

The Role of β 1-integrin in Mammary Stem and Progenitor Fate

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Safiah Olabi

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Abbreviations

4OHT	4hydroxytamoxifen
AKT	Serine-threonine protein kinase
APC	Allophycocyanin
Axin2	AXIS inhibition protein 2
BSA	Bovine serum albumin
cDNA	complementary DNA
CK	Cytokeratin
cMyc	Myelocytomatosis viral oncogene type C
CT	Cycle threshold
DAPI	4'-6-Diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EDU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
Elf5	E74-like factor 5
EPCAM	Epithelial cell adhesion molecule
ER	Oestrogen receptor
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
GBD	GTPase binding domain
GEF	Guanine Nucleotide Exchange Factor
GFP	Green fluorescent protein
GSK3bI	Glycogen synthase kinase 3 beta inhibitor
GSK3 β	Glycogen synthase kinase 3 beta
Hes1	hairy and enhancer of split-1
Hey	Hairy/enhancer-of-split related with YRPW motif protein
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ILK	Integrin-linked kinase
IPP	ILK, PINCH and Parvin
Itg	Integrin
JNK	Jun NH2-terminal kinase
kDa	Kilo Daltons
LB medium	Luria-Bertani medium
Lef1	Lymphoid Enhancer Binding Factor 1
MAPK	Mitogen-activated protein kinases
MaSC	Mammary epithelial stem cells
MECs	Mammary epithelial cells
MEF2	Myocyte enhancer factor-2

MRU	Mammary repopulating unit
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI3K	Phosphoinositide 3-Kinase
PolyHEMA	poly-2-hydroxyethyl methacrylate
PR	progesterone receptor
Prl	prolactin receptor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
Rho1	Ras-like GTP-binding protein Rho1
ROCK	Rho-associated protein kinase
shRNA	Short hairpin Ribose Nucleic Acid
SOC media	Super Optimal broth with Catabolite repression media
SOX9	SRY (sex determining region Y)-box 9
STDev	standard deviation
TCF	T cell factor
VSV	Vesicular stomatitis virus
Wnt	Wingless-type MMTV
WT	Wild type

Abstract

The mammary gland contains a subset of cells with regenerative capacity that is able to generate both luminal and myoepithelial mammary epithelial lineages. Those cells are described as mammary epithelial stem cells. The fate of stem cells is tightly controlled by their microenvironment and adhesion receptors on the stem cells play a vital role in the microenvironment–stem cell communication. They facilitate the interaction of stem cells with the extracellular matrix as well as adjacent cells, and they regulate stem cell homing to their niches, as well as stem cell proliferation, self-renewal, and differentiation. Stem cells express high levels of ECM binding adhesion receptors such as $\beta 1$ and $\alpha 6$ -integrins. Those integrins were used to isolate stem cells from the rest of the differentiated epithelial cells within the mammary gland. However, little is known about the role of those integrins in stem cell self-renewal and differentiation. This project aimed to understand how $\beta 1$ -integrin receptors contribute to stem cell behavior. To achieve this, FACS sorting method of stem cells, the organoid assay, and lentivirus knockdown of $\beta 1$ -integrin using shRNA were optimised. The organoid assay was used as an in-vitro test to assess for the frequency of bi-lineage and luminal progenitor cells in a given mammary epithelial population. It is known that bi-lineage cells produce solid organoids in culture while luminal progenitors produce hollow organoids. The frequency of solid and hollow organoids might therefore be an indication of the stem cells and luminal progenitor frequency respectively. My results showed that cells with the highest solid organoid forming ability were within the basal population, which is high for $\beta 1$ - and $\alpha 6$ -integrin. The $\beta 1$ -integrin signaling pathway was shown to be important for maintaining the organoid-forming population in basal and luminal populations. Knocking out $\beta 1$ -integrin in MECs resulted in abolishing their solid and hollow organoid-forming activity. Downstream of $\beta 1$ -integrin, I found that Rac1 but not ILK is important in $\beta 1$ -integrin maintenance of solid organoid-forming cells. Active Rac1 was able to rescue solid organoid formation but was not able to rescue hollow organoids in the $\beta 1$ -integrin knockdown cells. $\beta 1$ -integrin and Rac1 deletion resulted in the down regulation of Wnt/ β -catenin signaling, which is important for stem cells. This down regulation was rescued using active Rac1. Activating Wnt/ $\beta 1$ -catenin signaling in primary cells (using Wnt3a ligand or GSK3 β inhibitor) resulted in an increase in solid organoid and a decrease in hollow organoid formation. When activating Wnt signaling using GSK3I in $\beta 1$ -integrin knockdown cells, the solid organoid activity was rescued. However, Wnt3a did not rescue solid organoid formation in the $\beta 1$ -integrin knockdown cells. When active Rac1 was overexpressed in $\beta 1$ -integrin null cells, Wnt3a was able to activate solid organoid formation. When inhibiting Rac1 in primary MECs, solid but not hollow organoid activity was significantly decreased. Wnt3a or GSK3I addition did not rescue this reduction. Taken these results together, it can be concluded that $\beta 1$ integrin-Rac1 signaling play a role in controlling stem cells and this is might be achieved through controlling Wnt/ β -catenin signaling. These studies are important in understanding the role of integrins in mammary stem cells. They will also provide new insight on how integrins might be controlling breast cancer and thereby, help in providing new targets for cancer therapy.

