Cell-cell interactions during mammary gland development and breast cancer metastasis

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Abstract

Breast cancer arises in mammary epithelial tissue and causes mortality by metastasizing to various organs. While we have come to understand the various pathways regulating mammary gland development, tumor initiation and distant metastasis, our knowledge of how distinct subpopulations communicate with each other to facilitate such processes is rather limited in comparison. Epithelial tissue integrity of the mammary gland is established and maintained by numerous homo- and hetero-typic interactions among epithelial cells and between their stromal microenvironment. Furthering our understanding of how epithelial cells, both normal and malignant, regulate and rely on such cell-cell interactions may provide potential therapeutic targets to disrupt many critical processes in tumor initiation and distant metastasis. In the present dissertation, I investigated how mammary gland stem cells interact with each other and with their stromal microenvironment, and how metastatic breast cancer cells interact with their target stroma to facilitate their colonization of distant organs.

In the first study, we studied Perp, a tetra-span protein expressed on the cell surface of mammary gland stem cells (MaSCs), to elucidate its role in MaSC activity. We found that Perp expression is highly enriched in MaSCs and could potentially be used as a marker for isolating MaSCs. The suppression of Perp, however, did not affect MaSC activity in an in vivo transplantation setting, suggesting that Perp is not critical for mammary gland development, though it has been shown to be in skin development through a desmosome-dependent mechanism. Interestingly, Perp expression was elevated in tumors of spontaneous breast cancer models (MMTV-Wnt and -PyMT) and cancer cells...
with high Perp expression had higher tumor initiation potential. These results suggest that although both the skin and mammary glands are composed of epithelial cells, the mechanisms by which these cells establish and maintain desmosomes may be tissue-specific.

In the second study, we investigated the same molecule in the context of metastatic colonization to investigate whether Perp itself facilitates intercellular interactions between disseminating tumor cells and the stromal microenvironment provided by distant organs. Perp expression was enriched in epithelial-like metastatic breast cancer cell lines and the suppression of Perp significantly inhibited lung metastasis through a desmosome-independent mechanism. We also found that Perp is dynamically regulated during epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET), but Perp suppression alone did not affect these programs. Our results indicate that Perp is critical in successful distant metastasis of epithelial-like cancer cells and may be an effective target in blocking metastatic colonization in breast cancer patients.

In the third study, we searched for a vascular component to the MaSC microenvironment to modulate MaSC and/or tumor-initiating cell (TIC) activity through a putative (peri)vascular niche. We developed novel imaging methods to visualize MaSCs in their native microenvironment during mammary gland reconstitution, which we could enhance by inducing robust angiogenesis in the mammary fat pad. In addition, we have begun to distinguish specific subpopulations of endothelial cells that are modulated during pregnancy and tumorigenesis. Overall, our results suggest the existence of a (peri)vascular microenvironment for MaSCs. Further characterization of this niche during

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tumorigenesis may lead to the development of novel therapeutic strategies in breast cancer.
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Chapter 1. Introduction: Cell-cell Interactions in Cancer Progression
Mortality and morbidity of breast cancer

Breast cancer is the second leading cause of cancer-associated mortality in women, with 40,000 expected deaths in 2014 in the U.S. alone. Approximately 12.3% of women living in the U.S. will be diagnosed with breast cancer at some point during their lifetime, with 232,670 estimated new cases in 2014, but diagnosed patients with localized disease (61%) or regional spread to regional lymph nodes (32%) have a high chance of surviving 5 years or more after diagnosis (98.5% for localized and 84.6% for regional). Distant metastasis of breast cancer to various target organs, however, severely limits a patient’s 5-year relative survival to a meager 25%, highlighting the need to focus and improve the therapeutic options for patients with metastatic breast cancer (SEER Cancer Statistics Review, 2013; American Cancer Society, 2014; DeSantis et al., 2011).

Potential causes of breast cancer

As in most cancers, the cause of breast cancer can be inherited and/or from exposure to risk factors. A woman’s risk of breast cancer significantly increases if a first-degree relative has been diagnosed with breast cancer. Overall, approximately 5-10% of breast cancers can be linked to gene mutations inherited from either parent, but the mutations of breast cancer, early onset (BRCA) 1 and 2 genes are the most common by accounting for up to 25% of all hereditary breast cancers (Brose et al., 2002; Chen and Parmigiani, 2007; Malone et al., 2006). BRCA1 and BRCA2 genes produce proteins that serve as tumor suppressors by repairing damaged DNA, thereby contributing to the stability of the cell’s genetic material. This genome stability, however, can be disturbed by risk factors that are associated with the aging process and daily exposure to various carcinogens. In fact, 85%
of breast cancers occur in women who have no family history of breast cancer (Campeau et al., 2008; Easton, 1999; Liu et al., 2013).

**Forms and subtypes of breast cancer**

Breast cancer originates in the mammary gland, an organ with vastly diversified cell populations coordinating to perform highly-specified physiological functions. Depending on the origin, aggressiveness and molecular profiles of the cancer cells, breast cancer can be classified into various (sub)types. The mammary gland contains 15-20 lobes, which are composed of smaller sections called lobules. Stromal tissue, composed mostly of adipocytes and fibroblasts, interconnect these individual lobes, while ducts connect the lobes to the nipples (Osborne, 2010). While breast cancer is classified as a carcinoma, a cancer of epithelial origin, it can be divided largely into carcinoma *in situ* or invasive carcinoma within one of these two main structures of the inner breast – lobule and duct. The difference between *in situ* and invasive is that the cancer cells have not invaded from the site of origin into surrounding tissue. Invasive ductal carcinoma is the most common form of breast cancer and is diagnosed when the cancer has started in a milk duct of the breast, breaks through the wall of the duct, and grows into the fatty tissue of the breast. At this point, the invasive cancer cells can enter the blood/lymphatic vascular system and disseminate to various organs of the body (Dillon, 2010; Rosen, 2001). Breast cancer can be further classified into 18 distinct pathological subtypes based on histological type and grade, as well as the immunohistochemical characterization of receptors for estrogen, progesterone and human epidermal growth factor receptor 2 (HER2) (Dawson et al., 2013). While these distinct characteristics suggest potential therapeutic targets, molecular
profiling of the genetic composition of human breast cancer tumors has revealed four major classes of breast cancer – basal, luminal A, luminal B and HER2+ – and has provided a wealth of information for potential therapeutic molecular targeting (Brisken, 2013; Dawson et al., 2013; Perou et al., 2000; Sorlie et al., 2001).

**Cancer metastasis**

If cancer cells invade into surrounding tissue, intravasate into and survive in circulation, home and extravasate into the parenchyma of distant target organs, they have the potential to colonize these secondary sites to form metastatic lesions (Chambers et al., 2002; Gupta and Massague, 2006; Steeg, 2006). It has been observed that metastatic cancer cells derived from a particular metastasis site often display enhanced metastasis ability to that specific organ. With gene expression profiles showing distinct gene expression signatures for different organ-specific variants, we now know that specific genetic properties of tumor cells mediate organ-specific distributions of a primary tumor (Fidler, 1973; Kang, 2005; Kang et al., 2003; Minn et al., 2005a; Minn et al., 2005b). For breast cancer cells, metastasis occurs most frequently in the lungs and bone, and less frequently in the liver, brain and adrenal medulla (Lee, 1983). While therapeutic options for treating early-stage primary tumors can deliver relatively good outcomes for cancer patients, metastasis remains the most deadly feature of cancer, accounting for greater than 90% of cancer-related mortality (Fidler, 2003; Hanahan and Weinberg, 2011). The greatest challenge for treating metastatic disease lies in the disseminating properties of metastatic cancer cells. Instead of treating a localized area of cancerous tissue, either by surgical removal or radiation, oncologists need to approach metastatic disease
systemically. Once a primary tumor is formed, individual cancer cells need to detach from their neighbors and their surrounding stroma to start their metastatic journey. Invasive tumor cells increase their intrinsic mobility by adopting cellular programs, such as the epithelial-to-mesenchymal transition (EMT), and secrete multiple factors to degrade the rich extracellular matrix supporting primary tumor growth (Friedl et al., 2012; Friedl and Wolf, 2003). Intravasation of these cancer cells into the blood circulation allows them to disseminate systemically to distant target organs. Many cancer cell-derived factors have been identified to facilitate the binding/attachment of invasive cancer cells to endothelial cells, while other secreted factors compromise vascular integrity to enhance access into the circulation (Reymond et al., 2012; Sonoshita et al., 2011; Weis and Cheresh, 2011). As soon as cancer cells intravasate into blood vessels, they are exposed to tremendous amounts of shear stress and immunological attack. It has been shown in experimental metastasis settings that less than 0.02% of tumor cells can survive in circulation to form metastases (Chambers et al., 2002). Circulating tumor cells (CTCs) depend on contextual signaling to activate/maintain survival mechanisms to counteract stress induced by anoikis (Douma et al., 2004), shear stress and cytotoxic attack by various immune cells (Chao et al., 2012; Chen et al., 2011; Grabinski et al., 2011). This contextual and localized signaling also contributes to the homing and tethering of CTCs to activated endothelial cells of target organs, allowing the cells to extravasate from the circulation and successfully seed at their new home (Minn et al., 2005a; Padua et al., 2008; Valastyan and Weinberg, 2011). As in the case for intravasation into the circulation, cancer cells need to activate various integrins to attach to endothelial cells and secrete various factors to compromise vascular integrity, though
they employ different molecular programs and assistance from multiple cell types. Surviving the treacherous journey until successful extravasation does not guarantee colonization, however, as these cancer cells still need to find the appropriate “soil” to grow as seeds into secondary tumors. While most disseminated cancer cells fall into a state of dormancy to adapt to their new surroundings (Aguirre-Ghiso, 2007; Pantel and Brakenhoff, 2004), there is emerging evidence for the priming of target organs by the primary tumor or circulating tumor cells, thereby creating a “pre-metastatic niche” for incoming metastatic tumor cells (Psaila and Lyden, 2009). In lung metastasis, tumor-derived soluble factors or microvesicles released systemically recruit hematopoietic progenitor cells and macrophages from the bone marrow, which, along with cancer-associated fibroblasts and endothelial cells, remodel lung tissue to accelerate tumor cell adhesion, invasion and colonization (Grange et al., 2011; Hiratsuka et al., 2006; Hiratsuka et al., 2008; Kaplan et al., 2005; Peinado et al., 2012). Overall, disseminating cancer cells need to overcome all of these obstacles for successful distant metastasis, which is quite inefficient yet deadly. Once these aggressive cells have left the confines of a surgical/radiation field, they can be found in any or all of the aforementioned stages of metastasis, eluding conventional therapies by transitioning into a dormant state and/or constantly seeking favorable sites to colonize. Therefore, cancer metastasis still remains as the greatest obstacle to achieving cure for invasive malignancies.

**Mammary gland stem cells and tumor-initiating cells**

Mammary gland stem cells (MaSCs) are multi-potential adult stem cells capable of regenerating the entire mammary gland from a single cell. Following the traditional
definition of stem cells, MaSCs have the potential to self-renew to create a daughter cell identical to itself and also to differentiate into either a myoepithelial or luminal progenitor to eventually create the respective mature lineages of the mammary gland (Shackleton et al., 2006; Stingl et al., 2006; Visvader, 2009; Visvader and Smith, 2011). Ever since the initial isolation of a MaSC-enriched population using markers CD24 together with either CD29 or CD49f, after exclusion of hematopoietic, erythrocyte and endothelial lineages (CD45, TER119 and CD31, respectively, and referred to as “Lin”) (Shackleton et al., 2006; Stingl et al., 2006), many key signaling pathways involved in other adult stem cells have been identified to play key roles in MaSC maintenance and activity (Asselin-Labat et al., 2007; Dontu et al., 2003; Li et al., 2008). For example, Wnt signaling was first identified to play a role in MaSC activity when the first report to introduce MaSCs showed a significant expansion of the MaSC-enriched population (Lin\(^-\) CD24\(^+\) CD29\(^{hi}\)) in a transgenic mouse model overexpressing Wnt specifically in the mammary gland (Shackleton et al., 2006). This expansion was found to take place in the hyperplastic stage of tumorigenesis, in which massive tissue expansion precedes a detectable tumor. We now know that MaSCs express the receptors for Wnt molecules and can be stimulated by these ligands for self-renewal (Badders et al., 2009). Another well-characterized pathway regulating MaSC activity is the Notch signaling pathway. Unlike the Wnt pathway, Notch ligands can suppress MaSC expansion but lead to preferential expansion of the luminal progenitor population (Bouras et al., 2008). Many other pathways have been elucidated to act on stem/progenitor cells to dictate and fine-tune the fate of a mammary gland stem cell (Choi et al., 2009; Cicalese et al., 2009; Senoo et al., 2007; Yamaji et al., 2009), but the various sources of these regulatory signals remain to
be identified. In particular, the microenvironment in which these stem cells reside is poorly-defined. The mammary gland is largely composed of epithelial, endothelial, adipose, immune cells and fibroblasts (Korkaya et al., 2011). How each of these cell types interacts with MaSCs, either directly or indirectly, to regulate their activity requires further investigation. It is also important to characterize the intercellular interaction amongst stem/progenitor cells themselves, as these signaling activities may also dictate the cell’s ultimate fate.

The cancer stem cell (also known as tumor-initiating cell) hypothesis states that some cancers are organized into a hierarchy or subpopulations of tumorigenic cancer stem cells and their non-tumorigenic progeny. In other words, only a small fraction of cells within a heterogeneous tumor has the self-renewal ability to repopulate a new tumor (Meacham and Morrison, 2013). These tumorigenic cells, however, must also give rise to non-tumorigenic progeny to satisfy the cancer stem cell model. This hierarchical organization of tumorigenesis serves as the basis for the idea that cancer stem cells form heterogeneous tumors by undergoing epigenetic changes, which follows the clonal evolution theory, as well as differentiating into non-tumorigenic cells. The cancer stem cell hypothesis arose from many observations, including the resilience of certain cancer cells to conventional therapies (Bao et al., 2006; Diehn et al., 2009; Oravecz-Wilson et al., 2009), eventually leading to the relapse of disease, and experimental studies in which a limiting dilution of a specific population isolated from a tumor could give rise to the same kind of tumor indefinitely (Al-Hajj et al., 2003; Bonnet and Dick, 1997; Read et al., 2009). In furthering our understanding of normal MaSC biology, we hope to understand the biology underlying tumor initiation and relapse.
Epithelial origin of tumors and epithelial integrity

Carcinoma is defined as a cancer that is derived from cells that have developed the cytological appearance, histological appearance, or molecular characteristics of epithelial cells (Moore et al., 1967). Carcinomas are therefore most frequently found in the lung, prostate, colon and rectum, pancreas, ovaries and the breast. Each of these sites house epithelial cells with distinct molecular profiles and specialized function, but the relatively high susceptibility to oncogenic transformation lies in the rapid turnover of these cells for tissue homeostasis. Epithelial cells arise from the ectoderm and endoderm, the outer- and inner-most primary germ layers, respectively, of a developing embryo, and eventually assemble into epithelial sheets to form the skin and line most major cavities in the body, including the lung, intestine and mammary gland (Blanpain et al., 2007). As the primary function of epithelia is to create physical barriers to separate tissues, epithelial cells need to establish and maintain intercellular junctions composed of adherens, tight junctions and desmosomes (Niessen, 2007; Simpson et al., 2011). Tight junctions consist of the transmembrane proteins claudins, occludins and junctional adhesion molecules and are scaffolded by the cytoplasmic zonula occludens (ZO) proteins. These ZO proteins also link these junctions with the actin cytoskeleton of the cell. Adherens junctions are intercellular connections that coordinate the assembly and organization of the cortical cytoskeleton in epithelial cells. Of these, cadherins are transmembrane receptors that function through calcium-dependent homophilic and heterophilic interactions to mediate cell-cell contact and communication (Andrews et al., 2012; van Roy, 2014).

Epithelial(E)-cadherin is expressed in all normal mammary epithelial cells, providing a tight connection between epithelial cells by localizing and interacting with other
components of the adherens junction. The E-cadherin cytoplasmic tail interacts with proteins such as p120, γ-catenin and β-catenin, thereby providing a connection between the actin cytoskeleton and signaling pathways (Paredes et al., 2012; van Roy and Berx, 2008). Desmosomes are another class of intercellular adhesions that link to the intracellular network of keratin intermediate filaments. In a similar way to adherens junctions, desmosomes from neighboring cells are connected by members of the cadherin family, called desmogleins (DSGs) and desmocollins (DSCs) (Chitaev and Troyanovsky, 1997; Hobbs and Green, 2012; Nie et al., 2011; Spindler et al., 2009). With such specialized properties and characteristics, intercellular and intracellular molecules of epithelial cells interact with one another to form various adhesive complexes which establish and maintain epithelial integrity.

