lines did form spheres. Fibroblast cell lines have been cultured for years and may have evolved and adapted to survive even in suspension cultures. More importantly, the cytokines HIL-6 and GRO-α did not influence sphere formation in freshly isolated fibroblasts leading to false positives. In the context of the BM this was very crucial. Since, BM is a mesenchymal organ, it was essential that HIL-6 and GRO-α did not induce sphere formation in BM stromal or mesenchymal cells. These cytokines were therefore considered ideal in culturing DCCs *in vitro*.

4.1.2. Bone marrow DCC culture

Mammosphere conditions optimized for mammary epithelial sphere culture were tested to examine if these conditions could activate bone marrow DCC from BALB-neuT. Bone marrow DCCs in BALB-neuT express Her2 (Husemann, Geigl et al.

2008) and also EpCAM. Studies in BALB-neuT have identified Sca-1 (stem cell antigen-1) expressing cells enriched with tumor forming potential (Grange, Lanzardo et al. 2008). In yet another breast cancer mouse model carrying polyomavirus middle-T oncogene, a subpopulation of the DCCs that were largely responsible for dissemination and metastasis were found expressing Sca-1 (Weng, Penzner et al. 2012). Therefore, the bone marrow cells of BALB-neuT were enriched for Sca-1 in addition to EpCAM and Her2 (EHS) and thereby the DCCs in the BM confirmed by CK8/18. BALB-neuT BM cells either un-enriched or enriched for EHS cultured in the mammosphere medium failed to initiate growth in the DCCs. Although, the number of CK+ cells, identifying the DCCs, increased after enrichment with EHS they did not grow in the presence of GRO-α and HIL-6. Serum contains many factors that induce growth in cells and is routinely used in cell culture and, RPMI is a routine cell culture medium known to favor the growth of epithelial cells (Azzi, Bruno et al. ; Bussolati, Bruno et al. 2008). Since DCCs are epithelial derived, the BM cells were cultured in RPMI and FCS however, these conditions also could not trigger growth in DCCs. Even after 6 weeks in culture the DCCs failed to proliferate under these conditions. In conclusion, the conditions optimal for mammosphere formation from mammary epithelial cells are not optimal for DCC activation. Experiments evaluating the effect of BM in mammary epithelial cells (MEC) revealed that both BALB/c and BALB-neuT BM suppressed the growth of MECs. This suppressive effect however, was more significant in the presence of TBM than NSG BM cells on MECs. The suppressive effect of TBM and NBM conditioned medium on MECs suggests a role for BM cell secreted factors. It also suggests that the suppressive effect may not be a response of TBM cells due to the presence of DCCs or primary tumor in the BM but an innate property of the BM as NBM also had a similar effect on TMECs. NSG mice are deficient in mature T cells, B cells and natural killer cells and in several cytokine pathways (Shultz, Schweitzer et al. 1995; Shultz, Lyons et al. 2005). It has been reported that some of the immune cells secrete cytokines which exert a suppressive effect on the epithelial cells, especially the T-cells, like TGFβ, IFN, Oncostatin M and other factors (Huse, Quann et al. 2008). Since BM from NSG mice are deficient in T-cells, TMEC growth was not as affected as observed in NBM or TBM probably because they lacked secreted factors from T cells with growth inhibitory effect.

Sphere forming assay is a method used for enriching stem/progenitor cells, however this assay may not be suitable to activate quiescent cells resting in a G0 state. In one

of the studies published, quiescent neural stem cells were unable to form spheres in standard culture conditions and it was suggested that this could be due to the absence of key components of an *in vivo* niche that is required to activate the dormant stem cells (Pastrana, Silva-Vargas et al. 2011). Another possible explanation why DCCs were incapable of forming spheres in the mammosphere conditions could be that the *in vitro* conditions to activate DCCs may have lacked the key components of an *in vivo* niche.

Our results from coculture suggest that DCCs proliferate in the presence of primary mammary epithelial cells in direct co-cultures or in the presence of tumor cells in transwells. In the first instance, the activation of DCC in the cocultures could be attributed to cell-cell interactions and possibly through gap junctions. Gap junctions not only connect the neighboring cells but also aid in diffusion of small molecules such as amino acids, ions and nucleotides (Lampe and Lau 2004). In breast cancer, gap junctional intercellular communication has been shown to play an important role in providing cross-talk between MECs and cells in their surrounding microenvironment (El-Saghir, El-Habre et al. 2011). Connexins which form gap junctions not only act as channels between neighboring cells but also as signal complexes with a crucial role in regulating cell function and transformation. However, there is a caveat that goes along with the co-cultures, that is too many PKH26+ spheres. Since contact between the cells cannot be avoided in co-cultures, spheres containing both DCC and NMEC cells are possible leading to false positives.

In a transwell assay, DCCs and tumor cells were separated by a membrane with a pore size of 0.4 µm which allows only soluble secreted factors to pass through thereby preventing any intercellular communication. In this case, the activation of DCCs could have been through factors secreted by tumor cells of both TMEC and TUBO. Tumor cells are known to secrete many factors with proliferative effect on other cells. Proteomic studies of tissue interstitial fluids from breast tissue have shown that primary tissue secretes several cytokines which have an influence on cell proliferation. In tumor interstitial fluids, the secreted factor concentrations were higher in comparison to the normal breast tissue for some of the cytokines which are potential mitogens (Celis, Gromov et al. 2004). So, activation of DCCs in transwells was due to the factors secreted by TMEC or TUBO cells with growth inducing effect. Surprisingly, the normal mammary cells failed to activate DCC in a transwell

condition. Activation of DCCs by NMEC may require either 1) cell-cell interactions or 2) factors secreted by the cells in higher concentrations.