The University of Manchester

Safiah Olabi

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The Role of $\beta 1$ -integrin in Mammary Stem and Progenitor Fate

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Declaration

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Chapter1: General Introduction

1.1 The mammary gland structure and development

The mammary gland is an excellent tissue for understanding signaling and communication between adult stem cells and the microenvironment, because it has the unique property of completing its development after birth, specifically during puberty and pregnancy.

The mammary gland is a secretory organ made of ductal epithelial tree-like structures. Ducts are composed of hollow lumens surrounded by two layers of epithelial cells. The inner layer is made of luminal cells facing the central apical cavity and the outer layer is made of myoepithelial cells that surround the luminal cells and contact the basement membrane. The basement membrane is composed of extracellular matrix proteins (ECM) secreted by the myoepithelial cells. The BM provides structural support for the mammary gland and separates the epithelial cells from the nearby stromal cells Figure1.1. It also plays an important role in providing instructive cues that direct mammary epithelial cell behavior in response to different developmental needs (Muschler and Streuli, 2010) (Figure 1.1).

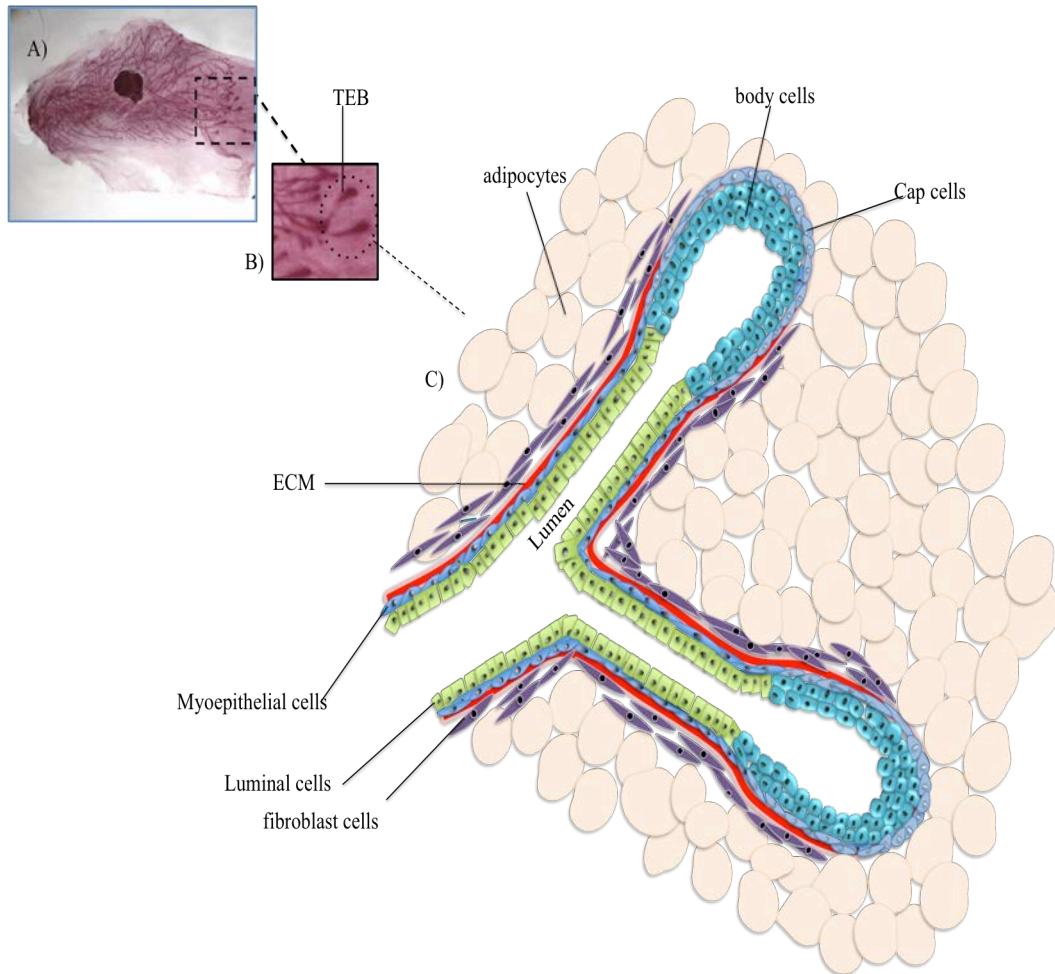


Figure 1.1 Cellular components of the mammary gland

A) Mammary fat pad of an adult virgin mouse showing the mammary gland tree structure. B) Enlarged image showing the terminal end bud at the end of the mammary duct. C) Different types of the mammary epithelial cells, their distribution in the mammary duct, and their position with respect to the basement membrane and inner lumen. ECM: extra cellular matrix; TEB: terminal end bud.

The embryonic mammary gland is initially visible as placode-like structures. It is specified along the ventral epidermis during embryonic development and progressively invades the underlying mesenchyme, called the mammary fat pad. In mouse, mammary development occurs from late in embryonic day 10 (E10) to birth at around E19 (Robinson, 2007). Around (E10) of mouse development the first mammary structure is produced and is marked by a lateral ectodermal thickening that protrudes slightly from the body wall. This structure is known as the milk line. On E11.5 the milk line disappears after breaking up into individual lens-shaped placodes and the underlying mammary mesenchyme starts to condense. In humans one pair of placodes is formed and in mouse five are formed. These will become the future sites of the mammary gland. During E11 and E12, the placodes sink deeper into the dermis to form small bulb-shaped buds and the mammary mesenchyme becomes organised in concentric layers around those buds. Between E13 and E15, no changes in shape occur and the cells in the bud only proliferate slightly.

On E15.5, the mammary epithelium starts to proliferate at the tip and the primary sprout pushes through the mammary mesenchyme towards the fat pad. At E16 the epithelial cells continue to proliferate in female mice and by E18.5 the elongating duct has invaded the fat pad and has formed a small primary ductal system that contains about 10–15 initial branches (Cowin and Wysolmerski, 2010; Robinson, 2007) Figure (1.2). Many important signaling pathways control this process such as the Wnt, Hedgehog, and FGF pathways (Boras-Granic et al., 2006; Chu et al., 2004; Hatsell and Cowin, 2006; Mailleux et al., 2002). The epithelial duct system then grows slowly after birth and remain dormant until puberty, when a second round of rapid expansion takes place (Hinck and Silberstein, 2005).