**Epithelial-to-mesenchymal and mesenchymal-to-epithelial transition**

Epithelial cells undergo a morphological transformation, known as epithelial-to-mesenchymal transition (EMT), to become motile (Tam and Weinberg, 2013). This developmental program has been shown to be important for cell migration during embryonic development, wound healing (fibrosis), and for cancer cells to leave the primary tumor to start their journey to their target organs (Thiery et al., 2009). When epithelial cells adopt the EMT program, they shed their epithelial characteristics, including cell-cell adhesion, planar and apical-basal polarity, and lack of motility, and acquire mesenchymal features, including motility, invasiveness and heightened resistance to apoptosis and chemotherapy (Polyak and Weinberg, 2009). The EMT gene expression program involves multiple transcription factors that regulate the transcriptional level of
epithelial and mesenchymal molecules (De Craene and Berx, 2013). A hallmark of EMT is the down-regulation of E-cadherin to destabilize adherens junctions. Accompanying repression of genes encoding for claudins, occludin, desmoplakin and plakophilin also contribute to the direct disintegration of the epithelial barrier (Lamouille et al., 2014). Destabilized epithelial cells are also induced to undergo changes in cytoskeletal architecture, to promote adhesion to mesenchymal cells and to alter the interaction of cells with the extracellular matrix (Yilmaz and Christofori, 2009). The up-regulation of genes associated with mesenchymal properties, including neural(N)-cadherin and fibronectin, greatly enhance the interaction of these epithelial cells with the surrounding stroma, thereby facilitating cell movement (Theveneau and Mayor, 2012; Wheelock et al., 2008). Although epithelial cells can receive heterotypical signals, specifically those released by the mesenchymal cells that constitute the stroma of normal and neoplastic tissues, to undergo EMT (Gao et al., 2012; Yang and Weinberg, 2008), the main and best understood inducer of this morphological transformation is the transforming growth factor-β (TGFβ) family of cytokines (Massague, 2008). TGFβ binds to tetrameric cell surface receptor complexes formed by Type I and Type II receptors, thereby causing the phosphorylation of the type I TGFβ receptor by the constitutively active Type II receptor, and induces a signal transduction pathway that ultimately results in the translocation of SMADs into the nucleus for transcriptional regulation of EMT factors. More specifically, activated TGFβ receptors phosphorylate the C termini of intracellular SMAD2 and/or SMAD3, which then form a trimeric complex with SMAD4, prior to nuclear translocation (Feng and Derynck, 2005; Massague, 2012). In order to achieve the hallmark of EMT – the suppression of E-cadherin expression – a battery of
transcriptional repressors, such as SNAI1, SNAI2, and ZEB2, directly represses E-cadherin transcription in response to EMT induction (De Craene and Berx, 2013; Peinado et al., 2007). Although there is relatively limited evidence for the reverse process of EMT, the mesenchymal-to-epithelial transition (MET) has emerged as a concept supporting the re-epithelialization of cells, which have undergone EMT, in the process of metastatic colonization (Lamouille et al., 2014; Nieto, 2013; Tsai and Yang, 2013). Indeed, mesenchymal but poorly metastatic cells colonize the lungs much more efficiently when they are forced into an epithelial-like state. It has been previously shown that in vivo induction of Twist1, a potent inducer of EMT, in squamous cell carcinoma cells promotes dissemination of tumor cells via an EMT-dependent manner (Tsai et al., 2012). However, the return of Twist1 to normal levels and the resulting reversion of these cells to their epithelial-like state greatly enhance metastatic colonization. MET can also be achieved by over-expressing a specific cluster micro-RNAs – miR-200s – that can suppress the ZEB transcription factors in the EMT program (Korpal et al., 2011; Lamouille et al., 2013). It has also been shown that ZEBs can repress miR-200s with the induction of EMT, so it is believed that MET occurs when the cell no longer receives reinforcing signals to stay in the mesenchymal state (Brabletz and Brabletz, 2010; Bracken et al., 2008; D'Amato et al., 2013). Studies showing the reversion of disseminated cancer cells from a mesenchymal-like to an epithelial-like cell at the secondary site imply that the MET program is actively initiated for metastatic colonization (Chaffer et al., 2006; Chaffer et al., 2007; Hugo et al., 2007). Therefore, targeting metastatic cancer cells before they can revert back to their epithelial state via the MET program is very appealing, especially since it has the potential to attack cells that have not yet fully
regained their familiar characteristics as malignant epithelial cells of the primary tumor, thereby preventing cancer relapse.

**EMT, Perp and desmosomes**

Before epithelial cells undergoing EMT acquire mesenchymal traits, they must detach from each other by down-regulating various adhesion molecules responsible for epithelial integrity. While E-cadherin expression is the distinguishing feature of the epithelial state, epithelial cells are also rich in desmosomal proteins which connect cell-cell contact sites at the plasma membrane and the intermediate filament cytoskeleton to promote tissue integrity and homeostasis (Dusek and Attardi, 2011; Savagner, 2001). Especially abundant in skin and the heart, desmosomes comprise three main protein families: cadherins, armadillo proteins and plakins (Nekrasova and Green, 2013). The composition of desmosomes in various tissues differs significantly based on the tissue- or differentiation-specific expression of the various isoforms of these desmosomal components. Desmosomal complexes have both extracellular and intracellular components to mediate cell-cell adhesion. Extracellular desmosomal cadherins, the desmogleins (DSG1-4) and the desmocollins (DSC1-3), mediate adhesion between apposing cells through interactions of their ectodomains. On the other side of the cell membrane, these cadherins bind to armadillo proteins junction plakoglobin (JUP) and plakophilins (PKP1-3), and bridge the cadherins to the intermediate filament cytoskeleton inside the cell. The final link between the intermediate filaments and the desmosomal complex at the plasma membrane is a plakin family member called desmoplakin (DSP). It has already been shown that EMT factors act directly on these various proteins to
disintegrate desmosomal complexes (Lamouille et al., 2014; Savagner et al., 1997). For example, both SNAI1 and ZEBs repress DSP and PKPs, along with E-cadherin suppression, during EMT (Aigner et al., 2007; Lamouille et al., 2014). The most critical desmosomal protein, however, does not fall under the three families of proteins described above. p53 apoptosis effector related to PMP-22 (Perp) is a tetraspan membrane protein that is transcriptionally activated by both p53 and p63 during DNA damage-induced apoptosis and development of stratified epithelia, respectively (Attardi et al., 2000; Dusek and Attardi, 2011). Not only are the expression profiles of p63 and Perp have been shown to be very similar in developing embryos and in stratified epithelia of adults, but both molecules also play critical roles in stratified epithelia development (Ihrie et al., 2005). Unlike in p63-deficient mice, which completely lack stratified epithelia, the loss of Perp in mice leads to postnatal mortality due to severe blistering of the skin. Perp localizes to desmosomes and it has been shown that Perp is required for proper desmosome assembly in skin epithelial cells. Desmosomal proteins in Perp -/- cells are expressed but are not localized to the plasma membrane, thereby compromising epithelial integrity in a desmosome-dependent manner. Although the upstream regulator of Perp expression has been identified to be p63 in the case of skin epithelial cells, the exact mechanism by which Perp mediates desmosome assembly is unknown. It has been speculated that Perp acts as a chaperone protein to facilitate the localization of desmosomal proteins to the plasma membrane or that it may contribute directly to cell-cell adhesion via homo- or hetero-typic interactions, but these hypotheses still remain to be proven.

**Perp, desmosomes and cancer**
As a target of p53 in the context of DNA damage-induced apoptosis, Perp has naturally received attention in cancer biology. There are multiple reports demonstrating Perp’s interaction with various effector molecules to facilitate apoptosis (Davies et al., 2009; Davies et al., 2011; Ihrie et al., 2003; Singaravelu et al., 2009), but these results seem to be largely context-dependent. On the other hand, cancer studies in the p63 context are quite limited. The absence of Perp in the skin has been shown to prevent papilloma formation (Marques et al., 2005), but it promotes UVB-induced squamous cell carcinoma initiation and progression (Beaudry et al., 2010). Similarly in breast cancer, Perp acts as a tumor suppressor and delays tumor incidence in a spontaneous breast cancer model (Dusek et al., 2012). However, the major difference between these two cancer types lies in the fact that desmosome assembly is not compromised in Perp-deficient mammary epithelial cells. Instead, desmosomal protein levels are decreased with Perp deficiency, suggesting that Perp may still contribute to desmosome function in the mammary gland, by supporting desmosomal protein stabilization, but to a lesser degree than in the skin. In both squamous cell carcinoma and breast cancer, it is still unclear whether the loss of Perp itself or the resulting change in desmosomes is driving tumor initiation and/or progression. The association between desmosomes and cancer has been proposed mostly based on correlational data, both positive and negative, of desmosome expression in various cancer types. Overall, high expression of some desmosomal proteins can be observed in certain cancer types (Brennan and Mahoney, 2009; Chen et al., 2007; Furukawa et al., 2005), whereas the expression of the same components is reduced in other cancers (Depondt et al., 1999; Oshiro et al., 2005; Shiina et al., 2005; Sobolik-Delmaire et al., 2007; Yashiro et al., 2006). More importantly, the invasion/metastatic
potential of the tumor can be either positively or negatively correlated to desmosome expression depending on the origin of cancer and the context in which the experiments were performed to test metastatic traits. Independent of their role in cellular adhesion, several desmosomal proteins have been shown to directly drive the cellular programs related to tumorigenesis. For example, JUP and PKPs can translocate to the nucleus and activate various β-catenin-LEF/TCF and other target genes to promote proliferation and transformation (Maeda et al., 2004; Schmidt et al., 1997; Sobolik-Delmaire et al., 2010; Zhurinsky et al., 2000). PKPs can also interact with translation-initiating factors to stimulate translation (Hofmann et al., 2006; Wolf et al., 2010). Since these desmosomal constituents need to be released from the plasma membrane for their transcriptional activity, the pre-requisite for desmosome disassembly and how this occurs leading up to a tumorigenic event remain to be explained. As mentioned previously, desmosome disassembly has been shown in the EMT context and these observations support the claim that desmosomes inhibit the invasion/metastatic potential of the primary tumor by preventing the detachment of malignant epithelial cells from each other. However, with the known role of Perp in desmosome assembly, whether Perp plays a (in)direct role in cancer metastasis remains an open question.
Chapter 2: Characterization of Perp in Mammary Gland Stem Cells
Introduction

Mammary stem cells have the potential to differentiate into luminal and myoepithelial/basal lineages to regenerate the entire mammary gland from a single cell (Visvader and Smith, 2011). While the master regulators of MaSC maintenance/activity have and continue to be identified and characterized, these studies do not provide sufficient information regarding the intercellular interaction between MaSCs and their neighboring cells. More specifically, our knowledge of the proteins expressed on the surface of MaSCs that mediate adhesion and consequential signaling amongst MaSCs, progenitors, mature epithelial cells, and stromal cells is rather limited compared to secreted protein signaling. The ultimate goal of this study was to identify and characterize extracellular molecules as potential targets to modify intercellular interactions between MaSCs and their neighboring cells to regulate MaSC activity. Does MaSC maintenance/activity rely on adherens, tight junctions and desmosomes? If so, how do they regulate molecular signaling to affect MaSC fate and activity?
Results

Gene expression profile analysis of the MaSC-enriched population

In order to identify putative gene candidates that are up-regulated in MaSCs compared to differentiated progenitors and progeny, the MaSC-enriched population (henceforth called “P4”) was isolated using fluorescence-activated cell sorting (FACS) based on Lin⁻ CD24⁺ CD29⁹hi expression. The luminal progenitor-enriched population (called “P5”) was isolated based on Lin⁻ CD24⁺ CD29⁹lo expression; other non-hematopoietic and endothelial cells of the mammary gland were also sorted based on Lin⁻ CD24⁻ expression (Figure 1-1A). These distinct populations were sorted from virgin and pregnant mice and subjected to microarray analysis. Both virgin and pregnant (1 day after parturition) statuses were analyzed, since it has been previously reported that the MaSC population expands significantly during pregnancy via hormonal signaling (Asselin-Labat et al., 2010; Joshi et al., 2010). Many surface proteins, including Delta-like ligand 1 (Dll1), G protein-coupled receptor 56 (Gpr56), and Perp, were significantly up-regulated in P4 compared to P5 and P6 in both situations (Figure 1-1B). Perp was chosen as a candidate for the following reasons: 1) it is a transmembrane protein expressed in epithelial cells and 2) its absence has been shown to disrupt proper desmosome assembly in skin epithelial cells and disrupt mammary gland homeostasis 3) it plays a role in tumor initiation/progression.
Figure 1-1. Surface markers up-regulated in the MaSC-enriched population. (A) Schematic diagram of P4 isolation by FACS. Mammary glands were isolated from 9-week-old virgin or pregnant (1 day after parturition) female mice and dissociated to create single-cell suspensions. Cells were labeled with appropriate FACS antibodies and subjected to sorting. (B) Expression data of candidate surface marker proteins. Selection criteria are outlined in the main text. P4 = CD24$^+$ CD29$^{hi}$, P5 = CD24$^+$ CD29$^{lo}$, P6 = CD24$^-$. All populations were sorted from the Lin$^-$ population.
**Perp is significantly up-regulated in the MaSC-enriched population**

The fact that Perp is a transmembrane protein expressed on the cell surface (Attardi et al., 2000) indicates that it can be used as a surface marker to isolate live cells using FACS. Using an antibody against Perp, Lin^- cells of the mammary gland were sorted based on Perp^{hi} and Perp^{lo} expression and compared to CD24 vs. CD29 expression. Consistent with the microarray data and validation of mRNA expression with quantitative real-time PCR (qRT-PCR) (Figure 1-2A), Perp^{hi} cells were significantly enriched in the P4 population, whereas Perp^{lo} cells were restricted to P5 and P6 populations (Figure 1-2B). This observation was further confirmed by sorting the P4 and P5 population by FACS and performing immunofluorescent (IF) staining with anti-Perp antibody on these respective populations. Perp expression was indeed present only in P4 cells and could not be seen in P5 cells with the same camera exposure (Figure 1-3A). Finally, Perp^{hi} and Perp^{lo} cells were isolated and stained with anti-smooth muscle actin (SMA; myoepithelial marker) and anti-cytokeratin 8 (K8; luminal marker) to validate the FACS profile data. P4 and P5 cells are SMA^+ and K8^+, respectively, and Perp^{hi} and Perp^{lo} cells indeed expressed the corresponding epithelial lineage markers (Figure 1-3B).

In order to characterize Perp expression and localization in the mammary gland, we stained adult virgin mammary glands with anti-Perp, -SMA and -K8 antibodies. Surprisingly, Perp expression was not restricted to the SMA^+ myoepithelial layer of the mammary duct but was expressed in the K8^+ luminal cells as well (Figure 1-3C). It should be noted that this finding does not disprove our previous claim that the MaSC-
Figure 1-2. Perp^{hi} cells are significantly enriched in the P4 population. (A) Expression levels of Perp in P4, P5 and P6 populations (normalized to P4) assessed by quantitative RT-PCR. Data is normalized to Gapdh and presented as mean ± s.e.m. **P < 0.001** (B) Flow cytometry analyses of Perp expression in P4 (green), P5 (orange) and P6 (gray) populations. Top panel, Perp^{hi} and Perp^{lo} populations from the Lin^- population are separated by top and bottom 5%. This histogram is overlaid with P4 (green), P5 (orange) and P6 (gray) Perp expression profiles. Bottom panel, P4, P5 and P6 population scatter dot plots (left) are superimposed with Perp^{hi} (red) and Perp^{lo} (blue) populations (middle).
Figure 1-3. Perp is expressed in myoepithelial and luminal populations of the mammary gland. (A) Immunofluorescence images of Perp expression in P4 and P5 populations. P4 and P5 populations were FACS-sorted, cyto-spun onto microscope slides and stained with anti-Perp antibody. (B) Immunofluorescence images of Perp$^{hi}$ and Perp$^{lo}$ cells stained for myoepithelial (SMA) and luminal (K8) markers. Perp$^{hi}$ and Perp$^{lo}$ cells were FACS-sorted, cyto-spun onto microscope slides and stained with anti-SMA and -K8 antibodies. (C) Immunofluorescence images of Perp expression in myoepithelial and luminal populations of the mammary gland. Adult mammary glands were sectioned and stained with anti-Perp, -SMA, and -K8 antibodies. Scale bars 50 µm.
enriched population has high Perp expression. Although both luminal and myoepithelial cells express Perp, Perp expression is noticeably higher in the SMA\textsuperscript{+} cells compared to the K8\textsuperscript{+} cells. Also, the P4 population is a subset of all SMA\textsuperscript{+} cells and the percentage of the P4 population within the entire mammary gland is \textasciitilde2\%. Since we do not have a single marker for MaSCs or for the P4 population, we cannot visualize the P4 population with IF staining. Therefore, all of these observations suggest that Perp is expressed in both myoepithelial and luminal lineages of the mammary gland but it is more abundantly expressed in the MaSC-enriched population.

**Perp is a surface marker for functional MaSCs**

In order to determine whether high Perp expression could be used as a single marker, as opposed to using CD24 and CD29 expression, to enrich for MaSCs, we isolated the MaSC-enriched P4, Perp\textsuperscript{hi} and Perp\textsuperscript{lo} cells for mammary gland reconstitution assays. MaSC potential/activity can be tested \textit{in vivo} by removing the endogenous mammary epithelium and injecting putative MaSCs into the adjacent “cleared” fat pad. P4, Perp\textsuperscript{hi} and Perp\textsuperscript{lo} cells were injected into the cleared fat pad in limiting numbers to assess the reconstitution frequency of these different populations. As expected from the results obtained above, Perp\textsuperscript{lo} cells had a significantly lower reconstitution frequency (1 putative MaSC in 7497 cells) compared to Perp\textsuperscript{hi} cells (1 in 246) (Figure 2-1A, B and Table 1). The reconstitution potential of Perp\textsuperscript{hi} cells was comparable to the P4 population (1 in 195), but Perp\textsuperscript{hi} cells were not further enriched for MaSCs.

Since Perp expression was found to be significantly higher in the P4 population, we investigated whether Perp expression within the P4 population could further enrich for
Figure 2-1. Perp$^\text{hi}$ cells are functional MaSCs. (A) Brightfield images of reconstituted mammary glands. P4, Perp$^\text{hi}$ and Perp$^\text{lo}$ populations were FACS-sorted and injected into cleared mammary fat pads. Reconstituted mammary glands were whole-mounted and stained with alum carmine. (B) Immunofluorescence images of myoepithelial and luminal populations in reconstituted mammary glands. Reconstituted mammary glands from P4 and Perp$^\text{hi}$ populations were sectioned and stained with anti-SMA (green) and -K8 (red) antibodies. Scale bars 1 mm and 50 µm, respectively.
Table 1. Reconstitution frequency of Perp expressing cells. Populations were FACS-sorted and injected into cleared mammary fat pads in limiting dilution. Successful reconstitution was scored based on the presence of epithelium, which was determined by alum carmine staining. Repopulating frequency was determined using the ELDA software. $P < 0.001$ (Perp$^{hi}$ vs. Perp$^{lo}$, 95% confidence interval).

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<th>125 cells</th>
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<td>0 of 14</td>
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<td>1 in 246</td>
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<tr>
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<td>4 of 14</td>
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<tr>
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<td>4 of 5</td>
<td>8 of 14</td>
<td>5 of 14</td>
<td>1 in 294</td>
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MaSCs. Perp\textsuperscript{hi} and Perp\textsuperscript{lo} cells within P4 were isolated and injected into the cleared fat pad in limiting numbers. Neither population (1 in 383 for Perp\textsuperscript{hi}; 1 in 294 for Perp\textsuperscript{lo}) enriched further for MaSCs compared to the unsorted P4 population (1 in 195), suggesting that Perp expression can only enrich for MaSCs within the Lin\textsuperscript{−} population and may have overlapping selective power with CD24/29 for MaSC enrichment.

\textit{Perp KD decreases MaSC activity in vitro but does not affect MaSC reconstitution potential in vivo}

Given that many surface proteins are uniquely expressed in specific subpopulations but do not contribute to any distinct function, we sought to investigate the functional basis of high Perp expression in MaSCs. Using short hairpin-mediated knockdown of \textit{Perp}, we stably suppressed \textit{Perp} expression in FACS-sorted P4 cells and subjected these transduced cells to \textit{in vitro} colony formation assays. MaSC potential can be tested in this way by embedding putative MaSCs in a 3D matrix using Matrigel. The number of spheroidal colonies formed in the \textit{in vitro} matrix positively correlates with the potential of these cells to reconstitute the mammary gland \textit{in vivo} (Shackleton et al., 2006). The efficiency of \textit{Perp} knockdown (KD) was confirmed using qRT-PCR and Western Blot analyses (Figure 3-1A and B). \textit{Perp} KD in MaSCs significantly reduced the number of colonies formed compared to the control and this could be rescued with \textit{Perp} over-expression (OE) (Figure 3-1C). These results suggest that Perp may play a role in positively regulating MaSC activity.

