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Reprogramming Cancer Cells *In Vivo*

Brian W. Booth[1]*, Sonia M Rosenfield[2] and Gilbert H Smith[2]

[1]*Institute for Biological Interfaces of Engineering, Clemson University, USA*
[2]*Mammary Stem Cell Biology Section (BRL), Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA*

Abstract

Tissue microenvironments have tremendous influence on both local cells and the surrounding tissues. Signals originating from the local microenvironment, both chemical and physical, help to regulate cell and tissue functions including proliferation, differentiation, wound healing, and tumorigenesis. Tumorigenesis is often defined as the result of multiple mutations that provide a growth advantage and lead to clonal expansion of a mutated population. Evidence is accumulating that demonstrates that local microenvironment impacts the behavior of cancer cells by favoring or inhibiting tumor progression. This review will discuss studies that demonstrate the potential of the mouse mammary microenvironment to reprogram tumor-derived cells into cells that contribute to the formation of a functional, tumor-free, mammary outgrowth. Mouse and human tumor cells, derived from different species and tumor types, are incorporated into regenerating mammary structures and differentiate in luminal, myoepithelial, and milkproducing secretory cells when incorporated into a competent mammary niche. These findings demonstrate that human or mouse cancers independent of their origin or differentiation state retain a subpopulation of cells with stem/progenitor activity that respond to the signals of a normal microenvironment and contribute their progeny to normal development, which suppresses their malignant phenotype. During this process, the normal mouse mammary cells are able to supply paracrine signals necessary for normal mammary gland development such as steroid receptor signals that the human and mouse cancer cells can not.

Keywords: Mammary; Niche; Reprogramming; Stem cell; Human/mouse cancer cells; Microenvironment; Tissue regeneration

Introduction

Pluripotent stem cells have the ability to self-renew and differentiate to form various cell types. The applications in which these cells can potentially be used are vast and include repair and regeneration following injury or disease. In order to avoid rejection by a patient's immune system, autologous cell based therapies are preferred. However the numbers of pluripotent stem cells within a given body are limited. Efforts have been made to create or expand the numbers of pluripotent stem cell and such efforts involved somatic cell nuclear transfer (SCNT). SCNT is a method used to revert a somatic cell to a totipotent state [1-4]. A similar early method of reprogramming somatic cells involved fusion of somatic cells with pluripotent cells. The main drawback of this method is the resulting pluripotent fused cell is tetraploid [5].

More recently a new approach to reprogramming somatic cells has been developed in which somatic cells are induced to express transcription factors (TFs) ectopically. Cells induced to express the TFs c-Myc, Klf4, Oct4, and Sox2 (or Nanog and Lin28 in lieu of c-Myc and Klf4) have been termed induced pluripotent stem cells (iPSCs) [6-8]. The ultimate goal using iPSCs is to devise differentiation strategies that drive these pluripotent cells toward a desired lineage for use in patient-specific treatments or new drug evaluation.

All three of the techniques listed above involve *in vitro* manipulation of cells. This chapter will focus on a fourth technique of reprogramming cells to adopt a different phenotype without the addition of exogenous factors or excessive *in vitro* manipulations. Cells are transplanted into an existing normal microenvironment where they receive the local, endogenous signals that drive cells to reprogram and develop a phenotype that matches the new microenvironment. This results in cells of a different origin being reprogrammed and incorporated into the local microenvironment with no deleterious effects on either the reprogrammed cells or the surrounding tissue.

Normal Microenvironment

The normal mammary gland is a diverse organ comprised of many cell types, each contributing to the overall dynamics of the environment. Epithelial, myoepithelial, endothelial, nerve cells, fibroblasts and adipocytes all influence the local microenvironments, niches, of the mammary gland through secreted factors, including extracellular components, and physical cues through cell-cell contacts [9]. The cells and associated physical and biochemical signals constitute the mammary stem cell niche or microenvironment. Somatic epithelial stem cells are present throughout the mammary gland as demonstrated by the fact that any portion of mammary epithelium, independent of age, can reform a functional mammary gland when transplanted into an epithelium-divested gland of a host animal [10-13].