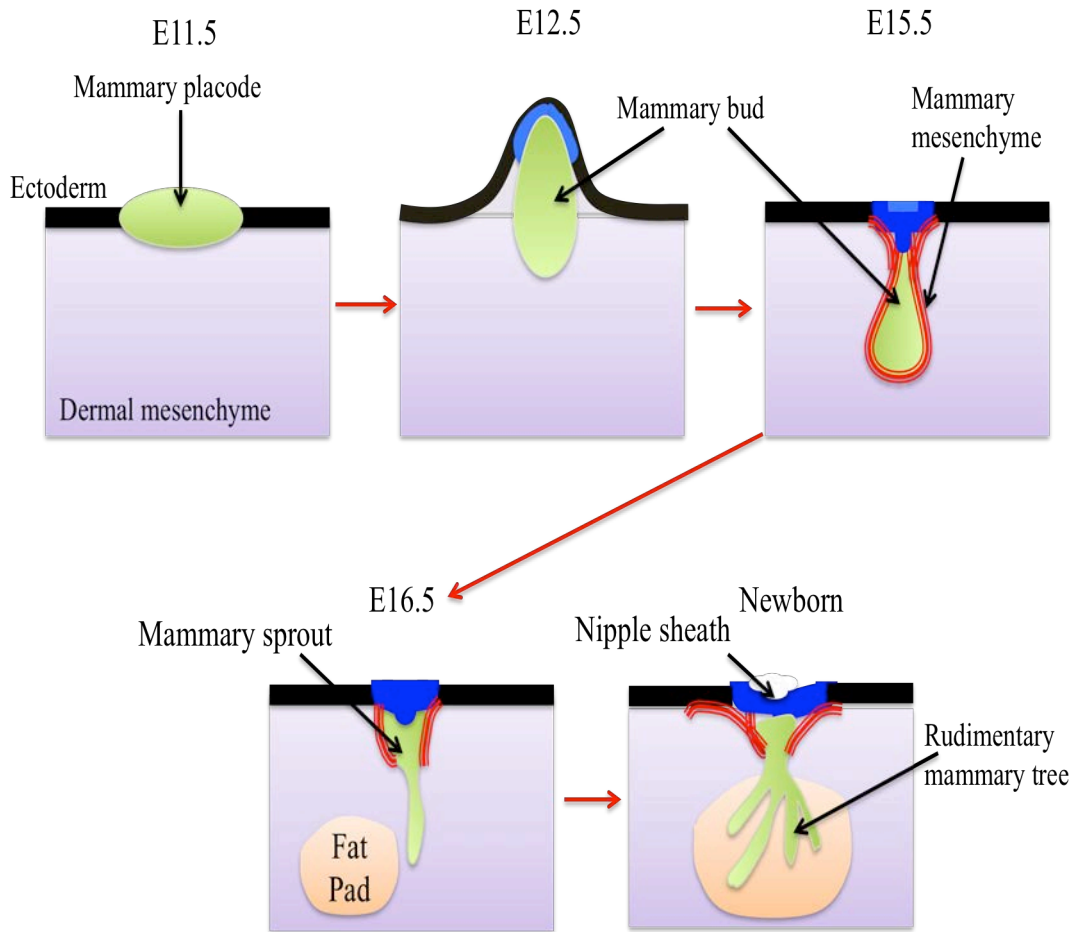


Figure 1.1 Developmental stages of the embryonic mammary gland

During E11 and E12, the placodes are visible and they sink deeper into the dermis to form the mammary bud, which is surrounded by the organized mammary mesenchyme. On E15.5, the mammary epithelium starts to proliferate at the tip and the primary bud and pushes towards the fat pad through the mammary mesenchyme. At E16.5- E18.5 the epithelial cells continue to proliferate in female and invade the fat pad until at birth where the rudimentary mammary tree is formed of small ductal system that contains about 10–15 initial branches. The nipple sheath will be also formed at that stage.

When the reproduction hormones are turned on at the onset of puberty, extensive ductal branching then occurs (Feng et al., 2007). The elongation and branching is initiated at bulb-like terminal end buds (TEBs) that are present at the tips of the mammary ducts (Figure 1.3). TEBs are motile structures that can invade the fatty stroma and undergo repeated dichotomous branching which involves ECM remodeling events (Hinck and Silberstein, 2005). TEBs contain outer undifferentiated cap cells that can differentiate into the myoepithelial layer, and inner multiple layers of body cells that can give rise to the luminal layer. The TEB also contain a small number of multipotent stem cells that are precursors for both luminal and myoepithelial cells (Kenney et al., 2001).