3D Matrigel colony formation assays are only an \textit{in vitro} surrogate to test the reconstitution potential of MaSCs. We therefore injected MaSCs stably knocked-down
Figure 3-1. Perp KD inhibits in vitro colony formation of MaSCs. (A) Expression levels of Perp in primary mammary epithelial cells with Perp KD, OE and KD+OE (normalized to pLKO and pLEIGW controls, respectively), assessed by qRT-PCR. Data is normalized to Gapdh levels. (B) Western blot showing Perp levels in primary mammary epithelial cells with Perp KD, OE and KD+OE relative to vector controls. β-actin was used as loading control. (C) Quantification of in vitro 3D Matrigel colony formation assays. FACS-sorted P4 cells were knocked-down for Perp and subjected to 3D Matrigel culture. Colonies exceeding 100 µm in diameter were quantified and normalized to vector control quantities. Data is presented as mean ± s.e.m. * P < 0.05, ** P < 0.01, *** P < 0.001 (Student’s t-test).
Table 2. Reconstitution frequency of *Perp KD* cells. FACS-sorted P4 cells were knocked-down for *Perp* and injected into cleared mammary fat pads in limiting dilution. Successful reconstitution was scored based on the presence of epithelium, which was determined by alum carmine staining. Repopulating frequency was determined using the ELDA software. $P = 0.0589$ (PLKO vs. *Perp* KD-A; 95% confidence interval), $P = 0.132$ (GIPZ vs. *Perp* KD-B; 95% confidence interval).

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<tr>
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<td>6 of 8</td>
<td>5 of 18</td>
<td>1 in 1110 cells</td>
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</table>
for Perp into the cleared mammary fat pad to test the functional basis of Perp in MaSCs during in vivo mammary gland reconstitution. In contrast to our in vitro findings, Perp KD in MaSCs did not decrease the reconstitution frequency; in fact, there was an insignificant trend in the opposite direction (Table 2). Not only was there no difference in the presence of reconstitution epithelium, but there were also no changes in the degree of reconstitution, based on the percentage of the cleared fat pad filled with reconstituted epithelium (data not shown). Therefore, we conclude that Perp is not a critical regulator of MaSC activity in vivo but is a surface marker for MaSCs.

Characterization of Perp expression in spontaneous breast cancer models

Since Perp is expressed in both luminal and myoepithelial lineages but is highly expressed in the MaSCs of the normal adult mammary gland, we wanted to characterize Perp expression in breast cancer. We chose two spontaneous breast cancer models, MMTV-Wnt (Tsukamoto et al., 1988) and MMTV-PyMT (Guy et al., 1992), to study Perp expression in both basal and luminal breast cancer models, respectively. In these spontaneous models, either Wnt or polyoma middle T virus (PyMT) is overexpressed using the murine mammary tumor virus (MMTV) promoter. It has already been shown that spontaneous tumors arising in MMTV-Wnt transgenic mice are basal-like based on histo-pathological analyses; MMTV-PyMT tumors are luminal-like. Using the same method as for the normal mammary gland, Perp\(^{hi}\) and Perp\(^{lo}\) cells were isolated from the primary tumors harvested from MMTV-Wnt and -PyMT mice and characterized based on their CD24 and CD29 expression. Interestingly, both tumor types showed Perp\(^{hi}\) cells to be enriched for the normal P4 expression profile (Lin\(^{-}\)CD24\(^{+}\)CD29\(^{hi}\)), whereas Perp\(^{lo}\)
cells were enriched for the P5 expression profile (Lin^− CD24^+ CD29^lo^) (Figure 4-1). It should be noted that the reverse analysis to confirm the Perp expression cannot be performed on these tumor samples, since the CD24 vs. CD29 profiles are not distinct in tumors as it is in the normal mammary gland.

In order to characterize the expression levels of Perp in these spontaneous breast cancer models, we analyzed mRNA and protein levels of these tumors in comparison to age-matched normal mammary glands. qRT-PCR and Western Blot analyses revealed that Perp mRNA and protein levels, respectively, were significantly higher in MMTV-Wnt tumors compared to the control (Figure 4-2A and C). Interestingly, Perp mRNA was not significantly higher in the MMTV-PyMT tumors but the protein level was much higher compared to the control.

To establish a connection between Perp and desmosomes in the spontaneous breast cancer models, we also analyzed several desmosomal components in these tumors. Although desmocollin-1 mRNA was significantly down-regulated in both MMTV-Wnt and -PyMT tumors, significant up-regulation of other desmosomal components, such as Dsg2, Dsp, Jup (hereafter referred to as Pg) and Pkp3, was observed for MMTV-Wnt tumors (Figure 4-2B). Surprisingly, with the exception of Pkp3, we did not see a significant change in these desmosomal components in the MMTV-PyMT tumors. It is important to note that the protein expression of some these desmosomal components reflected what was observed in mRNA expression. For example, the difference in expression levels of Dsg2 and Pg in the tumors compared to the control are significantly higher for the MMTV-Wnt than the MMTV-PyMT tumors (Figure 4-2C). Collectively,
Figure 4-1. Perp<sup>hi</sup> cells are enriched in the CD24<sup>+</sup> CD29<sup>hi</sup> population in spontaneous mammary tumors. Flow cytometry analyses of Perp<sup>hi</sup> (red) and Perp<sup>lo</sup> (blue) populations from the Lin<sup>-</sup> population (gray) in MMTV-Wnt and -PyMT primary tumors. Primary tumors from MMTV-Wnt (~5-month-old) and -PyMT (~12-week-old) transgenic mice were isolated and dissociated to create single-cell suspensions. Cells were labeled with appropriate antibodies and subjected to flow cytometry analysis. All populations were analyzed from the Lin<sup>-</sup> population.
Figure 4-2. Perp levels are elevated in MMTV-Wnt and -PyMT tumors. (A and B) Expression levels of Perp and desmosomal components in MMTV-Wnt and -PyMT tumors (normalized to normal mammary epithelial cells from age-matched mice) assessed by qRT-PCR. Data is normalized to Gapdh levels and presented as mean ± s.e.m. * P < 0.05, ** P < 0.01 (Student’s t-test). (C) Western blot of Perp levels in MMTV-Wnt and -PyMT tumors relative to normal mammary epithelial cells from age-matched mice. β-actin was used as loading control.
Figure 4-3. **Perp is expressed in myoepithelial and luminal populations of spontaneous mammary tumors.** Immunofluorescence images of myoepithelial (K14) and luminal (K8) populations in spontaneous mammary tumors. Primary tumors from *MMTV-Wnt* (~5-month-old) and *-PyMT* (12-week-old) mice were sectioned and stained with anti-Perp (green), -K14 (magenta) and -K8 (red) antibodies. Scale bar 50 µm.
these results suggest that Perp and desmosomal protein expression is particularly high in
*MMTV-Wnt* tumors, which are basal-like and have a well-defined tumor-initiating
population.

Finally, Perp expression was characterized in myoepithelial and luminal lineages of the
mammary tumors. Perp expression was once again found in both myoepithelial
(cytokeratin 14 (K14)) and luminal (K8) lineages (Figure 4-3). However, Perp expression
was much more co-localized with K14<sup>+</sup> cells compared to K8<sup>+</sup> cells, to a greater degree
of contrast than in the normal mammary gland.

**Tumor incidence is higher in *MMTV-Wnt* Perp<sup>hi</sup> cells**

As for the normal MaSCs, we sought to address whether Perp expression could be used
to isolate a population enriched for tumor-initiation potential in the spontaneous tumor
models. We therefore isolated Perp<sup>hi</sup> or Perp<sup>lo</sup> cells from the Lin<sup>−</sup> population and
subjected them to *in vitro* tumorsphere formation assays. Similar to *in vitro* colony
formation assays, tumorsphere formation assays test a single tumor cell’s potential to
give rise to a spheroidal colony, but these must be formed in anchorage-independent
conditions. Tumorsphere formation assays using Perp<sup>hi</sup> or Perp<sup>lo</sup> cells showed
significantly decreased tumorsphere number in the Perp<sup>lo</sup> population isolated from both
*MMTV-Wnt* and -*PyMT* tumors (Figure 5-1A). There was no difference in tumorsphere
size (Figure 5-1B), however, suggesting that the overall potential to form tumorspheres is
affected and not the expansion capability of the tumorspheres. These results suggest that
there is a positive correlation between Perp expression and the tumor-initiating potential
in *MMTV-Wnt* and -*PyMT* tumors.
Following the above observations, we then injected Perp\textsuperscript{hi} or Perp\textsuperscript{lo} cells into the mammary fat pad for tumor formation. Weekly tumor palpation revealed that tumor initiation was significantly delayed in Perp\textsuperscript{lo} compared to Perp\textsuperscript{hi} cells, indicating that the tumorigenic potential is greater in the Perp\textsuperscript{hi} cells (Figure 5-1C). However, since Perp\textsuperscript{lo} cells eventually gave rise to tumors in all of the recipients after the significant delay, we injected Perp\textsuperscript{hi} or Perp\textsuperscript{lo} cells in limiting numbers to test the tumor incidence. Lower cell number (500 cells) injections showed a higher rate of tumor incidence in Perp\textsuperscript{hi} cells (60%) compared to Perp\textsuperscript{lo} cells (25%), suggesting that the Perp\textsuperscript{hi} population is enriched for cells with higher tumor-initiating potential than in the Perp\textsuperscript{lo} population (Figure 5-1D). Overall, these results indicate that high Perp expression can be used as a surface marker for MaSCs and for tumor-initiating cells in basal-like tumors.
Figure 5-1. Perp$^{hi}$ cells have higher tumor initiation potential than Perp$^{lo}$ cells in MMTV-Wnt tumors. (A) Quantification of in vitro tumorsphere formation assays. Perp$^{hi}$ and Perp$^{lo}$ cells from MMTV-Wnt tumors were FACS-sorted and plated in low-attachment plates for tumorsphere formation. Colonies exceeding 100 µm in diameter were quantified and normalized to vector control quantities. (B) Measurements of in vitro tumorsphere diameter. Data is presented as mean ± s.e.m. $^*$ $P < 0.05$ (Student’s t -test). (C) Kaplan-Meier plot showing tumor initiation. Perp$^{hi}$ and Perp$^{lo}$ cells were FACS-sorted and injected into the mammary fat pad. $P < 0.001$ (log-rank test) (D) Tumor incidence of Perp$^{hi}$ and Perp$^{lo}$ cell limiting dilution injections. $P < 0.001$ (Perp$^{hi}$ vs. Perp$^{lo}$; 95% confidence interval).
Discussion

High Perp expression in mammary gland stem cells

We show here that high Perp expression is found in MaSCs and in a subpopulation of cells that have high tumor-initiating potential in a MMTV-Wnt spontaneous tumor model. While Perp was chosen for study based on its cell surface expression, its functional role in desmosome assembly and connection to tumorigenesis, we could not find a critical role for Perp in MaSC activity/function. Although Perp KD in MaSCs suppressed 3D Matrigel colony formation, suggesting that Perp may play a role in MaSC activity, this phenotype could not be observed in vivo during mammary gland reconstitution. One potential explanation for this discrepancy is that the in vitro colony formation assay is an isolated system with a relatively homogenous population, whereas the Perp KD MaSCs being injected into the cleared fat pad are exposed to a vast variety of cell types and signaling molecules. The 3D Matrigel colony formation assay determines MaSC activity by the ability of single cells to form clonogenic spheroids, but cells with similar stem cell potential may not reach the colony threshold due to diminished proliferation capacity. Therefore, whether the true self-renewal potential of Perp$^{hi}$ cells differs from Perp$^{lo}$ cells must be tested rigorously with serial colony formation assays. Since the cells being subjected to this assay were pre-selected for high Perp expression, these cells may rely on Perp to survive in an isolated state and/or to form such tightly-bound colonies. It has been previously reported that the loss of Perp in mammary epithelial cells, unlike in skin epithelial cells, does not lead to the mislocalization of desmosomes but rather results in decreased levels of desmosomal protein expression (Dusek et al., 2012). Although we did
not confirm this report in the MaSC-enriched population, decrease in desmosomal protein expression due to Perp KD is a potential explanation for the in vitro phenotype observed. We do not know whether Perp can directly facilitate cell-cell adhesion through a desmosome-independent mechanism, though Perp homotypic interactions have been previously proposed as a concept (Ihrie et al., 2005). Therefore, this also remains as an unaddressed hypothesis. When Perp\textsuperscript{hi} MaSCs are introduced into the in vivo mammary stroma for mammary gland reconstitution, Perp expression seems to be dispensable. In this context, Perp\textsuperscript{hi} cells are interacting with each other (through clonal expansion) as well as with other mammary gland stromal cells. Neither mechanism by which Perp may facilitate cell adhesion seems to affect the general process of MaSC reconstitution, as we did not observe any significant differences with Perp KD. Even if Perp KD in MaSCs does indeed lead to the significant decrease in the levels of desmosomal components, this phenotypic change does not seem to affect MaSC activity in vivo. These observations lead us to conclude that the expression of Perp and the putative functions of Perp in MaSCs are not critical to MaSC activity/function.

**Putative roles of Perp in tumor-initiating cells**

Profiling both basal- and luminal-like tumors based on their expression for the surface markers (Lin, CD24 and CD29) used to isolate the MaSC-enriched population revealed that Perp expression was enriched in the Lin\textsuperscript{−} CD24\textsuperscript{+} CD29\textsuperscript{hi} subpopulation. This observation is quite intriguing, as it suggests a tendency of Perp expression to be higher in the basal/myoepithelial than the luminal subpopulation of breast tumors. However, Perp protein levels between the \textit{MMTV-Wnt} (basal-like) and \textit{PyMT} (luminal-like) were
comparable, suggesting that the high expression of Perp is not restricted to basal-like cancers but is limited to a specific subpopulation within both basal- and luminal-like tumors. Whether this correlation has a functional basis, in terms of the relationship between Perp and CD29, remains to be explored.

Several markers for the isolation of the tumor-initiating population have been proposed in breast cancer. In human breast cancer, cells with the Lin−CD44+CD24low expression profile have high tumor initiation potential (Al-Hajj et al., 2003). Thy1.1 (Cho et al., 2008) and aldehyde dehydrogenase (ALDH) expression (Ginestier et al., 2007) have also been shown to enrich for this population in mouse tumors. Our results suggest that Perp can also be used to isolate TICs from MMTV-Wnt tumors, as Perphi cells gave higher incidence of tumor after injection into the mammary fat pad. This observation suggests that there is a functional basis for Perp in tumorigenesis, but this requires further investigation. Whether there is a further correlation between Perp expression and the aforementioned TIC markers should be addressed, as well as tumorigenesis studies in a Perp KD context. It has been recently shown that certain molecules expressed in normal stem cells as well as in TICs play a critical functional role only in the latter (Nakanishi et al., 2013). Based on lineage tracing models, Dclk1 was found to be expressed both in normal intestinal stem and tumor-initiating cells, but targeting the Dclk1+ population in a colorectal cancer model via toxin-mediated cell ablation showed its functional specificity to TICs, without any effect on adjacent normal stem cells. Therefore, further studies employing lineage tracing and genetic ablation models are critical to investigating the functional basis for Perp expression in MaSCs and TICs.
Is Perp a tumor-promoter or tumor-suppressor?

Recent studies have shown that Perp plays a tumor-suppressive role in cancer. This conclusion was reached by the fact that the loss of Perp facilitated tumor initiation and progression and was explained by the destabilization of desmosomal components due to the absence of Perp (Baron et al., 2012; Beaudry et al., 2010). An increase in immune cell infiltration and inflammation have also been observed to accompany Perp depletion (Dusek et al., 2012), but whether this effect directly contributes to tumor initiation/progression has not been fully addressed. Interestingly, it has been shown that the loss of p53, which is a transcriptional regulator of Perp, in enterocytes leads to the increase in intestinal permeability and thereby creates an inflammatory microenvironment to promote metastasis of colorectal tumors (Schwitalla et al., 2013). While the loss of Perp in a K14-specific p53 knockout (KO) model promotes tumor initiation in the mammary gland, it is important to note that this observation should not be generalized to other breast cancer models. Perp is transcriptionally regulated by both the p53 and p63 pathways, so the p53 KO background in this spontaneous breast cancer model may interfere with an accurate interpretation of Perp’s functional role. In addition, every spontaneous tumor model is unique in that the source driving tumorigenesis forces the resulting tumor into a specific context. Given that p53 was knocked-out in K14+ cells only, the target of tumorigenesis is already pre-defined. While our results also show that Perp is expressed in K14+ cells of a normal mammary gland, as well as in the MMTV-Wnt mammary tumor, whether this observation holds true in a p53 KO background requires further study. We did not find a functional basis for high Perp expression in MaSCs, as Perp KD in MaSCs did not affect mammary gland reconstitution. Also, we have not yet
addressed the functional role of Perp in our spontaneous breast cancer models. Further investigation of Perp playing a putative functional role in TIC activity would shed more light onto whether Perp is overall a tumor suppressor or also a tumor promoter in particular stages of tumor development. In conclusion, our study shows that Perp is highly expressed in MaSCs and in TICs of MMTV-Wnt tumors and provides preliminary evidence to question the functional role of Perp in mammary TICs.
Materials and Methods

Mammary epithelial cell preparation and FACS

Single cell suspensions were prepared from the mammary glands of wildtype FVB (9-week-old virgin or pregnant) or primary tumors from MMTV-Wnt (~5-month-old) or MMTV-PyMT (~12-week-old) after mechanical and enzymatic dissociation based on published protocols. Briefly, mammary glands were excised, minced using scalpels, and digested for 1hr in 300U/ml type 1A collagenase (Sigma) and 100U/ml hyaluronidase (Sigma). Cells were then treated with 0.25% trypsin/EDTA, dispase (Invitrogen)/DNase (Sigma), and ammonium chloride in succession. Between each treatment, cells were rinsed in MEGM (1:1 DMEM:F12 Ham supplemented with 5µg/ml insulin, 500ng/ml hydrocortisone, 10ng/ml EGF, 20ng/ml cholera toxin, 5% bovine calf serum, and 1 × penicillin/streptomycin). Afterwards, cells were resuspended in FACS buffer (PBS supplemented with 5% newborn calf serum plus 1 × penicillin/streptomycin) and filtered twice through 40µm nylon cell strainers. The following antibodies were used to label cells: biotin conjugated anti-TER119 (red blood cells, Cat # 553672, BD), biotin conjugated anti-CD31 (endothelial cells, Cat # 558737, BD), biotin conjugated anti-CD45 (hematopoietic cells, Cat # 553078, BD), CD29-FITC (Cat # MCA2298F, Serotec), CD24-PE (Cat # 553262, BD), and rabbit anti-Perp (Cat # ab5986, Abcam). PE-Cy7 conjugated Streptavidin (Cat # SA1012, Life Technologies) was used for secondary staining of lineage markers. Alexa Fluor 647 conjugated anti-rabbit IgG (Cat # A-21246, Life Technologies) was used for secondary staining of anti-Perp. For all staining, 50µl of antibody diluted 1:75 in FACS buffer was used per 1×10^6 cells, including single color
controls and combination staining. Control samples for four-color FACS included no-staining, propidium iodide (PI, cell viability dye) only, CD24-PE only, CD29-FITC only, Biotin-lineage-PE-Cy7 only, Perp-Alexa Fluor 647 only, as well as corresponding PE, FITC, PE-Cy7, and Alexa Fluor 647 single color fluorochrome conjugated antibody isotype controls. Both primary and secondary staining was conducted for 30 minutes at room temperature in FACS buffer. Between staining, cells were washed with 5ml FACS buffer. Cell sorting for transplantation experiments was done using a FACS Vantage SE w/DiVa (BD Biosciences). All cells were sorted into 1:1 Fetal Bovine Serum:MEGM.