Using this model it has been demonstrated that the mammary microenvironment can reprogram somatic stem cells originating in different tissues including testes, neural tissues and bone marrow [13-15]. Presumed stem cells isolated from these tissues are transplanted in concert with normal mammary epithelial cells (MECs) in predetermined ratios into epithelium-divested mammary glands where they reconstruct mammary epithelial stem cell niches. It is within these new niches that the foreign cells are incorporated, proliferate and differentiate into all mammary epithelial subtypes including milk secretory epithelial cells.

***Corresponding author:** Brian W Booth, Ph.D., Institute for Biological Interfaces of Engineering, Clemson University, 401-1 Rhodes Engineering Research Center, Clemson, SC 29634 USA, Tel: 864-656-4693; Fax: 864-656-4466; E-mail: brbooth@clemson.edu

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Evidence exists indicating that a normal microenvironment has the potential to inhibit tumor formation through the regulation of cancer cell within the niche [16]. Multiple animal models have demonstrated that tumorigenesis only proceeds in a tumor-permissive microenvironment [16,17]. When tumorigenic cells are located in a tumor non-permissive environment, for example during tissue development, tumorigenesis occurs less readily [18,19].

Interestingly, a specific correspondence between the microenvironment and the cancer cells is necessary for the malignant phenotype to be suppressed [20,21]. Within blastocysts, malignant transformation due to the injection of tumorigenic cells is suppressed only if the cancer cells are in contact with the trophectoderm cell layer [21]. The blastocyst is only able to suppress tumor formation when the number of tumor cells injected is below a given threshold [22]. These early studies indicate that there is a balance between the number of tumor cells and the surrounding microenvironment [23]. If the number of tumor cells is maintained below a threshold level, tumorigenesis is checked. However if the tumor cell number is able to break through the threshold, the physical and biochemical signals of the surrounding cells is unable to maintain the check and tumor formation and expansion proceeds [21].

Reprogramming Cancer Stem Cells

Cancer cells contribute to the regeneration of tumor-free mammary glands and respond to normal tissue-specific developmental signals.

Using the previously described technique of mammary tissues transplantation into the epithelium deprived mammary fat pad of juvenile immuno-compromised female mice [10-12], dispersed cells derived from both mouse and human tumors were found to respond to the mammary tissue specific signals to form a normal and functional mammary gland [24-26]. Tumor-derived cells were transplanted in concert with normal MECs in specific ratios [13-15,24-27]. In some cases, the recipient mice were allowed to complete a full-term pregnancy to allow observation of the fully mature and functional mammary structure [13-15,24-27].

The human analog of neu is HER2 [28]. Amplification and overexpression of HER2 is observed in 20-30% of human breast cancers and is inversely correlated with patient survival [29-31]. The mammary tumors that arise in these mice display similar features as HER2+ human breast cancers; thus this mouse model is an accepted model of human HER2+ breast cancer [32].

Cells were isolated from mammary tumors that arose in MMTV-neu transgenic mice that overexpress the neu (erbB2) oncoprotein [24,33]. In genetically engineered strains of mice that are highly susceptible to mammary tumorigenesis and exhibit accelerated tumor development in postpartum or multiparous females, the parity-identified, long-lived mammary epithelial cells (PI-MECs) serve as targets for neoplastic transformation [34,35]. PI-MECs are lobule-limited progenitors cells in the mammary stem cell hierarchy [34-37]. Int3/Notch4 tumorigenesis does not involve the PI-MECs [38].

The tumor-derived MMTV-neu cells were incorporated into the forming tumor- free mammary gland; proliferating and differentiating into both luminal epithelial cells and myoepithelial cells [24]. The redirected MMTV-neu cells did not differentiate into estrogen receptor (ER) or progesterone receptor (PR) expressing cells within the tumor-free mammary outgrowths (Figures 1A and 1B) [24]. Mammary tumors that arise in this transgenic model, as well as in parallel experiments where only MMTV-neu were transplanted in the epithelium divested mammary fat pad, are ER-negative and PR-negative by immunohistochemistry [33,39].