During pregnancy and lactation, the ducts contain cells that can differentiate and produce alveolar cells that are able to produce milk (Hennighausen and Robinson, 2005; Oakes et al., 2008a). During involution the mammary epithelium regresses and massive apoptosis events occur in the differentiated alveoli which causes reconstruction of the mammary tissue and restores it to a pre-pregnancy state (Stein et al., 2007) (Figure 1.3). These numerous changes during the mammary gland development will require the presence of cells with regenerative capacity known as mammary epithelial stem cells. During different developmental stages of the mammary gland, stem cells will maintain quiescence, proliferate, or differentiate into luminal and myoepithelial cells in response to environmental signals. Like other adult stem cells, these signals come from what is known as the stem cell niche.

A stem cell niche is defined as the anatomical location that encompasses the stem cells; then contain cellular, biochemical and mechanical cues necessary for stem cell maintenance (Walker et al., 2009). Stem cell niches have been divided into two categories: stromal niches (SNs), where stem cells' membranes directly come in contact with the stromal cells through cell-cell receptor interactions, and the epithelial niches (ENs), where stem cells are surrounded by ECM proteins that separate them from their neighboring stromal cells. In order for the stem cells to home to their specific niches and receive maintenance signals they need to express the correct adhesion molecules. These include cell-cell and ECM adhesion receptors (Xi, 2009).

The mammary stem cell niche is a type of epithelial niche that harbors the mammary stem cells. Those cells express high levels of ECM adhesion receptors that have

frequently been used for the identification and the sorting of those cells. However, the exact mechanisms by which those adhesion receptors control the stem cell-niche interaction to determine the stem and progenitor cell fate is still not fully understood.