**RNA extraction and quantitative real-time PCR**

Total RNA was extracted using the RNeasy Mini Kit (Cat # 74106, Qiagen) according to the manufacturer’s instructions. For mRNA analysis, cDNA was oligo(dT) primed from 1-2 µg of total RNA using a SuperScriptIII First-Strand Synthesis System (Cat # 18080-051, Invitrogen). cDNA from each sample was diluted 4-5 fold and real-time was performed in triplicates using Power SYBR green PCR master mix (Cat # 4367659, Applied Biosystems) on an ABI 7900HT series PCR machine. Expression levels were normalized to *GAPDH* expression. All analysis was performed using the SDS2.3 software.

**Microarrays for P4, P5 and P6 populations**

4 mice (~9-week-old) were used as donors for the wild-type CD24/CD29 isolation. For the pregnant mouse CD24/CD29 staining, cells were taken 1 day after parturition (~9-week-old). RNA was isolated using the Ambion mirVana miRNA isolation kit (without enrichment for small RNAs, Cat # AM1560, Life Technologies). RNA quality was
assessed using the Agilent Bioanalyzer 2100 using RNA Nano chips (Cat # 5067-1511, Agilent Technologies). Samples for the microarrays were prepared using the Agilent Quick Amp Labeling Kit (Cat # 5190-0424, Agilent Technologies) according to the manufacturer’s protocol. ~150ng of RNA from each population was labeled with Cy5 and hybridized to Agilent Whole Mouse Genome 4x44k oligo microarrays along with Cy3-labeled Mouse Universal Reference RNA (Cat # 740100, Stratagene). After hybridization, scanning was performed with an Agilent G2565BA scanner. Images were analyzed using Agilent Feature Extraction v9.5 software. The feature median signals normalized to the array median were used to calculate Cy5/Cy3 ratios. To identify P4-enriched surface markers, genes had to show at least 3-fold enriched expression in any one of the pair-wise comparisons between P4 and non-P4 populations. Heat maps of Log2 expression ratios were generated with MATLAB (MathWorks).

**Generation of stable Perp KD and Perp overexpressing mammary epithelial cells**

Short hairpin RNAs (shRNA), cloned into lentiviral vector pLKO.1-puro and pGIPZ-puro, targeting mPerp (Perp KD-A, Cat # TRCN0000112146; Perp KD-B, V2LMM_49774) were purchased from Sigma Aldrich and Open Biosystems, respectively. To overexpress Perp, the coding sequence was cloned into lentiviral vector pLEIGW. Lentivirus was produced by transfecting 293T cells with VSVG:deltaR8.9:shRNA or over-expression plasmid constructs at a ratio of 1:2.5:4. Virus was harvested 2-3 days after transfection, filtered, and used to spin-infect primary mammary epithelial cells at 1000g for 90 minutes in MEGM with 5 µg/ml polybrene.
Cells infected with empty vectors were used as negative controls for *in vitro* and *in vivo* experiments.

**Immunoblot analysis**

Cells were lysed in RIPA buffer and boiled in 1% SDS Laemmli sample buffer containing 3% β-mercaptoethanol for 10 minutes. Approximately 20 µg of protein was loaded per lane and resolved by SDS-polyacrylamide electrophoresis. Protein was transferred onto PVDF membranes, blocked and probing was carried out with rabbit anti-Perp (Cat # ab129083, Abcam) and mouse anti-beta Actin (Cat # ab6276, Abcam). Membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Cat # NA934V, GE Healthcare) or anti-mouse secondary antibody (Cat # NA931V, GE Healthcare) for 1h and signals were developed using the ECL method (GE Healthcare).

**3D Matrigel colony formation assay**

A thin layer of growth factor-reduced Matrigel was plated in a 24-well tissue culture plate and solidified, 10,000-20,000 FACS-sorted cells were resuspended in 50% Matrigel/50% MEGM and seeded on top. Cells were grown in MEGM for 10-14 days before colonies were scored. Images of colonies were taken on a Zeiss Axiovert 200M and colony diameter was measured using Zeiss imaging software.

**Tumorsphere formation assay**

10,000-20,000 FACS-sorted cells were cultured in 1:1 DMEM:F12 Ham supplemented with 20 ng/ml bFGF, 20ng/ml EGF, 4 ng/ml heparin, 50x B-27 in low-attachment plates.
Tumorspheres were counted 10-14 days after seeding. Images of tumorspheres were taken on a Zeiss Axiovert 200M and colony diameter was measured using Zeiss imaging software.

**Mammary fat pad transplantation**

All procedures involving mice, such as housing and care, and all experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) of Princeton University. After FACS, cells were centrifuged for 5 minutes at 1000g and then resuspended in 50% Matrigel/50% PBS. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/Kg) and xylazine (10mg/Kg). Cells were injected into cleared inguinal (#4) mammary fat pads according to standard injection procedures using Hamilton syringes. Briefly, a small incision was made to reveal the mammary gland, endogenous epithelium was surgically removed, and cells were injected directly into the cleared fat pad. The incision was subsequently closed with wound clips.

**Tumor xenografts**

Primary tumors were harvested and FACS-sorted with the same method used in mammary epithelial cell preparation. Sorted cells were resuspended in 50% PBS/50% Matrigel. For mammary fat pad primary tumor formation, female FVB mice at 6 weeks old were anesthetized by intraperitoneal injection of ketamine (100 mg/Kg) and xylazine (10 mg/Kg). Cells were injected directly into the mammary fat pad with the same method used in mammary fat pad transplantation. The site of injection was palpated weekly for tumor initiation and monitored by measurement of the tumor size.
Alum carmine staining of mammary glands

Excised mammary glands were fixed in 10% neutral-buffered formalin overnight at room temperature, washed for 15 minutes in 70%, 35%, 15% ethanol and distilled water, and then stained in alum carmine overnight at room temperature. Mammary glands were destained for 15 minutes in 70%, 95% and 100% ethanol, and transferred for long term storage into Histo-Clear II clearing agent (Cat # HS-200, National Diagnostics). Slides were mounted with Permount Mounting Medium (Cat # SP15-500, Fisher Scientific). Images were taken on a Zeiss Discovery V8 microscope.

Immunofluorescence

For staining of FACS-sorted cells, cells were mounted on microscope slides by cyto-spinning 25,000–50,000 cells/slide with a Cytospin 4 Cytocentrifuge. Cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes on ice and washed with PBS before blocking. For staining of tissue, the fourth and fifth mammary glands or primary tumors were excised, fixed with 4% PFA overnight at 4°C, embedded in paraffin or OCT, and sectioned at 6-10 µm thickness. The Mouse on Mouse (M.O.M.) Basic Kit (Cat # BMK-2202, Vector Laboratories) was used according to the manufacturer’s instructions to block and permeabilize the cells/tissue sections. Cells/sections were incubated with the following primary antibodies in blocking solution overnight at 4°C: rabbit anti-Perp (Cat # ab5986, Abcam), mouse anti-SMA (Cat # A 5228, Sigma-Aldrich), chicken anti-K14 (Cat # ab130102, Abcam) and rat anti-K8 (Cat # TROMA-I, DSHB). Cells/sections were washed 3 x 5 minutes with PBS and stained with the following secondary antibodies in blocking solution for 1 hour at room temperature: Alexa Fluor 594 goat anti-rabbit (Cat #
A11012, Life Technologies), Alexa Fluor 488 goat anti-mouse (Cat # A11029, Life Technologies), Alexa Fluor 633 goat anti-chicken (Cat # A21103) and Alexa Fluor 594 donkey anti-rat (Cat # A21209, Life Technologies). Cells/sections were washed 3 x 5 minutes with PBS, followed by DAPI nuclear staining for 5 minutes in room temperature, and washed for 5 minutes with PBS. Slides were mounted with VECTASHIELD Mounting Medium (Cat # H-1200, Vector Laboratories). Images were taken on either a Zeiss Axiovert 200M or Nikon A1 confocal microscope.

**Statistical Analysis**

Results were reported as mean ± s.e.m. (standard error of the mean). Two-sided independent student’s t-test without equal variance assumption was performed to analyze gene expression levels. A standard chi-square goodness of fit test was used to determine significance of repopulating frequency. The log-rank test was used to test for differences in tumor-free mammary gland univariate analysis. Statistical analyses were performed using R (www.r-project.org).
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Chapter 3: Perp Facilitates Lung Metastatic Colonization of Epithelial-Like Breast Cancer Cells
Introduction

The ZEB transcription factor family transcriptionally represses E-cadherin during EMT (Lamouille et al., 2014). miR-200s have been shown to prevent this process and the over-expression of this cluster of miRNAs in mesenchymal-like breast cancer cells leads to MET, which enhances the metastatic colonization of these cells in the lungs (Korpal et al., 2011). Although miR-200s also influence the tumor cell secretome by direct targeting of the Sec23a-mediated transport pathway, it is interesting to note that Perp expression is one of the most up-regulated genes upon miR-200 over-expression. Since Perp has been implicated in desmosome function in the skin and the disassembly of desmosomes occurs during EMT (Huang et al., 2012; Ihrie et al., 2005), we wanted to know whether these two processes could be linked in cancer metastasis and metastatic colonization. Is Perp downregulated during EMT to facilitate desmosome disassembly and subsequent metastasis? Moreover, is Perp-mediated desmosome reassembly critical for the complete reversion of breast cancer cells to an epithelial-like state during metastatic colonization? To address these questions, we investigated the role of Perp in breast cancer metastasis in the context of EMT/MET.
Results

Perp expression positively correlates with epithelial-like morphology in breast cancer cell lines

To investigate the role of Perp in breast cancer metastasis, we profiled Perp expression levels in two cancer cell lines series that model the progression of breast cancer. The 4T1 series, including 67NR, 169FARN, 4TO7 and 4T1, are near-isogenic mouse mammary tumor cell lines. Of these lines, only 4T1 cells are capable of spontaneously metastasizing and colonizing distant organs after orthotopic implantation (Aslakson and Miller, 1992; Yang et al., 2004). We found that Perp showed greatest expression in the highly metastatic 4T1 cells (Figure 6-1A and B), which was consistent with the acquisition of epithelial traits in 4T1 compared to the weakly metastatic 4TO7 cells. E-cadherin expression was used to quantify the epithelial characteristic of these cell lines and indeed showed highest expression in 4T1 cells. Similarly, elevated expression of PERP was also correlated with epithelial traits for the MCF10A human breast cancer progression series (Santner et al., 2001) and with greater metastatic ability. Although the parental MCF10AT cell line has relatively higher expression of PERP than its metastatic derivatives, the highly metastatic and more epithelial MCF10A1a derivative has higher expression of PERP than the MCF10A1h mesenchymal derivative. These observations collectively point to the possibility that Perp may be functionally involved in the successful completion of metastasis, particularly in the colonization step when metastatic cells revert to and rely on their epithelial state.
Figure 6-1. Perp expression correlates with epithelial-like morphology in breast cancer cells. (A) Expression levels of Perp and E-cadherin (Cdhl) in 4T1 and MCF10AT metastasis progression series assessed by quantitative RT-PCR. Data is normalized to Gapdh levels and presented as mean ± s.e.m. ** P < 0.01, *** P < 0.001 (Student’s t-test). (B) Western blot showing Perp and Cdhl expression. β-actin was used as loading control.
To investigate the clinical relevance of PERP expression, we performed a retrospective analysis on a series of breast tumor samples. *PERP* expression was significantly associated with poor distant metastasis-free survival (DMFS) as well as poor distant relapse-free survival (RFS) (Figure 6-2A). Furthermore, profiling of *PERP* levels in human no-evident disease (NED) and visceral metastasis (VM) relapse samples revealed higher expression in metastases, reinforcing the potential role of PERP in metastatic colonization (Figure 6-2B). Extensive subtype analysis showed *PERP* expression to be significantly higher in the basal compared to the non-basal subtype; *PERP* expression is also higher in estrogen receptor-negative (ER-) than in ER+ tumors (Figure 6-2C). It should be noted that while there is no correlation between *PERP* expression and RFS in ER- tumors, there is a positive correlation between high *PERP* expression and poor DMFS. Therefore, all of these results suggest that high *PERP* expression in epithelial-like metastatic cancer cells contributes to metastasis-associated mortality in breast cancer patients.

**Perp knockdown inhibits breast cancer metastatic colonization of the lungs**

To directly test the functional role of Perp in metastasis, we stably knocked down *Perp* using short-hairpin RNA (shRNA) in the epithelial-like, strongly-metastatic 4T1 cells. *Perp* knockdown (KD) did not lead to any changes in the epithelial-like cellular morphology (Figure 7-1A), though there were moderate changes in epithelial-to-mesenchymal transition (EMT) markers (Figure 7-1B and C). These results were expected, as Perp is not a transcriptional regulator that can activate/suppress EMT genes. Both sulforhodamine B colorimetric assay (Figure 7-2) and flow cytometry analysis
Figure 6-2. Patients with high PERP expression have poor prognosis in DMFS and RFS. (A) Kaplan-Meier curves showing the relationship of PERP expression levels with distant metastasis-free survival (left) and distant relapse-free survival (right). P values were computed by a log-rank test. (B) Expression levels of PERP in primary tumors of patients with no evident disease (NED) and visceral metastasis (VM) assessed by qRT-PCR. (C) Clinical correlation of PERP expression levels in primary tumors of patients with basal vs. non-basal or estrogen receptor(ER)-positive vs -negative. P values were computed by a Mann-Whitney test.
Figure 7.1. *Perp* KD in 4T1 cells does not affect epithelial-like morphology. (A) Phase contrast images of 4T1 cells knocked-down for *Perp*. Scale bar 300 µm. (B) Expression levels of *Perp* and EMT markers in *Perp* KD 4T1 cells relative to vector controls assessed by qRT-PCR. Data is normalized to *Gapdh* levels and presented as mean ± s.e.m. * P < 0.05, ** P < 0.01, *** P < 0.001 (Student’s t-test). (C) Western blot showing *Perp*, Cdh1 and Snai1 expression in *Perp* KD 4T1 cells relative to vector controls. β-actin was used as loading control.
Figure 7-2. *Perp* KD in 4T1 cells does not affect cell proliferation. Cell proliferation rate of *Perp* KD 4T1 cells, relative to control, assessed by the SRB assay.
using the proliferation marker Ki-67 (data not shown) showed no significant difference in the proliferation rate of these cells.

In order to test the potential role of Perp in tumorigenic potential in vivo, Perp KD and control 4T1 cells were injected into the mammary fat pad for primary tumor formation. Orthotopic implantation of Perp KD 4T1 cells showed no difference in rate of tumor growth or final tumor weight (Figure 7-3A and B), consistent with the in vitro proliferation assay results. Histological analysis of primary tumors did not show any obvious differences in cellular morphology or stromal composition (Figure 7-3C). 4T1 and EpRas cells are highly efficient in spontaneous pulmonary metastasis after orthotopic implantation. Therefore, control and Perp KD 4T1 cells, which were stably transduced with a lentiviral construct expressing the firefly-luciferase gene, were injected into the mammary fat pad to form primary tumors. In order to prolong the survival period of animals to allow for sufficient development of spontaneous lung metastasis, primary tumors were removed 2 weeks after implantation. Surprisingly, lungs isolated from mice with orthotopic firefly luciferase-labeled 4T1 tumors showed significantly reduced bioluminescence (BLI) signal in mice with Perp KD tumors (Figure 7-3D). We did not expect to see a difference in their spontaneous metastasis to the lungs, given that Perp KD alone did not affect the EMT program of 4T1 cells in vitro. Since these results strongly suggest that Perp may play a role specifically in metastasis, not in primary tumor initiation/progression, we proceeded to test the metastatic potential of Perp KD cells in distinct stages of distant metastasis.
Figure 7-3. *Perp* KD in 4T1 cells does not affect primary tumor growth but inhibits spontaneous pulmonary metastasis *in vivo*. (A) Measurements of *Perp* KD 4T1 primary tumor growth. *Perp* KD 4T1 cells were injected into the mammary fat pad of female BALB/c mice for primary tumor formation. Data is presented as mean ± s.e.m. *P* < 0.05 (Student’s t-test). (B) Final tumor weight of *Perp* KD 4T1 primary tumors. (C) H&E staining of *Perp* KD 4T1 primary tumors. Primary tumors were sectioned and stained with hematoxylin and eosin. (D) Bioluminescence quantification of *Perp* KD 4T1 spontaneous pulmonary metastasis. Luciferase-labeled 4T1 cells were knocked-down for *Perp* and injected into the mammary fat pad for primary tumor formation. Lungs were removed and bioluminescence was measured *ex vivo*. *P* values were computed with a Mann-Whitney test.
As metastasis is a multistep process, we aimed to elucidate the influence of Perp on early and late steps of metastasis. To determine whether invasion or intravasation is regulated by Perp, we analyzed whole blood samples for circulating tumor cells. Using the puromycin resistance gene present in the shRNA vector, we assessed the presence or absence of disseminating tumor cells in the circulation. In contrast to the results obtained from the 4T1 spontaneous metastasis assays, Perp KD had no effect on the efficiency of tumor cell entry into circulation from primary tumors (Figure 7-4A). There were no significant changes in EMT markers in Perp KD tumors (Figure 7-4B), consistent with in vitro results. Finally, the migratory and invasive potentials of Perp KD cells were directly tested in vitro using trans-well assays. As expected, Perp KD did not affect migration or invasion in vitro (Figure 7-4C), suggesting that Perp does not regulate the earlier stages of metastasis, notably the invasion and intravasation into the systemic circulation.