The cells derived from MMTV-neu-induced mammary tumors continued to express erbB2 however the active phosphorylated receptor was not detected [24]. Both ER and PR are required for the normal development of a normal mouse mammary gland [40-43]. However, the incorporated MMTV-neu cells formed functional milk protein secretory cells when the host animal was pregnant (Figure 1C). These results demonstrate not only that MMTV-neu tumor derived cells retain plasticity but also that signals that originate from the normal mammary microenvironment, including those emanating from ER+ and PR+ normal mammary cells, contribute to the reprogramming of the tumorigenic MMTV-neu cells.

In experiments using cell lines derived from human tumors similar results were obtained [25,26]. Cell lines derived from human testicular carcinoma (pluripotent NTERA2 cells) or triple negative breast cancer pleural effusion (MDA-MB-468, MDA-MB-231BRMS and MDA-MB-231 cells) were mixed with normal mouse MECs in fixed ratios and transplanted in the epithelium divested fat pad of athymic mice [25,26]. Again the tumorigenic phenotype of the human cancer cells was confirmed in experiments done in parallel where tumor cells were transplanted at low density in the absence of MECs [25,26]. Immunostaining for the human stem cell marker CD133, fluorescent in situ hybridization (FISH) for human specific genes, magnetic sorting for the surface expression of the human specific CD133 marker, and PCR analysis for the human specific sequence of the SRY gene identified NTERA2 cells within the tumor-free mammary outgrowths

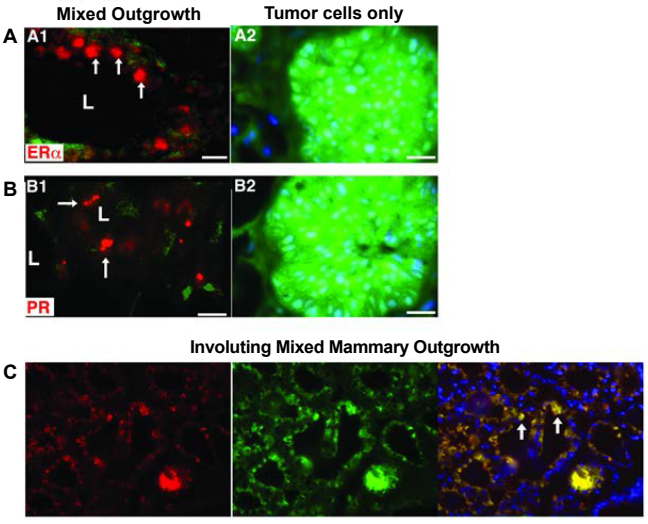

Figure 1: Tumor-derived cells differentiate into different cell lineages and contribute to the formation of a functional mammary gland. 1.0K tumor-derived cells were mixed with 50K normal FVB/N mammary epithelial cells and injected into the cleared fat pad of Nu/Nu female hosts. Tissue sections of mixed mammary outgrowths and mammary tumors were stained for β- gal (green) and ERα or PR (red). A1) Mixed mammary outgrowth showing ERα expression (red) indicated by arrows only in β-gal- cells. A2) Cross section of a β-gal+ mammary hyperplasia that is ERα-. B1) Mixed mammary outgrowth showing PR expression (arrows) only in β-gal- cells. B2) Cross-section of a β-gal+ mammary hyperplasia that is PR-. C) Cross section of a mixed mammary outgrowth at Day 2 postpartum demonstrating that tumor-derived cells, determined by β-gal (green) expression, become functional secretory epithelial cells and produces the milk protein β-casein (red). Arrows indicate tumor-derived cells that are producing β-casein. L-lumen. Sections A2, B2 and C were counterstained with DAPI. Scale bars = 20μm (A, B), 10μm (C). Figure from Booth et al., 2010.