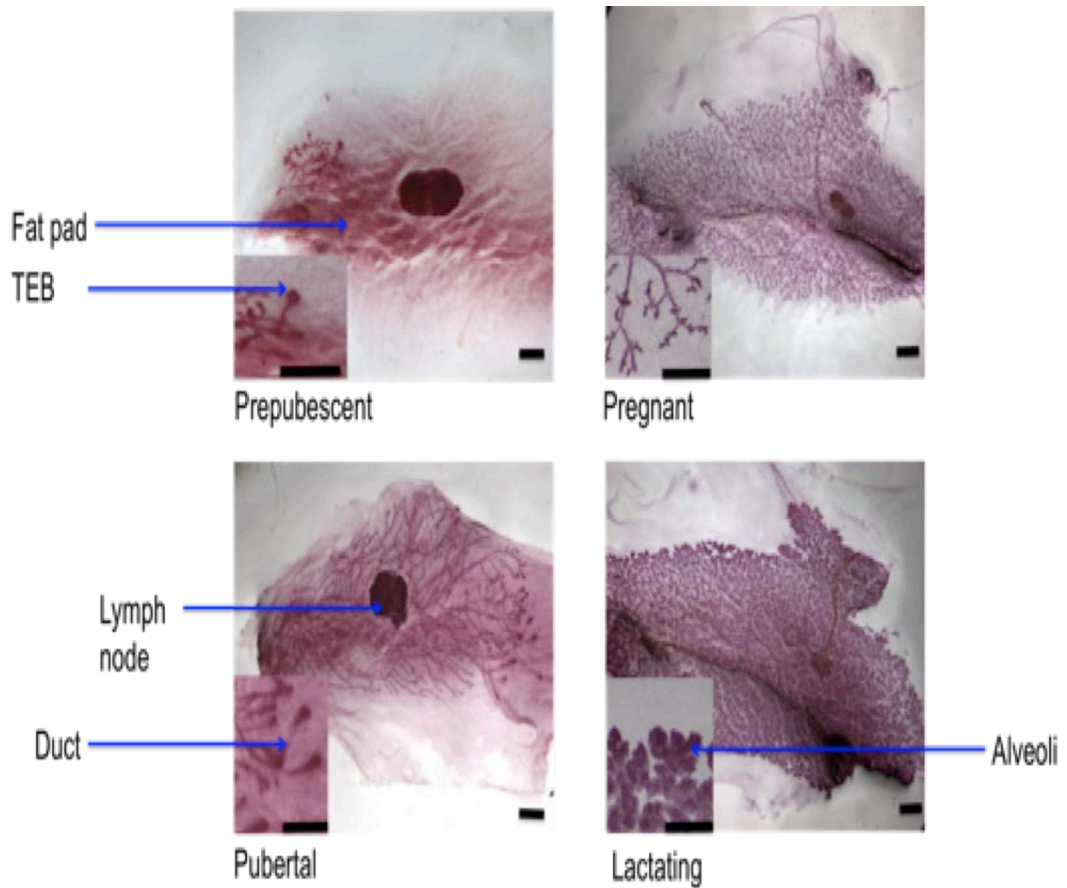


Figure 1.3 Developmental stages of the mammary gland

Mammary gland after birth is only made of rudimentary duct tree, which starts branching and invading the fat pad at puberty. During pregnancy ducts differentiate into milk secreting alveoli that enlarge during lactation. Images taken from Streuli's Lab, University of Manchester. Scale bar=1mm.

1.2 Mammary stem and progenitor cells

Mammary stem cells, termed MaSCs, are defined as cells that can self-renew and have the ability to differentiate into all cellular types of the mammary epithelium in order to generate a fully functional mammary gland with its ductal and lobular components. A single mammary stem cell is able to generate a whole mammary gland (Kordon and Smith, 1998). Progenitor cells, which originate from stem cells, are defined as those cells that have proliferative capacity and can differentiate into one particular cell type. The mammary epithelium has two types of progenitors, luminal and myoepithelial restricted progenitors (Shackleton et al., 2006; Stingl et al., 2006a; Wicha, 2006).

MaSCs are referred to as mammary repopulating units (MRU) in transplantation experiments where MaSCs are isolated from the primary tissue and transplanted in a clear mammary fat pad of immune-compromised mice to test for their stem cell properties. An MRU is a cell that can fully generate a functional mammary gland in a fat pad transplantation experiment. The prevalence of MRU within a specific population can be quantified statistically by limiting dilution assays (Stingl et al., 2006b).

The ability of stem cells to survive, engraft into a fat pad, and form a new mammary gland will depend on their ability to adhere and survive in the new microenvironment. These properties are dependent on cell surface adhesion receptors, particularly different types of integrins. This might explain why FACS sorting of primary MECs for basal cells (that express high levels of β 1-integrin) has shown to enrich for stem cells with multipotent capacity (Shackleton et al., 2006; Stingl et al., 2006a). There has been however some controversy in the literature regarding whether MRUs, counted from transplant assays, reflect the true number and characteristics of MaSCs under normal physiological conditions.

1.2.1 Multipotent or unipotent?