To test the functional role of Perp in metastatic colonization after tumor cells have already entered the circulation, we inoculated cells directly into venous circulation in mice and measured the incidence of pulmonary metastasis. Bioluminescence imaging of firefly luciferase-labeled cells showed significantly reduced metastasis burden for Perp KD 4T1 cells (Figure 7-5A and B). Notably, although the growth rates of BLI signal detected in the lungs were similar, significantly lower BLI signal was detected for Perp KD cells at Week 0.5 post-inoculation, suggesting that Perp may play a role in the initial seeding process of pulmonary metastasis. Quantification of macrometastases further validated these findings (Figure 7-6A and B). To test whether successful colonization of Perp KD cells was due to inefficient shRNA-mediated knockdown, metastatic nodules were analyzed for Perp mRNA expression. Surprisingly, Perp KD was maintained in the
Figure 7-4. *Perp* KD in 4T1 cells does not affect primary tumor cell entry into the circulation. (A) PCR analysis of circulating tumor cells from *Perp* KD 4T1 primary tumors. Genomic DNA was extracted from whole blood and genomic PCR analysis was performed using primers specific for *puromycin* drug resistance gene. (B) Expression levels of EMT markers in *Perp* KD 4T1 primary tumor cells assessed by qRT-PCR. (C) *In vitro* migration and invasion assays with *Perp* KD 4T1 cells. Growth factor-reduced Matrigel was used for invasion assays. Data is normalized to *Gapdh* levels and is presented as mean ± s.e.m. ** *P* < 0.01 (Student’s t-test).
Figure 7-5. Perp KD in 4T1 cells significantly inhibits pulmonary metastasis. (A) *In vivo* bioluminescence quantification of Perp KD 4T1 cells in the lungs, normalized to time point Day 0. Luciferase 4T1 cells were knocked-down for Perp and injected into the tail vein for experimental pulmonary metastasis. Data is presented as mean ± s.e.m. *P* < 0.05, **P* < 0.01, ***P* < 0.001 (Student’s t-test). (B) Images of *in vivo* bioluminescence quantification of Perp KD 4T1 cells in the lungs at Week 2 post-injection.
Figure 7-6. *Perp* KD in 4T1 cells significantly inhibits metastatic colonization of the lungs. (A) Brightfield images and H&E staining of metastatic colonies formed by *Perp* KD 4T1 cells at Week 2 post-injection. Scale bar 1 cm. (B) Quantification of *Perp* KD 4T1 macrometastases in the lungs. **P < 0.01 (Mann-Whitney test). (C) Expression levels of *Perp* and *Cdh1* in *Perp* KD 4T1 metastatic lung nodules assessed by qRT-PCR. Data is normalized to *Gapdh* levels and presented as mean ± s.e.m. **P < 0.01 (Student’s t-test).
Figure 7-7. Perp KD in EpRas cells significantly inhibits pulmonary metastasis. (A) *In vivo* bioluminescence quantification of Perp KD EpRas cells in the lungs, normalized to time point Day 0. Luciferase EpRas cells were knocked-down for Perp and injected into the tail vein for experimental pulmonary metastasis. Data is presented as mean ± s.e.m. * P < 0.05, ** P < 0.01, *** P < 0.001 (Student’s t-test). (B) Brightfield images of metastatic colonies formed by Perp KD EpRas cells. Scale bar 1 cm. (C) Quantification of Perp KD EpRas macrometastases in the lungs. *P* value was computed using a Mann-Whitney test.
metastatic nodules and E-cadherin levels remained unchanged (Figure 7-6C). This suggests that while Perp KD cells are still able to colonize the lungs, they are significantly less efficient in doing so. As seen in the primary tumor, these results confirm that the epithelial character of these lung-resident Perp KD cells has not been affected. We observed a stronger phenotype in the EpRas cell line (Figure 7-7A, B and C), suggesting that our observations are not cell line-specific. In summary, our results from both clinical and experimental analyses collectively show that Perp plays a critical role in distant colonization of epithelial-like breast cancer cells. However, the fact that Perp KD itself does not lead to any changes in the EMT context suggests that other genes or signaling pathways may be affected to compromise metastatic colonization.

**Perp and desmosomal components are down-regulated during EMT of breast cancer cells**

Desmosomal junctions are critical for epithelial integrity. Perp has been shown to play a critical role in desmosome assembly in the skin and Perp KO mice die from severe blistering (Ihrie et al., 2005). In order to address whether Perp KD-mediated desmosome disassembly led to decreased metastatic colonization, we analyzed several desmosome proteins in Perp KD cells. Perp KD did not lead to any changes in mRNA or protein levels of desmosome proteins (Figure 8-1A and B) and mis-localization of these desmosomal complex components could not be observed (Figure 8-2). Our observations using metastatic cancer cells are different from other reports, as it has been previously shown that desmosomal proteins are decreased in mammary epithelial cells of conditional Perp KO mice and the same study claims that Perp deficiency in breast cancer results in
Figure 8-1. *Perp* KD in 4T1 cells does not affect desmosome expression levels. (A) Expression levels of desmosomal components in *Perp* KD 4T1 cells (normalized to control) assessed by qRT-PCR. Data is normalized to *Gapdh* levels and presented as mean ± s.e.m. * P < 0.05, ** P < 0.01 (Student’s t-test). (B) Western blot showing desmosomal protein expression in *Perp* KD 4T1 cells. β-actin was used as loading control.
Figure 8-2. *Perp* KD in 4T1 cells does not affect desmosome localization. Immunofluorescence images of desmosomal proteins in *Perp* KD 4T1 cells. Cells were cultured on glass coverslips and stained with anti-Perp, -Cdh1, -Dsp, -Pg and -Pkp3 antibodies.
faster tumor initiation and progression based on a p53 spontaneous tumor model (Dusek et al., 2012). The authors also make a clinical correlation using human breast cancer cell lines, drawing a conclusion that PERP expression is low in human breast cancer cells compared to normal mammary epithelial cells. These findings, however, do not conflict with our results, since the authors do not show desmosomal localization in or desmosomal protein levels of the resulting tumor. One should also note that the human cell lines selected for this correlational analysis are already mesenchymal-like in nature and thereby express low levels of PERP. Therefore, whether the findings of Dusek et al. apply in the tumorigenic context remains unanswered and the different context could explain the differences in our findings.

Despite the lack of desmosomal localization or abundance upon Perp suppression, several of these components were highly expressed in the epithelial-like 4T1 and EpRas cells, suggesting a link between Perp and desmosomes in breast cancer cells. Although we did not observe significant changes in EMT markers with Perp KD alone, we wanted to know whether the EMT program modulates Perp expression, as well as desmosomes. In order to test whether Perp and desmosomes are affected during EMT in breast cancer cells, we treated EpRas cells with TGF-β to induce EMT. Perp and desmosomal components were significantly down-regulated in TGFβ-treated cells with accompanying changes in EMT markers (Figure 8-3A and B). Interestingly, several desmosome proteins showed mis-localization in cells undergoing EMT (Figure 8-4). In order to assess whether these changes were indeed through TGFβ-mediated EMT and not through independent effects of TGF-β signaling, SNAI1 was overexpressed in EpRas cells to induce the EMT program. Perp and other desmosomal components were significantly
down-regulated in SNAI1-overexpressing cells compared to the control, comparable to the levels with TGF-β treatment (Figure 8-5). These observations confirm the disassembly of desmosomal junctions between epithelial-like breast cancer cells while they adopt a mesenchymal-like morphology for motility via EMT. Although it has been shown in other studies that the disassembly of desmosomal junctions occurs during EMT in various contexts (Huang et al., 2012; Savagner et al., 1997; Yilmaz and Christofori, 2009), this is one of the first reports of Perp down-regulation in breast cancer cells during EMT. It is important to note that Perp KD in EpRas cells did not phenocopy the cells treated with TGF-β, suggesting that the down-regulation of Perp is not sufficient to induce EMT. It has already been shown in other studies that EMT factors can directly suppress the expression of desmosomal components (Aigner et al., 2007; Lamouille et al., 2014). To test whether enforced Perp expression is sufficient to prevent EMT, we overexpressed Perp in EpRas cells and treated them with TGF-β to induce EMT. Consistent with the Perp KD data, the over-expression of Perp was not sufficient to retain the expression and localization of desmosomal components (Figure 8-6). Therefore, Perp and desmosomal components are down-regulated during EMT-induced desmosome disassembly, but EMT-mediated Perp suppression does not seem to be the cause of desmosome disassembly, since Perp KD alone was insufficient to cause desmosome disassembly. These findings suggest that Perp may have a desmosome-independent role in the epithelial state of breast cancer cells.
Figure 8-3. TGFβ-induced EMT leads to a decrease in desmosome expression levels. (A) Expression levels of desmosomal components in TGFβ-treated EpRas cells (normalized to PBS-treated cells) assessed by qRT-PCR. Data is normalized to Gapdh levels and presented as mean ± s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student’s t-test). (B) Western blot showing desmosomal protein expression in TGFβ-treated EpRas cells relative to PBS-treated cells. β-actin was used as loading control.
Figure 8-4. TGFβ-induced EMT leads to disassembly of desmosomes.
Immunofluorescence images of Cdh1 and desmosomal components in TGFβ-treated EpRas cells. Scale bar 50 μm.
Figure 8-5. *Snail* over-expression in EpRas cells leads to a decrease in desmosome expression levels. Expression levels of desmosomal components in *Snail* OE EpRas cells (normalized to control cells) assessed by qRT-PCR. Data is normalized to *Gapdh* levels and presented as mean ± s.e.m. **$P < 0.01$ (Student’s t-test).
Figure 8-6. Over-expression of *Perp* does not inhibit TGFβ-induced disassembly of desmosomes. Immunofluorescence images of *Perp* OE EpRas cells treated with TGF-β compared to controls treated with PBS. Scale bar 50 μm.
Perp knockdown inhibits breast cancer MET-induced metastatic colonization of the lungs

In order to determine whether Perp may play a role in the reverse process of EMT, mesenchymal-to-epithelial transition (MET), we overexpressed miR-200s in the mesenchymal-like 4TO7 cells. We have previously shown that miR-200 over-expression significantly enhances the metastatic colonization efficiency of 4TO7 cells (Korpal et al., 2011). miR-200s suppress ZEBs and thereby relieve mesenchymal-like cells from the suppression of the epithelial program. miR-200 over-expression induced a transition to epithelial-like morphology (data not shown) with a corresponding increase in E-cadherin. Interestingly, miR-200 over-expression also significantly increased Perp expression (Figure 9-1A and B), suggesting that Perp expression may be suppressed by ZEBs in the mesenchymal-like 4TO7 cells. Binding motif analyses of the various EMT transcription factors revealed that ZEBs, as well as SNAI1 do indeed have the potential to bind to the promoter region of Perp (data not shown). In order to validate these predictions, we generated stable Zeb1 and Zeb2 knock-downs in the 4TO7 cell line and measured Perp mRNA expression. Although both Zeb knock-downs resulted in increased Perp expression, Zeb2 KD led to a significantly higher increase compared to Zeb1 KD (Figure 9-1C). Therefore, these results suggest that ZEBs may transcriptionally repress Perp expression and the increase of Perp upon miR-200 OE may be through ZEBs.

Although it has been previously shown that miR-200 over-expression can affect the tumor cell secretome by suppressing Sec23a-mediated secretion of metastasis suppressors, such as Igfbp4 and Tinagl1 (Korpal et al., 2011), we sought to test the functional impact
Figure 9-1. miR-200 over-expression in 4TO7 cells leads to an increase in Perp expression. (A) Expression levels of Cdh1 and Perp in miR-200 OE 4TO7 cells (C1+C2) (normalized to vector controls) assessed by qRT-PCR. (B) Western blot showing Cdh1 and Perp expression in miR-200 OE 4TO7 cells relative to vector controls. (C) Expression levels of Zeb1, Zeb2, Cdh1 and Perp in Zeb1 and Zeb2 KD 4TO7 cells (normalized to vector controls) assessed by qRT-PCR. Data is normalized to Gapdh levels and presented as mean ± s.e.m. * P < 0.05, ** P < 0.01, *** P < 0.001 (Student’s t-test).
Figure 9-2. *Perp* KD in miR-200 OE 4TO7 cells significantly inhibits pulmonary metastasis. (A) Expression levels of *Perp* and EMT factors in miR-200 OE 4TO7 cells knocked-down for *Perp* assessed by qRT-PCR. Data is normalized to *Gapdh* levels. (B) *In vivo* bioluminescence quantification of miR-200 OE+*Perp* KD 4TO7 cells in the lungs, normalized to time point Day 0. (C) Images of *in vivo* bioluminescence quantification of miR-200 OE+*Perp* KD 4TO7 cells in the lungs at Week 1.5 post-injection. (D) Brightfield images of metastatic colonies formed by miR-200 OE+*Perp* KD 4TO7 cells at Week 1.5 post-injection. Scale bar 1 cm. Data is presented as mean ± s.e.m. * P < 0.05, ** P < 0.01, *** P < 0.001 (Student’s t-test).
of Perp up-regulation. miR200-overexpressing 4TO7 cells labeled with firefly luciferase were stably knocked down for Perp (Figure 9-2A) and inoculated directly into circulation to test for pulmonary metastasis. Compared to the control, Perp KD in miR200-overexpressing cells significantly inhibited pulmonary metastasis (Figure 9-2B, C and D). Interestingly, similar to 4T1 and EpRas cells, suppression of Perp in these cells affected the initial seeding step and affected the earlier stages of metastatic colonization. Overall, our results show that the expression of Perp and desmosomal components is dynamically regulated during the EMT/MET process and the suppression of Perp can inhibit MET-induced metastatic colonization. However, the functional role of Perp in breast cancer cell metastasis is likely to be independent of its role in skin epithelial integrity.

**Novel pathways affected by Perp KD in breast cancer metastatic colonization of the lungs**

To identify functional gene and pathway targets of Perp, we performed microarray analysis on Perp KD 4T1 cells and corresponding lung metastatic nodules. Although we observed global changes in gene expression in the *in vivo* lung metastatic nodules, substantially less gene expression change was evident in the *in vitro* samples (Figure 10-1A), suggesting a potential role of Perp in mediating the interaction of tumor cells with the lung stroma. We therefore pre-screened for genes that were greater than 2-fold up-regulated in the *in vivo*-isolated control samples compared to the *in vitro* control sample and then selected only the genes that were greater than 2-fold down-regulated in the *in vivo*-isolated Perp KD samples compared to the *in vivo*-isolated control samples (Figure 10-1B). These would represent genes that could potentially be required for *in vivo*
survival and/or interaction with the lung stroma that are down-regulated by Perp KD. Gene ontology analysis of this gene list revealed that numerous extracellular proteins are down-regulated \textit{in vivo} with Perp KD. More interestingly, several candidates involved in carbohydrate binding and cellular response to toxin exposure were found to be down-regulated in Perp KD cells (Table 3).

In order to investigate whether the changes in extracellular proteins, including Perp, and carbohydrate binding due to Perp KD affect the initial binding of cancer cells to the lung stromal cells, we performed a co-culture binding assay. Lung stromal cells were isolated and cultured \textit{in vitro} until complete confluency was reached. Firefly luciferase-labeled control and Perp KD 4T1 cells were seeded on top of the lung stromal cells for 1 hour before unbound cells were washed away. Perp KD showed significant reduction of bound cancer cells after co-culture (Figure 10-2), suggesting that Perp itself or consequential changes upon Perp KD inhibits cell-cell binding between cancer cells and the lung stroma.
Figure 10-1. *Perp* KD in 4T1 cells leads to global changes in gene expression *in vitro* and *in vivo*. (A) Venn diagram showing number of genes changing greater than 2-fold between control and *Perp* KD 4T1 *in vitro* and/or *in vivo*. (B) Heat map showing genes changing greater than 2-fold between control 4T1 *in vitro* vs. *in vivo* (left) and *in vivo* control vs. *in vivo* *Perp* KD.
Table 3. Functional gene sets enriched in *in vivo* 4T1 control samples relative to Perp KD samples.

<table>
<thead>
<tr>
<th>Cellular component</th>
<th># of genes</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular space</td>
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<td>0.019</td>
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<tr>
<td>Extracellular region</td>
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<td>0.028</td>
</tr>
<tr>
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<td>0.034</td>
</tr>
<tr>
<td>Protein-lipid complex</td>
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<td>0.034</td>
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<tr>
<td>Extracellular region part</td>
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<td>0.044</td>
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<td>0.034</td>
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<table>
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<tr>
<th>Biological process</th>
<th># of genes</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to toxin</td>
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<td>0.044</td>
</tr>
</tbody>
</table>
Figure 10-2. *Perp* KD in 4T1 cells significantly inhibits adhesion to lung stromal cells *in vitro*. Luciferase-labeled 4T1 cells were knocked-down for *Perp* and seeded on top of primary lung stromal cells for 1 hr. Data is presented as mean ± s.e.m. *P* < 0.05, **P** < 0.01 (Student’s t-test).
Discussion

Perp facilitates breast cancer lung metastasis

Although it is well known that desmosomes are abundant in epithelial cells (Dusek and Attardi, 2011), we show that Perp expression levels positively correlate with the levels of primary epithelial marker E-cadherin. Within the isogenic cell line series with different metastatic potential, where E-cadherin expression is highest in the most metastatic cell line, Perp expression was indeed higher in the more metastatic cell line, suggesting a link between the metastatic potential and Perp expression. However, Perp expression itself does not seem to play a significant role in maintaining the epithelial state of these cells, as Perp KD did not lead to any major changes in the EMT program. On the other hand, EMT induction led to a significant down-regulation of Perp and other desmosomal components. These results suggest that Perp expression is tightly linked to the epithelial state of breast cancer cells and is dynamically regulated by EMT factors.

We show here that the suppression of Perp significantly compromises breast cancer lung metastasis. While the role of Perp in breast cancer initiation and progression within the primary tumor is still being addressed, our results show that Perp plays a critical role in facilitating the early process of metastatic colonization in the lungs. The previously established role of Perp in cellular adhesion via desmosome assembly (Ihrie et al., 2005) does not seem to apply to our experimental system, as Perp KD did not lead to desmosome disassembly or decrease in protein levels of desmosomal components in 4T1 and EpRas breast cancer cells. Therefore, homo- and/or hetero-typic interaction between
Perp and lung stromal cells, independent of Perp-mediated desmosome assembly, may be the underlying mechanism for the observed phenotype.