(Figure 2A) [25]. In order to determine if human cancer cell progeny formed secretory mammary epithelial cells, mammary outgrowths were removed following a full-term pregnancy at Day 2 of lactation [25,26]. Outgrowths were found to completely fill the mammary fat pad and exhibited extensive development of secretory acini [25,26]. When the host animals that received the tumor-free NTERA2/MEC transplants became pregnant, the differentiated male NTERA2 cells identified by human-specific immunocytochemical staining of CD133 gave rise to both basal and secretory mammary cells that produced the human milk proteins α-lactalbumin and lysozyme and did not show increased ploidy (Figure 2 A4, B-D) [25]. These findings provide evidence that human cancer cells are redirected from their malignant phenotype to differentiate into a "normal" mammary epithelial cell progeny during the regeneration of the mammary gland. Interestingly, the differentiated male NTERA2 cells retained the expression of the marker CD133 as the tumor cells.

In normal mammary outgrowths comprised of human breast cancer cells and normal mouse MECs, CD44 enriched as well as CD44 depleted human breast cancer cells differentiated into luminal

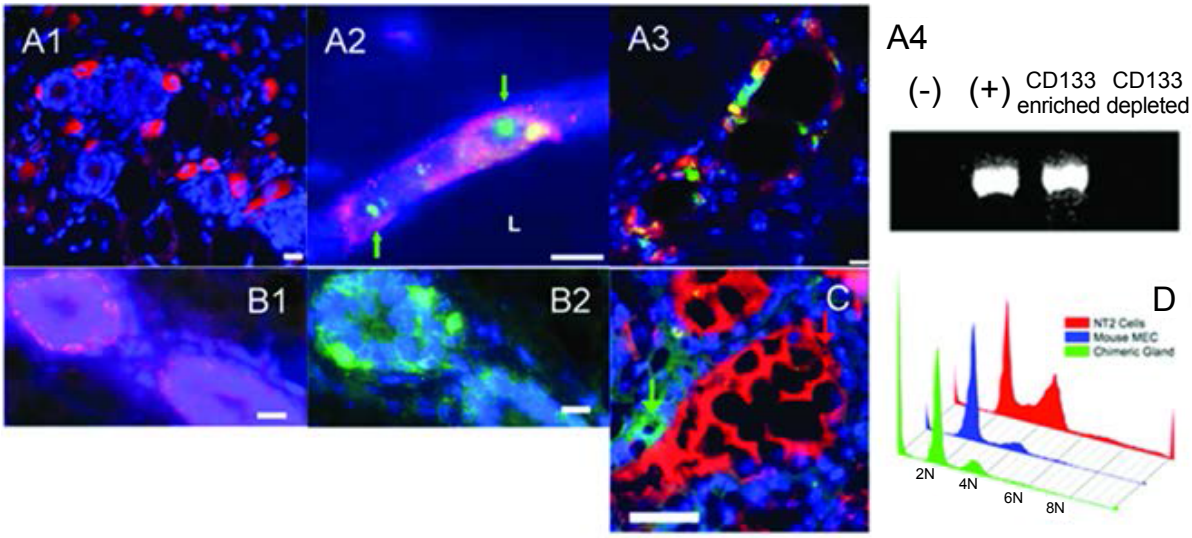

Figure 2: Human cancer cells identified in the tumor-free mixed mammary outgrowths are hormonal responsive, secrete human milk proteins in the mammary ducts and do not show increased ploidy. A1) Human-specific immunocytochemical staining for CD133 (red) shows CD133-positive NTERA2 cells present within the confines of the fat pad containing regenerated mammary ducts. A2) Human-specific fluorescent in situ hybridization (green, nuclear; identified with green arrows) and human-specific immunocytochemical staining for CD133 (red) shows NTERA2 cells present within the mammary outgrowths. A3) CD133-positive NTERA2 cells (red) differentiate into ERα (green) luminal epithelial cells. A4) PCR shows human specific Y chromosome present in NTERA2 cells prior to transplantation (+) as well as in the CD133enriched fraction obtained by magnetic sorting but not in normal mouse mammary epithelial cells (-) and the CD133depleted fraction. B1-B2) Basal cells express human K14 (red) and mouse K14 (green) in consecutive sections of the same duct. C) Immunocytochemical staining of mixed mammary outgrowth for human α-lactalbumin (green) and mouse caseins (red). D) Flow cytometry of propidium iodide stained cells demonstrate that mixed mammary outgrowths (green) do not contain a greater proportion of cells with abnormal ploidy as compared to cultures of mouse mammary epithelial cells (blue) or NTERA2 (red). All sections are counterstained with DAPI (blue). Scale bars A1) 20 μm, A2) and A3) 15 μm, B1) and B2) 10 μm, and C) 25 μm. Figure from Rosenfield and Smith, 2013.

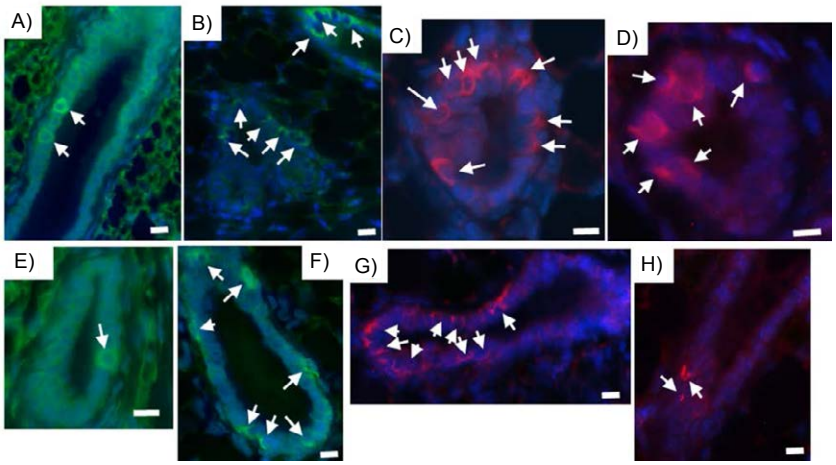

Figure 3: CD44 enriched as well as CD44 depleted human breast cancer cell populations are able to integrate and contribute their progeny to mixed mammary outgrowths. Immunocytochemical staining of mixed mammary outgrowths obtained from the inoculation of either CD44 enriched or CD44 depleted MDA-MB-231-GFP breast cancer cells (10K) and mouse mammary epithelial cells (50K) A, E) Human keratin 8 (green); B, F) human keratin 14 (green); C, G) mouse keratin 14 (red); D, H) human mitochondria (red). A-D) CD44 enriched; E-H) CD44 depleted. Scale bars = 10 μm. Figure from Rosenfield and Smith, 2013.

mammary epithelial cells, basal epithelial cells, and myoepithelial cells as determined by expression of human keratin 8, keratin 5, or keratin 14 respectively (Figure 3) [26]. These findings led to the conclusion that human breast cancer cells independently from their metastaic state retain a cell population that can give rise to ductal and alveolar progenitors that are able to respond to the differentiation stimuli of the regenerating mammary gland *in vivo*.

When transplanted with normal MECs, the NTERA2 cells differentiated into ER and PR expressing cells (Figures 2 and 3) [25]. The human breast cancer cell lines did not express ER or PR in resulting mammary outgrowths [26]. Presumably, the lack of nuclear receptor signaling of the human cancer cells is overcome by the signaling of the surrounding normal nuclear receptors expressing mammary epithelial cells. Interestingly, the percentage of primary outgrowths derived from human breast cancer cells/MEC mixed ratio cell population (≥ 50%) was lower compared to the percentage of primary outgrowths derived from NTERA2/MEC mixed ratio cell population (> 80%) [25,26]. It is possible that the differentiated state of the human breast cancer cells is the cause for the decreased efficiency of the primary outgrowths generation.