Transplant assays were considered for many decades as the gold-standard assay for measuring stem cells frequency (Smalley et al., 2012). They have proven to be particularly useful for assessing stem cell capacity in gene-specific loss-or gain of function studies. Transplant assays have also suggested the presence of multipotent stem

cells in the adult mammary gland that have the capacity to self-renewal and are responsible for the mammary epithelium maintenance during different stages of development (Stingl et al., 2006b; Wicha, 2006).

Transplantations of sorted basal cells, that have the (EpCAM^{low}, β 1-or α 6-integrin^{high}) FACS profile, showed that there are stem cells with multipotent capacity within the basal population that can generate both luminal and myoepithelial lineages. The luminal population however, which is (EpCAM^{high}, β 1-or α 6-integrin^{low}), did not contain multipotent stem cells but only luminal-restricted progenitors, therefore failed to generate mammary glands in transplant assay (Shackleton et al., 2006; Stingl et al., 2006a).

In-vitro colony- and organoid-forming stem cell assays confirmed the bi-lineage potential of some cells within the basal population (Gu et al., 2013; Guo et al., 2012; Stingl, 2009). Transplanting colonies that were formed in-vitro from basal cells generated functional mammary glands in mice. Culturing basal cells in the presence of agents that disrupt actin–myosin interactions, such as the Rho protein kinase (ROCK) inhibitor (Y27632), increased the MRU potential of basal cells (Prater et al., 2014). Based on these transplant experiments and in-vitro colony forming assays, the existence of multipotent stem cells in the adult mammary gland became more evident.

Transplant assays however, were based on the assumption that cells dissociated from their tissue context would retain cell autonomous properties similar to those observed in the intact tissue. Transplant assays were therefore often criticised as being misleading in extrapolating the self-renewal and differentiation potential of stem cells, as the transplantation procedure might force stem cells to differentiate into lineages, which they usually do not contribute to under physiological conditions. This suggested that the MRUs from the sorted basal population might be artefacts of the transplant assays (Van Keymeulen et al., 2011).