**The role of Perp in the mesenchymal-to-epithelial transition**

Having observed the positive correlation between Perp expression and the epithelial character of breast cancer cells, it was not surprising to us that the induction of MET in mesenchymal-like, poorly metastatic cells resulted in a significant increase in Perp expression. We cannot conclude at this point whether this increase is an indirect response of miR200-mediated suppression of Zebs, which can potentially bind to the promoter region of *Perp* to down-regulate transcriptional activity, or is merely a correlational increase based on an independent program related to the epithelial state. We were, however, surprised to observe that the suppression of *Perp* in the epithelial-like cells, which were forced to undergo MET, significantly compromised metastatic colonization of the lungs. Once again, *Perp* KD in these cells did not affect the EMT program, meaning that the decrease in lung metastasis was not due to the inhibition of the MET process. These results further support our claim that epithelial-like cells rely on Perp expression for successful metastatic colonization.

In characterizing every distinct stage of metastatic progression, we sought to investigate the role of Perp in different contexts, with a particular interest in the metastatic colonization stages of the process. Our results from the tumor initiation/progression studies differ from previous reports because we did not use spontaneous tumor models, in which a specific sub-population is pre-selected to give rise to tumor, as well as be the target for *Perp* depletion. Our study uses a relatively homogenous population of
epithelial-like cancer cell lines to induce tumorigenesis and to study primary tumor progression. The general idea of Perp being a tumor suppressor lies in the fact that Perp is traditionally viewed as an apoptosis effector and/or a critical desmosomal protein (Dusek and Attardi, 2011). In the cell adhesion context, several reports of desmosome disassembly during the EMT process (Huang et al., 2012; Savagner et al., 1997; Yilmaz and Christofori, 2009) further supports the claim that the expression of Perp and the resulting maintenance of desmosomes would inhibit cell motility, thereby suppressing local invasion of the primary tumor. Conversely, one would hypothesize that desmosome disassembly and the consequential compromise in epithelial integrity can facilitate cell motility and tumor invasion. We do not think Perp suppression in epithelial-like breast cancers facilitates tumor invasion and intravasation into the circulation, since we did not observe any differences in cell migration or invasion and could not detect more circulating tumor cells in the Perp KD tumor-bearing mice.

We employed an experimental metastasis setting in which we introduced Perp KD tumor cells directly into the circulation, bypassing all the steps prior to intravasation. It is between the entry into circulation and the beginnings of secondary tumor growth that Perp seems to play a critical role for successful metastatic colonization. It is important to note that temporal bioluminescence imaging provides very useful information in assessing which step could be most affected by the suppression of Perp. Given that the rates of increase in bioluminescence signal between Perp KD and control are very similar, we do not think the ability of Perp KD cells to expand at the secondary site is affected. However, the drastic difference seen at the initial time point of measurement, when circulating tumor cells have successfully seeded in the lungs, suggests one or a
combination of the following: *Perp* KD cells have 1) low survival in circulation 2) low efficiency of extravasation from circulation 3) low survival in the lung stroma. We have not addressed each of these processes in detail, but we do not think the survival in circulation is affected based on our analysis of CTCs. Our results from the co-culture binding experiments, however, indicate that the low attachment efficiency of the tumor cells may affect both extravasation from the circulation as well as the survival in the lung stroma. In addition, Perp-mediated cellular signaling between Perp-positive dormant tumor cells and their potential niche is a possibility that should be explored in the context of breast cancer relapse.
Materials and Methods

Cell culture

67NR, 168FARN, 4TO7, 4T1 and EpRas parental and derivative cells were maintained in complete media: Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing 10% fetal bovine serum, supplemented with 1mM L-glutamine and penicillin/streptomycin (Pen/Strep, Gibco). Perp KD and miR-200 overexpressing lines, along with their respective vector controls, were maintained in the same media as parental cells. MCF10AT, MCF10A1h and MCF10Aha cells were maintained in 1:1 DMEM:Ham’s F12 medium (Invitrogen) containing 5% horse serum, supplemented with 20ng/ml EGF, 0.5µg/ml hydrocortisone, 10µg/ml insulin, 1% Pen/Strep and 50ng/ml cholera toxin.

Generation of stable Perp knockdown and Snai1, miR-200 overexpressing lines

Short hairpin RNAs (shRNA), cloned into lentiviral vector pLKO.1-puro, targeting mPerp (KD #1, Cat # TRCN0000112146; KD #2, Cat # TRCN0000112145) were purchased from Sigma Aldrich. To overexpress all five miR-200 family miRNAs simultaneously, genomic fragments encoding cluster 1 (miR-200b/200a/429) and cluster 2 (miR-200c/141) were cloned into pMSCV-puro and pMSCV-hygro retroviral vectors respectively. To overexpress Snai1, the coding sequence was cloned into pLEX-puro lentiviral vector. For bioluminescent imaging and co-culture adhesion assays, 4TO7 and 4T1 cells were labeled with pLmPGK-FiG and pLEIGW-LiG, respectively. Lentivirus was produced by transfecting 293T cells with VSVG:deltaR8.9:shRNA or overexpression plasmid constructs at a ratio of 1:2.5:4. Virus was harvested 2-3 days after
transfection, filtered, and used to infect cell cultures in the presence of 5 µg/ml polybrene. Retrovirus was produced by transfecting the vectors into the H29 packaging cell line and harvested 48h after transfection, filtered, and used to infect cell cultures in the presence of 5 µg/ml polybrene. Cells infected with empty vectors were used as negative controls for \textit{in vitro} and \textit{in vivo} experiments. In all cases, infected cells were selected with appropriate drugs in the media and at least 1000 independent clones were pooled to generate stable cell lines to avoid clonal variations.

\textbf{Primary tumor cell culture}

Single cell suspensions were prepared from primary tumors after mechanical and enzymatic dissociation based on published protocols. Briefly, primary tumors were excised, minced using scalpels, and digested for 1hr in 300U/ml type 1A collagenase (Sigma) and 100U/ml hyaluronidase (Sigma). Cells were then treated with 0.25% trypsin/EDTA, dispase (Invitrogen)/DNase (Sigma), and ammonium chloride in succession. Between each treatment, cells were rinsed in MEGM (1:1 DMEM:F12 Ham supplemented with 5µg/ml insulin, 500ng/ml hydrocortisone, 10ng/ml EGF, 20ng/ml cholera toxin, 5% bovine calf serum, and 1× penicillin/streptomycin). Dissociated tumor cells were cultured in complete media and stromal cells were depleted using 4 µg/ml puromycin.

\textbf{RNA extraction and quantitative real-time PCR}

Total RNA was extracted using the RNeasy Mini Kit (Cat # 74106, Qiagen) according to the manufacturer’s instructions. For mRNA analysis, cDNA was oligo(dT) primed from 1-2 µg of total RNA using a SuperScriptIII First-Strand Synthesis System (Cat # 18080-88
cDNA from each sample was diluted 4-5 fold and real-time was performed in triplicates using Power SYBR green PCR master mix (Cat # 4367659, Applied Biosystems) on an ABI 7900HT series PCR machine. Expression levels were normalized to GAPDH expression. All analysis was performed using the SDS2.3 software.

**Immunoblot analysis**

Cells were lysed in RIPA buffer and boiled in 1% SDS Laemmli sample buffer containing 3% β-mercaptoethanol for 10 minutes. Approximately 20 µg of protein was loaded per lane and resolved by SDS-polyacrylamide electrophoresis. Protein was transferred onto PVDF membranes, blocked and probing was carried out with rabbit anti-Perp (Cat # ab129083, Abcam), mouse anti-E-Cadherin (Cat # 610181, BD Transduction), rabbit anti-Desmoglein 2 (Cat # ab150372, Abcam), mouse anti-gamma Catenin (Cat # ab12083, Abcam), rabbit anti-Plakophilin 3 (Cat # ab109441, Abcam), mouse anti-Snail (Cat # 3895, Cell Signaling), and mouse anti-beta Actin (Cat # ab6276, Abcam). Membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Cat # NA934V, GE Healthcare) or anti-mouse secondary antibody (Cat # NA931V, GE Healthcare) for 1h and signals were developed using the ECL method (GE Healthcare).

**Immunofluorescence**

Cells were seeded onto gelatin coated glass coverslips and placed in 24-well plates. 48h subsequent to seeding, media was aspirated and cells were washed with PBS before fixation with cold 4% PFA for 20 minutes or ice cold methanol for 10 minutes. Following
PBS washes, the Mouse on Mouse (M.O.M.) Basic Kit (Cat # BMK-2202, Vector Laboratories) and 0.3% Triton X-100 were used according to the manufacturer’s instructions to block and permeabilize the cells. Cells were incubated with the following primary antibodies in blocking solution overnight at 4°C: rabbit anti-Perp (Cat # ab5986, Abcam), mouse anti-E-Cadherin (Cat # 610181, BD Transduction), rabbit anti-Desmoglein 2 (Cat # ab150372, Abcam), mouse anti-Desmoplakin (Cat # ab16434, Abcam), mouse anti-gamma Catenin (Cat # ab12083, Abcam), rabbit anti-Plakophilin 3 (Cat # ab109441, Abcam). Cells were washed 3 x 5 minutes with PBS and stained with the following secondary antibodies in blocking solution for 1 hour at room temperature: Alexa Fluor 594 goat anti-rabbit (Cat # A11012, Life Technologies) and Alexa Fluor 568 goat anti-mouse (Cat # A11031, Life Technologies). Cells/sections were washed 3 x 5 minutes with PBS, followed by DAPI nuclear staining for 5 minutes in room temperatures, and washed for 5 minutes with PBS. Slides were mounted with VECTASHIELD Mounting Medium (Cat # H-1200, Vector Laboratories). Images were taken on either a Zeiss Axiovert 200M or Nikon A1 confocal microscope.

**Cell proliferation assay**

100 cells were seeded in a 96-well plate and cultured in complete media. Cell growth was measured using the SRB assay. Briefly, cells were fixed at certain time-points with 10% TCA for 1 hr at 4°C, washed with distilled water and air-dried at room temperature. All samples were stained with 0.057% SRB solution for 30 minutes and rinsed with 1% acetic acid. Protein-bound dye was solubilized in 10 mM Tris base and OD was measured at 530 nm.
**Transwell migration and invasion assays**

Control and *Perp* KD cells were trypsinized and 1x10⁵ cells were resuspended in serum-free media and placed in inserts (Costar) containing 8-µm pores with (invasion assay) or without (migration assay) Matrigel (1 mg/ml). These inserts were placed in wells with serum-containing media. 12h post-seeding, serum-containing media was aspirated and cells that had passed through the pores were trypsinized. Number of cells was counted using a hemacytometer.

**Co-culture binding assay**

Lungs were excised from wildtype female BALB/c mice and the single cell suspensions were prepared using the same method as in primary tumor cell culture. Cells were cultured in complete media until complete confluency. 10⁵ F_LUC-labeled control and *Perp* KD 4T1 cells were seeded on top and allowed to bind for 1 hr. Unbound cells were washed with warm PBS multiple times before luciferase assays were performed using previously described procedures.

**Tumor xenografts**

All procedures involving mice, such as housing and care, and all experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) of Princeton University. Cells were harvested from subconfluent cell culture plates, washed with PBS twice, and resuspended at the appropriate concentration in PBS for tail vein injection or in 50% PBS/50% Matrigel for mammary fat pad injection. For primary tumor formation, female BALB/c mice at 6 weeks old were anesthetized by intraperitoneal injection of
ketamine (100 mg/Kg) and xylazine (10 mg/Kg). A small incision was made to reveal the mammary gland and 50,000 cells resuspended in 10 µl PBS/Matrigel were injected directly into the mammary fat pad. The incision was subsequently closed with wound clips. The primary tumor growth was monitored weekly by measurement of the tumor size. For pulmonary metastasis, 100,000 cells in 0.1 ml PBS were injected on day 0 into the lateral tail vein of 4-week-old, female BALB/c mice using 27G needles as previously described.

**Bioluminescent imaging and analysis**

We injected anesthetized mice retro-orbitally with 75 mg kg⁻¹ D-luciferin and acquired bioluminescence images by the IVIS Imaging System. We performed analysis by measuring photon flux of a region of interest (ROI) drawn around a bioluminescence signal of a metastasis lesion to be measured. We normalized weekly measurements to the initial measurement at time-point day 0. For *ex vivo* imaging of the lungs, excised lungs were placed in 6-well plates in PBS. D-luciferin was added directly into the PBS prior to imaging with the IVIS Imaging System.

**Quantification of tumor cells in circulation**

Whole blood was collected from the retroorbital sinus weekly from mice orthotopically injected with tumor cells in the mammary gland. Genomic DNA was isolated from 50 µl of whole blood using the DNeasy Blood & Tissue kit (Cat # 69506, Qiagen) and genomic PCR was performed using primers designed to amplify a region of the puromycin resistance gene (part of the pLKO.1 backbone stably integrated in *Perp* KD lines).
Quantification of macrometastases

The number of overt macrometastases was counted manually on fixed lungs. Non parametric Mann-Whitney test was performed to assess statistical differences between groups.

Isolation of lung metastatic nodules

Lungs were excised and briefly rinsed in PBS. Approximately 10 individual lung metastatic nodules were excised with scalpels and pooled together. Tissue was homogenized directly in RNA extraction buffer using a Dounce tissue grinder (Cat # P0485, Sigma-Aldrich).

Microarrays for in vitro and in vivo Perp KD samples

Total RNA was extracted using the RNeasy Mini Kit (Cat # 74106, Qiagen) according to the manufacturer’s instructions. RNA quality was assessed using the Agilent Bioanalyzer 2100 using RNA Nano chips (Cat # 5067-1511, Agilent Technologies). Samples for the microarrays were prepared using the Agilent Quick Amp Labeling Kit (Cat # 5190-0424, Agilent Technologies) according to the manufacturer’s protocol. ~150ng of RNA from each population was labeled with Cy5 and hybridized to Agilent Whole Mouse Genome 4x44k oligo microarrays along with Cy3-labeled Mouse Universal Reference RNA (Cat # 740100, Stratagene). After hybridization, scanning was performed with an Agilent G2565BA scanner. Images were analyzed using Agilent Feature Extraction v9.5 software. The feature median signals normalized to the array median were used to calculate
Cy5/Cy3 ratios. Expression heatmaps were generated in GeneSpring GX (Agilent Technologies).

**Analysis of primary tumors**

Women with resected breast cancer were selected from patients followed from 1995 to 2010 in IRCCS IRST, Meldola, Italy. Tumor specimens were de-identified and were considered exempt samples in accordance with the institutional review board of the Loca Ethic Committee, Forli, Italy. Tumor specimens were fixed in formalin and frozen. Tissues collected were from 9 primary breast tumors from patients with no relapse and 10 primary breast tumors from patients with visceral metastases.

**Clinical dataset survival analysis**

Similar to the process described in Gyorffy et al (2010), we combined a series of breast cancer gene expression datasets with reported distant metastasis- or relapse-free survival values (http://www.kmplot.com/index.php?p=3).

**Statistical Analysis**

Results were reported as mean ± s.e.m. (standard error of the mean). Two-sided independent student’s t-test without equal variance assumption or the Mann-Whitney test was performed to analyze gene expression levels, differences in number of tumor colonies and nodules, time-points of *in vivo* luciferase assays and end-points of *in vitro* assays. The log-rank test was used to test for differences in survival. Statistical analyses were performed using R (www.r-project.org).
Acknowledgements

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Chapter 4: Investigation of a Vascular Component to the MaSC Microenvironment
Introduction

Although recent studies have characterized major signaling pathways governing MaSC activity (Makarem et al., 2013), our knowledge of how MaSCs are regulated via interactions with their putative stromal microenvironment is quite limited. The best understood microenvironment of an adult stem cell population is that of hematopoietic stem cells (HSCs). These multi-potential adult stem cells with the potential to regenerate the entire blood system are known to reside as a quiescent population on the osteoblastic surface of the bone (Morrison and Scadden, 2014). Various osteoblast-specific factors, such as osteopontin, signal to the HSCs to maintain their activity as stem cells. However, it has been recently shown that HSCs move to endothelial cells of the blood vessels to transition into a more active state (Sugiyama et al., 2006). Perivascular cells covering the blood vessels express a variety of signaling factors to provide a perivascular niche for HSCs. Several studies have shown that HSC activity can be modulated by perturbing these intercellular interactions between HSCs and the niche constituents (Ding et al., 2012; Kunisaki et al., 2013), introducing novel therapeutic targets for disrupting potential interactions between leukemic stem cells and their fostering microenvironment. In comparison to the HSC microenvironment, the MaSC microenvironment is still an emerging concept. Previous studies have shown that adipocytes (Couldrey et al., 2002) and various immune cells (Coussens and Pollard, 2011; Reed and Schwertzfeger, 2010), notably macrophages (Dai et al., 2002), are critical for mammary gland development, but the direct signaling of these populations to MaSCs have not been addressed. With increasing knowledge of MaSC regulators and of the expression profiles of various
mammary stromal cells, functional links need to be established and interconnected to understand an intricate network of MaSC activity regulation.

Endothelial cells assemble together to create blood and lymphatic vessels for nutrient/oxygen supply and drainage. Vasculogenesis occurs in the early developing embryo and describes the creation of the fundamental network of vessels (Olsson et al., 2006; Parker et al., 2004). Early endothelial precursors arise from the endoderm germ layer (Lammert and Axnick, 2012; Strilic et al., 2009) and whether a progenitor population determined for endothelial differentiation exists is still an ongoing debate (Pearson, 2010). Angiogenesis, on the other hand, is the sprouting of pre-existing blood vessels to create an intricate network of blood vessels (Augustin et al., 2009; Koh, 2013). Angiogenesis occurs in various stages of development (Herbert and Stainier, 2011; Jeltsch et al., 2013), but it has been the focus of study in cancer progression. It has been previously shown that the secretion of vascular endothelial growth factors and angiopoietins from tumor cells induces angiogenesis in tumors to increase nutrient supply to the tumor and also facilitate its metastasis to distant organs (Carmeliet et al., 2009; Nagy and Dvorak, 2012; Saharinen et al., 2011). Although the link between tumor progression and angiogenesis has been linked, whether blood endothelial cells can contribute to the initiation and/or relapse of tumor via a tumor-initiating population remains to be explored. Our goal for this study was to search for a putative vascular niche of normal MaSCs and, if found, characterize the signaling factors involved in MaSC activity regulation. With these findings, we hope to further our understanding of vascular biology in the context of tumor initiation and relapse, ultimately revealing potential therapeutic targets in the microenvironment of tumor-initiating cells.
Results

Development of novel imaging methods to characterize vasculature in the mammary gland

Due to the abundance of adipose tissue in the murine mammary gland, it has traditionally been difficult to perform wholemount IF analysis on mammary gland tissue. Adipocytes prevent sufficient antibody penetration and generate a high amount of background fluorescence. We therefore developed a novel tissue processing/staining method to overcome these obstacles and image the entire mammary gland. This was a critical step to visualize the 3D spatial orientation of the vascular network in the mammary gland and its relation to MaSCs. By employing cold acetone fixation and methyl benzoate dehydration, we were able to remove a significant amount of adipocytes from the mammary gland while preserving the vascular structure. Wholemount IF staining of mammary glands isolated from K14-H2B-GFP mice, which expresses GFP in the histones of cells expressing K14, with anti-CD31 (endothelial) antibodies showed an intricate network of CD31+ vessels surrounding K14+ mammary epithelium (Figure 11-1A). While all K14+ cells are not MaSCs, MaSCs are known to be predominantly K14+. Many of the K14-H2B-GFP+ were found to be directly in contact with CD31+ endothelial cells (Figure 11-1B), suggesting that K14+ MaSCs may directly interact with neighboring endothelial cells.