Not only were cancer cell progeny from both mouse and human tumors identified in primary mammary outgrowths but also in secondary mammary outgrowths resulting from transplantation of tissue fragments of the primary outgrowth into the cleared fat pads of new recipient mice. No tumors arose in any secondary transplantation [24-26]. It was observed that the number of human cells in the secondary mammary outgrowth was significantly higher than the number in primary outgrowths [25]. An estimated a 60-660-fold increase in the number of human cancer-derived cells in the secondary outgrowths compared to the primary outgrowths occurred [25]. Interestingly, when the MMTV-neu transgenic cells that were incorporated into normal mammary outgrowths were isolated from the normal mammary epithelial cells by magnetic cell sorting based on expression of erbB2, and then transplanted alone into the epithelium-divested mammary fat pads, mammary tumors arose (Figure 4) [24]. This suggests that signals arising from the normal MECs are required for the reprogramming of the tumor-derived cells.

Additional work showed that the signals that originate from the MECs are able to drive differentiation of not only tumor derived cells to a mammary phenotype but also of cells of different origins such as epithelial, bone marrow and neuronal cells derived of other epithelial and mesenchymal origins [13-15,24-27].

Overall, these data demonstrated that tumors independent from their origin (mouse or human), differentiation (embryonic or somatic) or metastatic state contain a subpopulation of cancer cells that are able to self-renew, integrate, and contribute their progeny to the tumor-free mammary outgrowths during transplantations studies.

Cell-cell fusion

One issue brought forward regarding these results is the possibility of cell-cell fusion. Cancer cells can combine with normal somatic cells during metastases and during normal tissue development and regeneration normal somatic cells such as myoblasts and macrophages are known to engage in cell-cell fusion [44,45]. Human —mouse cell fusion has been a factor in accurate interpretation of results in tissue regeneration experiments where human cells have been transplanted into murine host animals [46-48].

In experiments outlined above, evidence indicates that cell-cell fusion is not occurring. FISH analysis demonstrates that cells containing XY chromosomes (male) are next to XX cells (female) [25]. Analysis of DNA content shows that cells recovered from the mammary outgrowths only contain 2n or 4n; no aneuploidy or polyploidy was evident in any cellular preparations when either cancer–derived cells or normal cells of non-mammary origin were used in the mixing transplantation experiments (Figure 2D) [14].

Niche signals influence tumor-initiating cells

At one time cancer was believed to arise from clonal expansion [49,50]. This may be true for a small subset of tumors but evidence is mounting indicating that most tumors are comprised of heterogeneous cell populations representing different stages of differentiation [51,52]. Each stage of differentiation is characterized by different capacities for self-renewal, proliferation and expansion, and differentiation [51,52]. Within the heterogeneous population of cells in a given tumor there exists a subset of cells termed "tumor-initiating cells" (TICs) or "cancer stems cells" (CSCs) [52]. This rare population of cells has garnered much attention in recent years as efforts to isolate and study these cells have intensified. TICs represent an undifferentiated cell population thought to be responsible for tumor formation, progression, and recurrence. The TIC population possesses the stem cell characteristic of self-renewal leading to the name CSC [52].

Genomic profile comparisons between normal epithelial cells of the colon and colon cancer cells revealed similarities suggesting that the colon cancer cells retain at least the potential of a normal phenotype even when in a cancer-prone environment [51].