It was shown that during EMT epithelial markers like cytokeratins and EpCAM are down regulated in disseminating or circulating cancer cells (Mikolajczyk, Millar et al. 2011; Gorges, Tinhofer et al. 2012). According to these studies, the number of DCCs could be higher than those detected by CK staining. However, our data suggests otherwise (table 8). In transwell cultures the number of DCC derived spheres never exceeded the number of CK8/18 positive cells detected per million EpCAM, Her2 or Sca-1 enriched cells. These results are still preliminary and need to be examined further.

4.2. Functional and molecular characterization of the *in vitro* activated DCCs

Although DCC presence in the BM predicts poor outcome, not all DCCs are capable of initiating metastasis (Klein 2009) . Proliferation of DCCs *in vitro* does not imply that they are tumorigenic. However, the *in vitro* proliferating DCC spheres were tumorigenic *in vivo* suggesting that a population of activated DCCs had tumor initiating cells in the spheres that were transplanted in mice. The cells isolated from the *in vivo* tumors when cultured could expand *in vitro* in routine cell culture conditions to generate continuous cell lines. RPMI + 10% FCS has been used by many groups especially to maintain epithelial cells. RPMI was shown to favor the growth of epithelial cells. In human renal cell carcinomas, when CD105+ cells were cultured in RPMI + 10% FCS they differentiated in to epithelial cells confirmed by expression of epithelial markers CK7, pan CK and E-cadherin (Azzi, Bruno et al. 2011). DCC cell lines were generated by combining low centrifugation speeds for epithelial cell isolation (see materials and methods) and serum-free medium for eliminating stromal or fibroblast contamination (Kulkarni and McCulloch 1994). Morphological examination of the DCC cell lines generated contained minimal stromal cell contamination and CK8/18 and Her2 staining of the cells was comparable to CK8/18 staining in TUBO cells. TUBO cell line is an epithelial cell line derived from primary tumors of BALB-neuT and is positive for both CK8/18 and Her2. The DCC cell lines showed cobblestone morphology in culture growing as tight compact colonies, similar to TUBO cell line cultures, typical for epithelial cell lines in

culture. The tumors, the isolated tumor cells and the cell lines expressed CK8/18 confirming their epithelial identity moreover, the presence of Her2 transgene in spheres and its expression in tumors and cell lines is an evidence for their DCC origin. Array CGH of DCC cell lines showed that they shared a few aberrations with TUBO cells. However, many genomic aberrations were unique to the DCC cell lines. The DCC cell lines were heterogeneous in their aberrant genomic profile. Cell lines derived from DCCs propagated in the presence of TUBO shared deletions on chromosome 4 and 14 with TUBO cells. On the other hand DCC cell lines 90-17 and 4072 cell lines were derived from DCCs cultured in the presence TMECs were different to the rest of the DCC cell lines. Cell line 90-17 established from tumors generated by sub-cutaneous and 4072 from mammary fat pad transplantation of DCC spheres. DCC cell line 4072 was highly aberrant with aberrations seen on all the chromosomes with only one deletion on chromosome 4. Cells from 90-17 were quite different from 4072 and were less aberrant but deletion on chromosome 4 was common to both the cell lines. Cluster analysis showed that, 4072 and 90-17 were closer to each other and to 3940 and 3941 than 3942 and 3943. Furthermore, 3940, 3943, 4072 and 90-17 were grouped together with TUBO. The DCC cell lines 3940 and 3943 were closer to TUBO than 4072 and 90-17 while 3941 and 3942 were clustered in two separate groups and were away from the rest of the cell lines suggesting they are different from TUBO and other DCC derived cell lines. TUBO cell line was established from the primary tumors of BALB-neuT. Since DCCs are also derived from primary tumors of BALB-neuT, it is possible that a few aberrations are shared between the TUBO and the DCC cell lines.

The DCC cell lines were derived from transplanted mice which had metastasis representing M1 stage of breast cancer, where one could argue that the DCCs were already in a proliferating state and hence were able to propagate *in vitro* and *in vivo*. However, DCCs from 8 week old BALB-neuT, representing M0 stage, when cultured in the presence of TMECs in a transwell were also able to proliferate and form spheres. Unfortunately, the mice transplanted with the DCC spheres of BALB-neuT mouse died of lymphoma preventing us from verifying their ability to initiate tumors *in vivo*. Still, BALB-neuT BM sphere shared some aberrations with TUBO and the DCC cell lines, especially on chromosomes 4 and 14. The aberrant genomic profile

indicates that the sphere has tumor cells but their tumorigenic ability still requires to be validated.

It was unfortunate that the mouse injected with DCC spheres died of lymphoma and *in vivo* malignancy of the spheres could not be evaluated. Array CGH performed on one of the DCC spheres showed an aberrant genomic profile. Furthermore, the DCC sphere shared aberrations on chromosomes 4 and 14 with TUBO and the DCC cell lines, 3940, 3941, 3942 and 3943. This suggests that sphere contained tumor cells but their tumorigenic ability still needs to be evaluated.

Together, in the current doctoral project we have shown that the conditions optimal for the growth of mammospheres *in vitro* are not optimal for *in vitro* propagation of bone marrow DCC. This study shows that 1) bone marrow has a suppressive effect on mammary cancer cells and 2) BM derived disseminated cancer cells from the mouse can be propagated *in vitro* in the presence of tumor cells. We could also show that the DCC activation could be mediated through tumor cell secreted factors in a transwell assay. The activated DCCs were tumorigenic *in vivo*, could further expand *in vitro* as cell lines and displayed an aberrant genomic profile suggesting that these were tumor cells.

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