In order to characterize the vascular network during mammary gland reconstitution, we developed a novel intravital imaging method using two-photon microscopy. Two-photon microscopy enables the imaging of live cells and provides deep penetration of tissue (Li
et al., 2013; Weigert et al., 2013). With this technology, we sought to track injected MaSCs and endothelial cells in the cleared mammary fat pad throughout the regeneration process in real-time. This was achieved by imaging the site of injection of the same animal at multiple timepoints, re-exposing and re-mounting the same mammary gland on an imaging platform while the animal is anesthetized. The MaSC-enriched population was isolated from actin-GFP⁺ donors and the CD31⁺ endothelial cells from actin-DsRed⁺ donors. Both cell populations were mixed together before injection into the cleared mammary fat pad for mammary gland reconstitution (Figure 11-2). These GFP⁺ cells could be seen in the cleared fat pad right after injection. Surprisingly, imaging of the same animal 7 days post-injection showed spheroidal colonies (Figure 11-3A), which are reminiscent of in vitro 3D Matrigel colonies. With multiple imaging sessions up to 6 weeks post-injection, we could observe elongation of these colonies, which eventually became mammary ducts of the reconstituted mammary gland. We could also see an extensive vascular network created by the injected DsRed⁺ cells (Figure 11-3B). Interestingly, GFP⁺ colonies at 7 days post-injection were physically attached to the DsRed⁺ endothelial cells. Upon closer observation of the various regions of the reconstituting mammary gland, we could see a positive correlation between vascular density and the number of colonies. These observations suggest that MaSCs physically interact with blood vessels in vivo and may receive positive regulatory signals for MaSC activity during mammary gland reconstitution. We therefore proceeded to test this hypothesis with functional studies.
Figure 11-1. Visualization of intricate vascular network surrounding mammary epithelium. (A) Immunofluorescence images of endothelial cells and myoepithelial cells in the mammary gland. Mammary glands from K14-H2B-GFP mice were wholemount-stained with anti-CD31 (endothelial) antibody. (B) Magnified view of vascular network surrounding a mammary duct. Scale bar 70 µm.
Figure 11-2. Schematic diagram of novel intravital imaging method. FACS-sorted P4 and CD31$^+$ populations from actin-GFP$^+$ and -DsRed$^+$ mice were mixed 1:10 and injected into the cleared mammary fat pad for imaging. Multiple imaging sessions were performed on the same animal over 6 weeks.
Figure 11-3. MaSCs form spheroidal colonies and attach to CD31\(^+\) vessels during mammary gland reconstitution. (A) Intravital images of GFP\(^+\) P4 in a cleared mammary fat pad of the same animal at Day 0 and Day 7 post-injection. (B) Intravital images of GFP\(^+\) P4 and DsRed\(^+\) endothelial cells in a cleared mammary fat pad at Day 7 post-injection. Scale bars 20 \(\mu\)m.
Ubiquitous *COMP-ANG1* over-expression leads to an increase in lobular structures in the mammary gland

A family of growth factors known as angiopoietins can stimulate endothelial cells and induce angiogenesis (Koh, 2013). A modified version of angiopoietin-1, called COMP-ANG1, can form multimers to stimulate Tie-2 phosphorylation in endothelial cells (Cho et al., 2004; Cho et al., 2006). Compared to the native ANG-1, COMP-ANG1 is soluble, stable and is far more efficient in inducing angiogenesis. We used this recombinant protein to test whether the induction of angiogenesis would affect MaSC activity. Since a mammary gland-specific animal model was not available, we used an inducible *Rosa26-COMP-ANG1* over-expression mouse model to induce angiogenesis (Hato et al., 2009). Tamoxifen injection into these animals leads to the over-expression of the recombinant COMP-ANG1 protein in every cell of the body, since the *Rosa26* promoter is ubiquitously expressed. Induction of *COMP-ANG1* over-expression during the pubertal stages of mouse development led to a significant morphological change in the mammary gland. Compared to the control mice, which were also treated with tamoxifen but did not have recombinant protein transgene, *Rosa26-COMP-ANG1* mice had mammary glands full of lobular structures (Figure 12-1A and B), reminiscent of hyperplastic mammary glands in the *MMTV-Wnt* model. Flow cytometry analysis of these mammary glands showed a drastic change in the CD24 vs. CD29 profile of Lin− cells (Figure 12-1C). Although the P4 and P6 percentages remained similar to the control, *Rosa26-COMP-ANG1* mammary glands had significantly less of the luminal progenitor-enriched population. This was due to a dramatic shift of this population in the CD24 vs. CD29 profile rather than the isolated decrease of this specific population. Altogether, these
Figure 12-1. Ubiquitous COMP-ANG1 over-expression leads to a significant increase in lobular structures in the mammary gland. (A) Brightfield images of mammary glands from Rosa26-COMP-ANG1 transgenic and control mice. Mammary glands were whole-mounted and stained with alum carmine. Scale bar 1 mm. (B) Immunofluorescence images of myoepithelial and endothelial cells in mammary glands from Rosa26-COMP-ANG1 transgenic and control mice. Mammary glands were wholemount-stained with anti-SMA and -CD31 antibodies. Scale bar 100 μm. (C) Flow cytometry analyses of P4, P5 and P6 populations in mammary glands from Rosa26-COMP-ANG1 transgenic and control mice.
results suggest that COMP-ANG1 over-expression in the mammary gland drastically changes the morphology of mammary glands. We did not see a significant expansion of the P4 population, as observed in MMTV-Wnt hyperplastic mammary glands, but there was a significant shift of the P5 population toward the P4 population based on the CD24 vs. CD29 profile, accompanied by a dramatic increase in lobular structures. It is important to note that this over-expression system forces every cell in the mammary gland to overexpress COMP-ANG1. Therefore, all lineages of the mammary epithelium and other stromal cells can be affected. While this experimental system and these results do not address the impact of angiogenesis on the MaSCs directly, we cannot rule out that the resulting phenotype is MaSC-dependent. For example, the quality rather than the quantity of MaSC may have changed with COMP-ANG1 over-expression, thereby affecting the differentiation potential of MaSCs. With these results from a ubiquitous animal model, we decided to move onto a more isolated and controlled experimental system to test the effects of angiogenesis induction on MaSC activity.

**COMP-ANG1 treatment increases MaSC activity *in vivo***

Instead of overexpressing the potent angiogenic factor COMP-ANG1 ubiquitously in the system, we sought to treat the MaSCs directly with the recombinant protein. The MaSC-enriched population was isolated and mixed with COMP-ANG1 prior to injection into the cleared mammary fat pad. Whereas only 33.3% of injected fat pads had reconstituted epithelium in the control, 100% of injected fat pads had successful reconstitution with COMP-ANG1 treatment (Figure 13-1). It is important to note that these injections were performed on contra-lateral sides of the animal, meaning that the control and COMP-
ANG1 treatments were performed in the same recipient. Unlike previous reports in which COMP-ANG1 was distributed systemically into the circulation, localized angiogenic effects could be observed with the co-injection of the recombinant protein into the cleared mammary fat pad. This could be due to the embedding of COMP-ANG1 in the injection medium, Matrigel, which would prevent efficient dissemination in tissue. COMP-ANG1-treated reconstituted mammary glands had significantly more CD31$^+$ vessels than in the control glands (Figure 13-2A). Interestingly, upon closer observation of the successfully reconstituted mammary glands, more terminal end buds (TEBs) were observed with COMP-ANG1 treatment. Based on label-retention and functional studies in the mammary gland, it has been proposed that the relatively quiescent MaSCs reside in the TEB structure of the developing mammary duct (Bai and Rohrschneider, 2010; Kenney et al., 2001). The increased presence of TEBs in the COMP-ANG1-treated reconstituted mammary glands suggests that MaSC number and/or activity is increased.

It has been previously shown that a particular founder line of the firefly luciferase reporter mouse model has significantly enriched luciferase activity in MaSCs (Tiede et al., 2009). Luciferase expression can be used to isolate cells with greater reconstitution potential than the P4 population and therefore this animal model provides a way to track MaSC activity in vivo using non-invasive bioluminescence imaging. We used this firefly luciferase reporter mouse model as donors to isolate the MaSC-enriched population and treated these cells with COMP-ANG1 before cleared mammary fat pad injection. Consistent with the observation that TEB number is increased in COMP-ANG1-treated mammary glands, COMP-ANG1 treatment led to significantly higher bioluminescence in the reconstituting mammary glands (Figure 13-2B), suggesting that MaSC number is
increased with COMP-ANG1 treatment. These results suggest that MaSC activity is positively regulated by COMP-ANG1 stimulation and thereby enhances the MaSC potential to reconstitute the mammary gland. However, these findings cannot yet claim that MaSC potential is being affected by through a putative vascular niche, as the increase in vasculature and increased MaSC activity may simply be correlational or MaSC may be directly regulated by COMP-ANG1 signaling.

In order to address the second issue, we isolated the MaSC-enriched population and treated these cells with COMP-ANG1 before subjecting them to 3D Matrigel colony formation assays. COMP-ANG1 treatment did not affect MaSC activity in vitro, as observed by no differences in colony number or colony size (Figure 13-3). Since this isolated system only subjects the MaSC-enriched population to COMP-ANG1 stimulation, these results suggest that the phenotype observed is dependent on other cell types of the mammary gland stroma. Therefore, COMP-ANG1 treatment likely enhances MaSC reconstitution of the mammary gland by stimulating non-MaSC cells in the stroma.

**Characterization of perivascular cells in the mammary gland**

In addition to increasing endothelial proliferation and survival, ANG-1 has been shown to increase pericytic coverage of blood vessels (Gaengel et al., 2009). Various perivascular cells provide coverage and signaling functions to maintain endothelial integrity and homeostasis (Crisan et al., 2012; Takakura, 2011). Pericytes wrap around blood vessels and regulate capillary blood flow, the clearance of cellular debris and phagocytosis, and the permeability of blood vessels, particularly that of the blood-brain barrier.
**Figure 13-1.** COMP-ANG-1 treatment enhances mammary gland reconstitution.
Reconstitution rate of COMP-ANG-1-treated MaSCs compared to MaSCs treated with BSA (top). Successful reconstitution was scored based on the presence of epithelium, which was determined by alum carmine staining. Brightfield images of reconstituted mammary glands (bottom). Reconstituted mammary glands were whole-mounted and stained with alum carmine. Scale bar 5 mm.
Figure 13-2. COMP-ANG-1 treatment increases MaSC quantity in vivo. (A) Immunofluorescence images showing reconstituted mammary epithelium and CD31⁺ vessels from COMP-ANG-1-treated MaSCs. Cleared mammary fat pad injections were performed on contralateral sides of the recipient (top, BSA control; bottom, COMP-ANG-1). Scale bar 200 µm. (B) Bioluminescence quantification of reconstituting mammary glands from COMP-ANG-1-treated MaSCs relative to BSA control. Data is presented as mean ± s.e.m. ** P < 0.01, *** P < 0.001 (Student’s t-test).
Figure 13-3. COMP-ANG-1 treatment does not directly affect MaSC activity. (A) Quantification of *in vitro* 3D Matrigel colony formation assays of P4 cells treated with COMP-ANG-1 (CA-1), ANG-2 or vascular endothelial growth factor (VEGF)-A. (B) Measurements of *in vitro* 3D Matrigel colony formation assays. Data is presented as mean ± s.e.m.
It has been shown in numerous studies that perivascular cells, such as Nestin- and leptin receptor-positive cells, interact with HSCs via signaling molecules to establish and maintain a perivascular niche (Ding et al., 2012; Mendez-Ferrer et al., 2010). We used a well-established marker for pericytes, platelet-derived growth factor receptor β (PDGFR-β) (Lindahl et al., 1997; Olson and Soriano, 2011), to characterize the perivascular coverage of the vessels surrounding mammary epithelia. IF analysis of normal mammary glands showed an extensive coverage of vasculature by the PDGFRβ+ cells (Figure 14-1). Interestingly, there was a high concentration of these cells around TEB structures, irrespective of the presence of vasculature.

To further characterize the PDGFRβ+ population in the mammary gland, we isolated these cells and performed qRT-PCR for factors that have already been implicated in the HSC perivascular niche (Morrison and Scadden, 2014). Compared to PDGFRβ− cells, PDGFRβ+ cells had significantly higher expression of various signaling molecules, such as delta-like ligand 1 (Dll1), insulin growth factor binding protein 2 (Igfbp2), kit ligand (Kitl), intercellular adhesion molecule 1 (Icam1), pleiotrophin (Ptn) and vascular cell adhesion molecule 1 (Vcam1) (Figure 14-2A). Tie-2 receptor expression was also higher, suggesting that these cells have the potential to respond to ANG-1 stimulation. Several of these factors, such as Igfbp2, Kitl and Ptn, are secreted molecules. To test whether these secreted factors, among others, could affect MaSC activity binding to their corresponding receptors, we treated isolated MaSCs with conditioned media from cultured PDGFRβ+ cells. Surprisingly, treated MaSCs led to more 3D Matrigel colony formation compared to MaSCs that were treated with conditioned media from PDGFRβ− cells (Figure 14-2B),
Figure 14-1. Characterization of perivascular coverage of vasculature surrounding mammary duct terminal end buds (TEBs). Immunofluorescence images of a mammary duct TEB, surrounding CD31⁺ vessels and PDGFRβ⁺ cells. Mammary glands from 6-week-old female mice were wholemount-stained for anti-SMA, -CD31 and -PDGFRβ antibodies. Scale bar 50 µm.
Figure 14-2. PDGFRβ⁺ cells express secreted factors that may increase MaSC activity. (A) Expression levels of candidate factors in PDGFRβ⁺ relative to PDGFRβ⁻ cells from the mammary gland assessed by qRT-PCR. (B) Brightfield images of 3D Matrigel colony formation assays with conditioned media treatment from PDGFRβ⁺ or PDGFRβ⁻ cells. FACS-sorted P4 cells were plated in 3D Matrigel and concentrated conditioned media was added to the media. Scale bar 5 mm.
suggesting that PDGFRβ<sup>+</sup> cells secreted factors that positively regulate MaSC activity in vitro.

**Vascular characterization of mammary glands during pregnancy and tumorigenesis**

In order to understand vascular biology in the various contexts in which MaSC activity is changing under physiological conditions, we sought to characterize the vascular network in the mammary gland during pregnancy and tumorigenesis. The MaSC population undergoes massive expansion during pregnancy to provide an ample source of progenitors/progeny that facilitate the transformation of the mammary gland (Visvader and Smith, 2011). As mentioned previously, this expansion has also been reported in the hyperplastic stages of the MMTV-Wnt model (Shackleton et al., 2006), in which the over-expression of Wnt is stimulating the MaSCs directly to promote their self-renewal. In both of these physiological conditions, it has already been reported that the vascular network undergoes significant remodeling to facilitate the changes in nutrients, oxygen supply and recruitment of various cell types (Djonov et al., 2001). Recent findings in the HSC field have shown that the vascular niche can be further subdivided into arteriolar and sinusoidal microenvironments, where the former induces quiescence in HSCs (Kunisaki et al., 2013). With this outlook, we wanted to know whether changes in specific endothelial subpopulations, rather than the overall endothelial population, accompany the drastic changes in MaSC activity and whether these specific subpopulations provide a functional microenvironment. Mammary glands from pregnant (~9-week-old; 1 day after parturition) mice and MMTV-Wnt transgenic mice at either the hyperplastic stage (~3-month-old) or tumor stage (~5-month-old) were subjected to flow
cytometry analyses for CD31+ cells, which we further profiled based on stem cell antigen 1 (Sca-1) and Tie-2 expression. These markers were chosen since they are highly expressed in arteriolar vessels in the bone marrow (Kunisaki et al., 2013). As expected, there was a significant increase in CD31+ cells in the pregnant mammary glands and MMTV-Wnt tumors compared to the virgin and normal controls, respectively (Figure 15-1 and 15-2), reflecting the increase in vasculature under these conditions. Interestingly, pregnant mammary glands had a significant increase in the Sca1+ Tie2- population, whereas both hyperplastic glands and tumors had a significant decrease in this population. While these observations are merely correlational at this point, the functional basis of the specific subpopulation requires further investigation.
Figure 15-1. Subpopulation characterization of CD31\(^+\) cells in the mammary gland during pregnancy. Flow cytometry analyses of the CD31\(^+\) population in mammary glands from pregnant mice (1 day after parturition), relative to virgin mice controls.
Figure 15-2. Subpopulation characterization of CD31+ cells in the mammary gland during tumorigenesis. Flow cytometry analyses of the CD31+ population in hyperplastic (~3 month old) mammary glands and in tumor (~5-month-old), relative to normal age-matched controls.
Discussion

Modulating MaSC reconstitution potential through a putative vascular microenvironment

We show here that MaSCs physically associate with blood vessels in vivo and that the increase in vascular density leads to an increased potential of MaSCs to reconstitute the mammary gland. While both of these observations were made in an experimental reconstitution setting, in which inflammation and wound healing-dependent angiogenesis occur, this method allowed us to test MaSC activity while modulating the surrounding in vivo microenvironment. It is important to note that increasing angiogenesis in the reconstituting mammary gland also means that there is a corresponding increase in nutrient supply. Until we identify the MaSC activity-promoting factors provided by the putative vascular niche and perform functional assays, we cannot exclude the possibility that the enhanced MaSC activity is simply due to an increase in nutrient supply. In addition, other stromal cells, particularly various immune cells, express the Tie-2 receptor and can therefore be stimulated by COMP-ANG1. One should also consider that the changes in vasculature could, in turn, induce other stromal cells to undergo transformation. Identification and characterization of these additional stromal components is critical to furthering our understanding of the vascular component to the putative MaSC niche.