Both human and mouse cancer cells of embryonic (NTERA2) or somatic (MMTV-neu, MDA-MB-231) origin respond to the signals originating from the mammary niche resulting in the differentiation of the cancer cells thus reducing their tumorigenic potential [25,26]. Signals initiated by MECs are required for the reprogramming of the cancer cells. When the cancer cells are transplanted without the MECs, tumors arise indicating that signals from the mammary stroma that includes adipocytes, endothelial and fibroblasts alone are not sufficient to induce the reprogramming of the cancer cells [24-26]. Breast cancer cells enriched for the CSC marker CD44 have greater tumorigenic potential compared CD44-depleted cells [53]. When breast cancer cells are sorted based on expression of CD44 and placed in non-adherent culture conditions, an *in vitro* stem cell growth assay, the CD44-depleted population demonstrated a delay in sphere formation compared to the CD44+ fraction [26]. However there was not a significant difference in overall number of spheres formed. Both the CD44+ and CD44-depleted fractions were reprogrammed when transplanted with normal MECs but the CD44-depleted fraction showed a decreased proliferation rate [26]. These observations indicate one of two possibilities: 1) TICs are more readily reprogrammable or 2) CD44+ reprogrammed cells have a higher proliferative rate. Since CD44 is an accepted cancer stem cell marker and stem cells are believed to be relatively quiescent, the second possibility is less likely.

In mammary outgrowths comprised of normal MECs and MMTV-neu tumor-derived cells, the transgenic erbB2 is not phosphorylated [24]. Hyperactivation of erbB2 is responsible for tumor formation in MMTV-neu transgenic mice and in human HER2+ breast cancer [29-32]. The inactivation of erbB2 in mammary outgrowths comprised of normal MECs and MMTV-neu tumor-derived cells is proposed to be a mechanism involved in the reprogramming of the MMTV-neu cells in the mammary microenvironment. The data indicate that the expression and phosphorylation patterns of EGFR are not altered in the tumor-

derived cells that are redirected during mammary regeneration unlike the transgenically overexpressed erbB2 which experiences attenuated phosphorylation [24]. Phosphorylated EGFR is also prevalent in erbB2-induced human breast tumors [55]. ErbB3 is required for erbB2-induced neoplastic changes in breast epithelium [56] while erbB2 is required for erbB4 activation by erbB4-specific ligands in breast cancer cells [57]. ErbB2 tyrosine kinase activity is required for neuregulin-2b to induce proliferation while tyrosine kinase activity of neuregulin-2b's preferred binding partner erbB4 is not necessary [57]. Changes in the activation of these receptors will alter the activity of intracellular signaling cascades involved in tumorigenesis and/or homeostasis. The erbB2/erbB3 heterodimer acts as the oncogenic unit driving proliferation of breast cancer cells [58]. Both erbB2 and erbB3 rely on heterodimerization for activation as there are no conventional ligands that bind erbB2 and erbB3 does not have intrinsic kinase activity [59]. In breast cancer patients the presence of erbB4 is associated with increased sensitivity to Herceptin [60]. Herceptin targets erbB2 but not erbB3, therefore the presence of erbB4 leads to competition in dimerization leading to more erbB2/erbB4 dimers that initiate differentiation; as opposed to erbB2/erbB3 dimers that initiate growth and proliferation [59]. Preliminary results indicate that within MMTV-neu-induced mammary tumors erbB3 is active but in reprogrammed mammary outgrowths little phosphorylated erbB3 is present (data not shown). These observations match *in vitro* experiments where MMTV-neu cells are mixed with the normal mouse mammary epithelial cell line COMMA-D in ratios used in transplantation experiments. Immunostaining and Western Blot analyses indicate a decrease in activation of both erbB2 and erbB3 in MMTV-neu cells cultured in the presence of the COMMA-D cells (data not shown). This reduction in activation is proportional to the number of cancer cells and normal cells seeded adding further proof that normal MECs provide signals that reprogram cancer cells.

Ongoing experiments are further investigating the mechanism through which the normal microenvironment suppresses tumorigenesis and if the tumor cells can be terminally reprogrammed to a normal phenotype. In an attempt to answer the question of whether the tumor-derived cells undergo permanent or just temporary changes by the tumor non-permissive microenvironment, MMTVneu- positive cells were isolated by magnetic sorting from secondary mammary outgrowths based on surface expression of erbB2 and subsequently transplanted in the cleared fat pad without normal MECs [24]. Only the recovered reprogrammed MMTV-neu-positive cells formed mammary tumors suggesting that the reprogrammed cancer cells retain their tumorigenic potential and that their inherent tumorigenicity is attenuated through interaction with the normal mammary microenvironment (Figure 4). Similar studies are currently being planned to isolate human cancer cells from the tumor-free mammary outgrowths. The redirected NTERA 2 human cancer cells will be isolated by magnetic sorting based on the surface expression of human specific CD133, while breast cancer cell lines will be isolated by magnetic sorting based on the surface expression of human specific EpCAM and CD44.

Conclusion

In addition to the reprogramming of stem cells via transfection of stem cell genes resulting in iPSCs [6,7], stem cells are reprogrammed through interactions with normal stem cell niches [13-15,27]. Both normal stem cells and CSCs are influenced by the normal mammary microenvironment [13-15,24-27]. Mouse and human CSCs from either embryonic origin or somatic origin are reprogrammed to a non-tumorigenic phenotype and provide progeny that proliferate

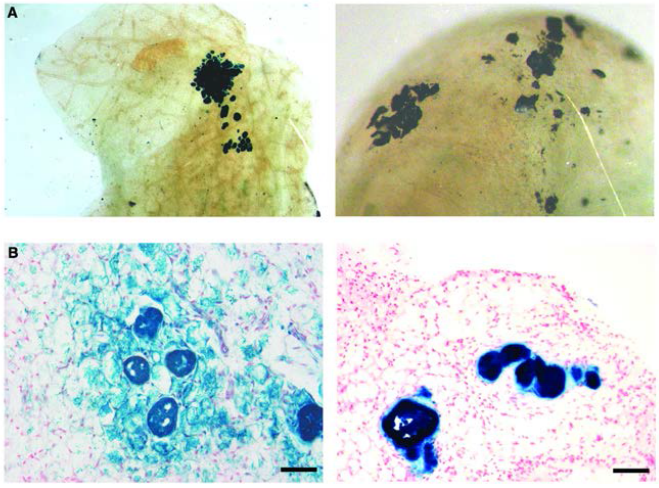

Figure 4: Recovered erbB2+ cells form mammary tumors. Dissociated cells from mixed mammary outgrowths were magnetically sorted for erbB2 prior to transplantation. A) ErbB2+ fraction formed mammary tumors when 1000 cells were transplanted even when fat pad contained host outgrowth (unstained ducts-add arrows). Tumors from two separate mammary recipients are shown. B) Cross sections analyses of mammary outgrowths shown in (A) demonstrating entire mammary tumors consist of lacZ+ cells. Sections counterstained with nuclear fast red. Scale bars = 100μm. Figure from Booth et al., 2010.

and differentiate due to interaction with normal mammary niche [13-15,27]. The progeny of CSCs differentiate into luminal epithelial cells, myoepithelial, and functional milk producing secretory epithelial cells [24-26]. The expansion of the tumor-derived cells was not due to cellular fusion [25]. Overall, the results indicate that normal mammary microenvironments have the capacity to influence tumorigenic cells and reprogram the cancer cells to adopt a non-cancer phenotype regardless of the origin, differentiation or metastatic state of the cancer cells [24-26]. Studies where the redirected tumor-suppressed MMTV-neu were isolated from the chimeric mammary outgrowths and subsequently transplanted in the cleared fat pad without normal MECs suggested that tumorigenesis of MMTV-neu tumor-derived cells is restrained by the surrounding normal niche and that signals that originate from the normal MECs coordinate the reprogramming of the CSCs. Similar studies are currently carried on to determine if the human cancer cells are temporarily or permanently redirected by the mammary niche towards a non-tumor phenotype. Additional current studies are focusing on the identification of key factors of the mammary niche in reprogramming of cells of both non-mammary origin and tumor-derived cells. Whether these reprogramming signals are chemical, physical, or a combination is still under investigation.

Acknowledgments

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