A putative perivascular microenvironment for MaSCs

Endothelial cells do not act alone to form functional vessels but rely on various perivascular cells for support. We decided to focus on pericytes, as these cells have been
shown to respond to ANG-1 as well as secrete a battery of signaling molecules (Gaengel et al., 2009). While pericytic coverage was abundant on mammary gland vasculature, we also noticed PDGFRβ+ cells surrounding mammary duct TEBs, a well-known physical location of MaSCs (Bai and Rohrschneider, 2010; Kenney et al., 2001). Since these cells are not attached to CD31+ endothelial cells, whether they are indeed pericytes or a distinct population remains to be determined. More importantly, what factors are secreted by this population to increase MaSC activity are currently being investigated. Finally, if perivascular coverage and the characteristics of perivascular cells change during pregnancy and/or tumorigenesis, we need to understand how such modifications in the perivascular niche contribute to aberrant behavior in normal MaSCs.

In addition, we can segregate the CD31+ population in the mammary gland into two distinct subpopulations based on Sca-1 expression. Although the Sca-1+ population has been characterized in the HSC vascular niche (Kunisaki et al., 2013), whether this identity is conserved in the mammary gland remains unknown. Given the correlational observations of this population with pregnancy and tumorigenesis, with the subpopulation increasing with pregnancy but decreasing in hyperplasia and tumor, these cells may play differing roles in pregnancy vs. tumorigenesis or their function may change in these different physiological conditions. Further characterization and functional studies using cell depletion models may provide sufficient evidence to target specific constituents or subpopulations of the mammary tumor perivascular niche.
Materials and Methods

Mammary epithelial cell preparation and FACS

Single cell suspensions were prepared from the mammary glands of actin-GFP, -DsRed, virgin (~9-month-old), pregnant (~9-month-old; 1 day after parturition), normal (age-matched), MMTV-Wnt transgenic (~3 or 5-month-old), and luciferase transgenic animals after mechanical and enzymatic dissociation based on published protocols. Briefly, mammary glands were excised, minced using scalpels, and digested for 1hr in 300U/ml type 1A collagenase (Sigma) and 100U/ml hyaluronidase (Sigma). Cells were then treated with 0.25% trypsin/EDTA, dispase (Invitrogen)/DNase (Sigma), and ammonium chloride in succession. Between each treatment, cells were rinsed in MEGM (1:1 DMEM:F12 Ham supplemented with 5µg/ml insulin, 500ng/ml hydrocortisone, 10ng/ml EGF, 20ng/ml cholera toxin, 5% bovine calf serum, and 1× penicillin/streptomycin). Afterwards, cells were resuspended in FACS buffer (PBS supplemented with 5% newborn calf serum plus 1× penicillin/streptomycin) and filtered twice through 40µm nylon cell strainers. The following antibodies were used to label cells: biotin conjugated anti-TER119 (red blood cells, Cat # 553672, BD), biotin conjugated anti-CD31 (endothelial cells, Cat # 558737, BD), biotin conjugated anti-CD45 (hematopoietic cells, Cat # 553078, BD), CD29-Alexa Fluor 647 (Cat # MCA2298A647, Serotec), CD24-PE (Cat # 553262, BD), Ly-6A/E-PE-Cy7 (Cat # 558162, BD), Tie-2-APC (Cat # 124010, BD), CD140b-APC (Cat # 17-1402-80, BD). PE-Cy7 (Cat # SA1012, Life Technologies) or PerCP-Cy5.5 (Cat # 551419, BD) conjugated Streptavidin was used for secondary staining of lineage markers or anti-CD31, respectively. For all staining, 50µl of antibody
diluted 1:75 in FACS buffer was used per $1 \times 10^6$ cells, including single color controls and combination staining. Control samples for four-color FACS included no-staining, GFP or DsRed only, propidium iodide (PI, cell viability dye) or DAPI only, CD24-PE only, CD29-APC only, Biotin-lineage-PE-Cy7 only, Biotin-CD31-PerCP-Cy5.5 only, as well as corresponding PE, APC, PE-Cy7, and PerCP-Cy5.5 single color fluorochrome conjugated antibody isotype controls. Both primary and secondary staining was conducted for 30 minutes at room temperature in FACS buffer. Between staining, cells were washed with 5ml FACS buffer. Cell sorting for transplantation experiments was done using a FACSVantage SE w/DiVa (BD Biosciences). All cells were sorted into 1:1 Fetal Bovine Serum:MEGM.

**RNA extraction and quantitative real-time PCR**

Total RNA was extracted using the RNeasy Mini Kit (Cat # 74106, Qiagen) according to the manufacturer’s instructions. For mRNA analysis, cDNA was oligo(dT) primed from 1-2 µg of total RNA using a SuperScriptIII First-Strand Synthesis System (Cat # 18080-051, Invitrogen). cDNA from each sample was diluted 4-5 fold and real-time was performed in triplicates using Power SYBR green PCR master mix (Cat # 4367659, Applied Biosystems) on an ABI 7900HT series PCR machine. Expression levels were normalized to *GAPDH* expression. All analysis was performed using the SDS2.3 software.

**Whole-mount immunofluorescent staining**

Fourth and fifth mammary glands were excised and mounted directly on glass microscope slides before being fixed in ice cold acetone overnight at -20°C. Mounted
mammary glands were dehydrated in methyl benzoate for 2 hrs and cleared in xylene for 2 hrs at room temperature. Tissues were rehydrated in PBS and blocked with 1% BSA, 5% horse serum, and 0.8% Triton X-100 in PBS for 1 hr at room temperature. Whole-mounds were incubated with the following primary antibodies in blocking buffer overnight at 4°C: hamster anti-CD31 (Cat # MAB1398Z, Millipore), mouse anti-SMA (Cat # A 5228, Sigma-Aldrich), rabbit anti-K14 (Cat # ab53115, Abcam), and rat anti-CD140b (gift from Gou Young Koh). Whole-mounds were washed 5 x 5 minutes with PBS and stained with the following secondary antibodies in blocking solution overnight at 4°C: Alexa Fluor 594 goat anti-hamster (Cat # A21113, Life Technologies), Alexa Fluor 488 goat anti-mouse (Cat # A11029, Life Technologies), Alexa Fluor 488 donkey anti-rabbit (Cat # A21206, Life Technologies), and Alexa Fluor 647 goat anti-rat (Cat # A21247, Life Technologies). Whole-mounds were washed 5 x 5 minutes with PBS, followed by DAPI nuclear staining for 5 minutes in room temperature, and washed for 5 minutes with PBS. Slides were mounted with VECTASHIELD Mounting Medium (Cat # H-1200, Vector Laboratories). Images were taken on a Nikon A1 confocal microscope.

**Conditioned media concentration**

FACS-sorted cells were cultured in complete media in 10-cm plates until complete confluency was reached. Cells were cultured in serum-free media for 24 hrs before cultured media was collected and concentrated in Ultracel-3K centrifugal filters (Cat # UFC900324, Amicon). Protein concentrations of conditioned media were measured using a DC Protein Assay kit (Cat # 500-0111, Bio-Rad) according to the manufacturer’s instructions.
3D Matrigel colony formation assay

A thin layer of growth factor-reduced Matrigel was plated in a 24-well tissue culture plate and solidified, 10,000-20,000 FACS-sorted cells were resuspended in 50% Matrigel/50% MEGM and seeded on top. Cells were grown in MEGM, supplemented with concentrated conditioned media, for 10-14 days before colonies were scored. Images of colonies were taken on a Zeiss Axiovert 200M.

Intravital imaging of developing mammary glands

All procedures involving mice, such as housing and care, and all experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) of Princeton University. After FACS, cells were centrifuged for 5 minutes at 1000g and then resuspended in 50% Matrigel/50% PBS. P4 cells and CD31\(^+\) cells were mixed 1:10. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/Kg) and xylazine (10mg/Kg). Cells were injected into cleared inguinal (#4) mammary fat pads according to standard injection procedures using Hamilton syringes. Briefly, a small incision was made to reveal the mammary gland, endogenous epithelium was surgically removed, and cells were injected directly into the cleared fat pad. Immediately after injection, animals were mounted on a heated imaging platform of a two-photon microscope. Hydration of live tissue was maintained with sterile PBS throughout the imaging session. A sterile coverslip was placed on top of the injection site to create a separate water immersion field for high magnification objectives (40x and 60x). The incision was subsequently closed with sterile non-absorbable silk sutures (Cat # 100-6830, Henry Schein). Subsequent imaging sessions of the same animals were performed by re-opening the
Two-photon imaging was performed on a custom, home-built two-photon microscope, which was built around a commercial upright microscope frame (Olympus BX51WI) and used two GaAsP PMTs (Hamamatsu H10770PA-40 SEL) for detection. Excitation was provided by a Coherent Chameleon Ultra II femtosecond Ti:Sapphire laser. The PMT signal was amplified by two high-speed, low noise current pre-amplifiers (Stanford Research Systems). Image acquisition and microscope control was performed by open-source ScanImage software (HHMI Janelia Farms) and National Instruments DAQ hardware. Images were taken using ScanImage software (HHMI Janelia Farms).

**Mammary fat pad transplantation**

After FACS, cells were centrifuged for 5 minutes at 1000g and then resuspended in 50% Matrigel/50% PBS with 100 µg/ml COMP-ANG1 recombinant protein or BSA. Cells were injected into cleared mammary fat pads as described above.

**Induction of Rosa26-COMP-ANG1 over-expression**

To overexpress Ang1 globally in a tamoxifen-dependent manner, COMP-Ang1-Tg mice were intercrossed with Rosa26-CreERT2 mice. Tamoxifen (Sigma-Aldrich) was dissolved in corn oil (Sigma-Aldrich), and the resulting tamoxifen solution was injected into the peritoneal cavity of 5-week-old female mice in two dosages for a total of 2 mg.

**Alum carmine staining of mammary glands**

Excised mammary glands were fixed in 10% neutral-buffered formalin overnight at room temperature, washed for 15 minutes in 70%, 35%, 15% ethanol and distilled water, and
then stained in alum carmine overnight at room temperature. Mammary glands were
destained for 15 minutes in 70%, 95% and 100% ethanol, and transferred for long term
storage into Histo-Clear II clearing agent (Cat # HS-200, National Diagnostics). Slides
were mounted with Permount Mounting Medium (Cat # SP15-500, Fisher Scientific).
Images were taken on a Zeiss Discovery V8 microscope.

Bioluminescent imaging and analysis

We injected anesthetized mice retro-orbitally with 75 mg kg\(^{-1}\) D-luciferin and acquired
bioluminescence images by the IVIS Imaging System. We performed analysis by
measuring photon flux of a region of interest (ROI) drawn around a bioluminescence
signal of mammary fat pad outgrowth to be measured. We normalized weekly
measurements to the initial measurement at time-point day 0.

Statistical Analysis

Results were reported as mean ± s.e.m. (standard error of the mean). Two-sided
independent student’s t-test without equal variance assumption was performed to analyze
time-points of \textit{in vivo} luciferase assays and end-points of \textit{in vitro} assays. Statistical
analyses were performed using R (www.r-project.org).
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Chapter 5. Summary
The work presented in this dissertation investigates specific cell-cell interactions that could potentially mediate mammary gland development and breast cancer metastasis. By applying multidisciplinary approaches and developing novel techniques, we were able to shed light on the following areas of mammary gland biology: 1) Although Perp expression is highly enriched in MaSCs and TICs, it does not play a critical role in desmosome-mediated epithelial integrity in normal mammary gland development, 2) Perp expression is highly enriched in epithelial-like breast cancer cells and is dynamically regulated during EMT and MET, 3) Perp facilitates breast cancer metastatic colonization of the lungs, possibly by mediating cancer cell-lung stroma adhesion, 4) endothelial cells and neighboring perivascular cells, which are constituents of the mammary gland stroma, have the potential to modulate MaSC activity through a putative (peri)vascular stem cell microenvironment.

**Epithelial integrity in mammary gland development and tumorigenesis**

Mammary gland stem cells self-renew and differentiate in specified lineages during development to generate an extensive ductal network of functional mammary epithelium. The mammary epithelial integrity, provided by numerous intercellular adhesion molecules, is critical for the specialized functions of the mammary gland, such as milk production and secretion. This integrity is also essential for malignant cells to create and maintain a tumor, while these characteristics are shed during local invasion and distant metastasis. Epithelial integrity during cancer metastasis, however, is not uni-directional in this manner, as metastatic cells, which have undergone a morphological transformation, revert and regain their epithelial-like traits to create a secondary tumor at the site of
metastasis. Therefore, furthering our understanding of mammary epithelial integrity and its dynamic regulation during mammary gland development and breast cancer metastasis may provide novel therapeutic targets to disrupt the integrity of malignancy.

Since Perp is a critical mediator of desmosome assembly in skin epithelial cells and is essential for skin integrity, we studied this cell-surface molecule in the context of mammary gland stem cells. We show here that the high expression of Perp in MaSCs does not play a functional role that is critical for mammary gland development. Perp suppression does not compromise the potential of MaSCs to reconstitute the mammary gland and mammary epithelial integrity is intact. While we do not understand why Perp is not critical, unlike in skin, for mammary epithelial integrity, we do not propose desmosome-mediated cell-cell interactions are dispensable in mammary epithelium. Desmosomes are quite abundant in the mammary epithelial cells and the expression of several desmosomal components, such as Dsp and Pkp3, correlate with Perp expression and are indeed enriched in MaSCs (data not shown). The possibility that another molecule, which is specific to mammary epithelial cells, may play a more critical role in proper desmosome assembly requires investigation. The high expression of Perp was once again observed in tumor cells with high potential of tumor initiation. Although the functional basis of this observation remains untested, we hope that further knowledge of Perp in the context of mammary tumorigenesis and/or relapse will be useful.

Our study introduces two concepts that have the potential to transform our perception of epithelial integrity in mammary gland development: 1) the mechanisms by which epithelial integrity is maintained and established may be different in the mammary gland
compared to the skin and 2) molecules expressed on the cell surface of normal MaSCs may be used as biomarkers for TICs. The therapeutic benefits that could be derived from these concepts are complementary. For example, enhancing our ability to isolate TICs with specific biomarkers, which may or may not have a functional basis, will lead to a better understanding of the molecular profile of this tumorigenic population and thereby identify TIC-specific molecular targets. In being able to distinguish some of these targets with high mammary gland specificity, one could receive highly specified treatments in which only an organ- and tumor-specific cells are targeted. Furthermore, if these targets are intercellular mediators between a cancer cell and stromal cell, treatment may provide prevention of relapse by inhibiting any favorable interactions between these cell types.

**Targeting epithelial integrity in breast cancer metastasis**

Disseminating cancer cells undergo a disintegration of epithelial integrity to leave their site of origin and re-establish integrity at the site of secondary tumor formation. An effective treatment of metastatic disease is especially difficult, as the systemic dissemination of disease through the circulation makes it virtually impossible to completely remove all cancer cells from the patient’s body. Primary, or even secondary, tumors can be surgically removed to reduce the tumor burden, but disseminating cancer cells can escape this method of treatment and eventually colonize the same and/or distant organ via relapse and/or metastasis. Therefore, targeting metastatic cancer cells during the stages of integrity re-establishment via MET may lead to the most effective treatment of metastatic disease.
We investigated Perp in the context of breast cancer metastasis for the following reasons: 1) desmosomes must disassemble during EMT-mediated invasion of metastatic cancer cells, 2) the involvement of Perp in desmosome assembly in skin epithelial cells, 3) patients with high PERP expression have poor prognosis in distant metastasis-free survival. We show here that Perp expression in epithelial-like breast cancer cell lines positively correlates with their metastatic potential and epithelial traits. More importantly, the suppression of Perp, which is dynamically regulated in EMT and MET, significantly compromises metastatic colonization of the lungs. As in the case of normal mammary epithelial cells, Perp does not seem to play a critical role in proper desmosome assembly in breast cancer cells, confirming the functional distinction between the mammary gland and skin. Our findings are particularly interesting in the context of EMT/MET, since this is the first report, to our knowledge, that Perp is transcriptionally regulated, reflecting desmosome disassembly/reassembly, during these programs. However, suppression of Perp does not lead to the disassembly of desmosomes. Therefore, Perp most likely has an independent function as a cell-surface molecule in breast cancer metastasis.

Our study of Perp in breast cancer metastasis proposes a novel therapeutic target to inhibit metastatic colonization and/or relapse of breast cancer. We do not yet understand the molecular mechanism mediating Perp’s functional role in breast cancer metastasis. Our preliminary evidence shows that Perp alone may act as an adhesion molecule to facilitate the interaction between Perp-expressing cancer cell and a lung stromal cell, but which specific cell type of the lung and the identity of the binding partner remain elusive. Targeting Perp directly as a cell-surface molecule may not be ideal, as it plays a critical function in maintaining skin epithelial integrity. Therefore, we are currently searching for
interactive partners of Perp in the lung stroma to identify target organ-specific, as well as metastasis-specific, molecules that could be targeted without compromising normal physiological functions.

**Targeting the (peri)vascular component of the stromal microenvironment**

In this way, our study of a putative (peri)vascular niche for MaSCs is a more directed attempt to distinguish the normal vs. tumor microenvironment, specifically in the tumorigenic/relapse context. Angiogenesis has always been a highlighted cancer biology subfield, as tumor progression relies heavily on the abundance of nutrients and growth factors. The concept of vasculature influencing tumor initiation and relapse, however, is just emerging. Also, anti-angiogenic therapy has proven to be largely effective in reducing primary tumor growth and local invasion, but the effects of such therapies have also been shown to increase metastasis and relapse. Furthering our understanding of vascular biology in the context of MaSCs and TICs may provide insight into how we could target tumor angiogenesis to encompass all aspects of breast cancer.

In our study, we have developed novel imaging methods to investigate/characterize an intricate vascular network in the mammary gland. Our methods provide two elements that are critical to development: spatial and temporal. To our knowledge, this is the first study to report *in vivo* imaging of MaSCs during mammary gland reconstitution. With this new method, we are now able to study the dynamics of MaSC activity in their native stromal microenvironment. In particular, cell-cell interactions can be observed throughout the entire developmental process and experimental inhibition of these interactions can be visualized in a dynamic manner. By employing these new methods and modulating the
host vasculature with a potent variant of ANG-1, we show here that MaSC potential to reconstitute the mammary gland is enhanced via the induction of angiogenesis. We also identify/characterize various subpopulations of perivascular and endothelial cells that may positively influence MaSCs and TICs. Although further investigation to understand the molecular mechanisms mediating the intercellular communication between our target populations and their surrounding stroma, we propose the existence of a (peri)vascular component to the MaSC microenvironment.

Our findings have the potential to introduce a novel subfield in mammary stem cell biology. Cancer relapse is thought to occur through a quiescent/dormant subpopulation of cancer cells, which receive specific signals from ideal microenvironments to reinitiate their active state. We hope that this study will contribute to our understanding of such tumorigenic microenvironments and unveil novel therapeutic targets within the vascular component of a TIC niche.
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