It was therefore important to use in-situ approaches, such as lineage tracing, alongside with transplant assays for studying stem cell functions. Lineage tracing allows the stem

cells and progenitors fate to be tracked in the context of development, tissue maintenance, and disease.

Lineage tracing experiments in the mammary gland involved the expression of Cre-recombinase under the control of a specific gene promoter. A reporter gene, which its expression is driven by Cre-recombinase, was used to irreversibly label cells (that express the gene of interest) and their progeny. The contribution of specific cells to the mammary tissue at defined developmental time points was interrogated at clonal densities (Rios et al., 2014; Sale and Pavelic, 2015). By using an inducible system, the timing and the proportion of cells that undergo genetic recombination was refined. One mechanism to achieve that, included the use of tamoxifen-inducible Cre-recombinase (CreER) (Feil et al., 1996), and the more refined CreER^{T2} (Indra et al., 1999).

Common promoters that were used to track specific cells in lineage tracing experiments were CK5, CK14, and Acta2 for tracing basal cells and Lgr4, Elf5, CK8 and CK18 for tracing luminal cells (Prater et al., 2014; Rios et al., 2014; Van Keymeulen et al., 2011). Lineage tracing of basal cells using CK14 and CK5 promoters has brought a debate into the field on whether basal stem cells are unipotent or multipotent. Transplantation assays showed that basal cells but not luminal cells contain multipotent stem cells that are able to reconstitute a fully functional mammary gland. These findings were challenged by a lineage tracing study which showed that multipotent CK14 and CK5 cells only exist in the embryonic mammary bud (Van Keymeulen et al., 2011). After birth however, the mammary gland is maintained by uni-potent long-lived progenitors that can give rise to only basal or luminal cells under normal physiological conditions (Van Keymeulen et al., 2011).

In order to clarify the discrepancy between results obtained from transplantation assays lineage tracing experiments, (Van Keymeulen et al., 2011) performed experiments where equal ratios of myoepithelial-YFP-labeled cells and unlabeled luminal cells were transplanted into NOD/SCID mice. Those cells were able to reconstitute a fully functional mammary gland but myoepithelial cells were exclusively labeled with YFP and luminal cells remained unlabeled. This showed that myoepithelial cells and luminal cells came from uni-potent basal and luminal progenitors respectively. When the luminal/basal cell ratio was decreased to 1/5, which is ten times lower than

physiological conditions, basal stem cells gave rise to both myoepithelial and luminal lineages. The study therefore suggested that transplanting sorted basal cells (in the absence of luminal cells) might cause them to differentiate into lineages that they don't normally give rise to in-situ. These findings suggested that bi-potent MaSCs only exist in the embryonic and not in postnatal mammary glands (Van Keymeulen et al., 2011).

When the mammary cells' fate was tracked using CK8 and CK18 promoter, cells that expressed those genes gave rise to purely luminal cells. This was the case for cells tracked at birth, at 4-weeks, in adulthood, and during pregnancy. These findings showed that luminal cells expressing CK8 and CK18 contain luminal restricted progenitors that can only give rise only to luminal cells (Van Keymeulen et al., 2011).

These results suggested that both types of lineage-restricted progenitors possess a sustained self-renewal potential and are not progressively replaced by multipotent stem cells in the developing mammary gland. Those findings however, were not able to rule out the presence of a multipotent stem cell that was not targeted by the multiple induced-Cre lines. (Van Keymeulen et al., 2011).

Similar findings were achieved when tracing cells using *Acta2* gene, which is a marker for myoepithelial cells. Freshly sorted myoepithelial cells expressing this gene had MRU capacity in transplant assays. However, lineage tracing experiments using *Acta2-Cre-ER^{T2};Rosa26LacZ* mice, demonstrated that myoepithelial cells in the intact virgin and pregnant mouse mammary glands, contain cells that function as unipotent long-lived progenitors and contribute to the basal cell layer only during different stages of mammary gland development (Prater et al., 2014).

An interesting lineage-tracing study added further layers of complexity through the discovery that Wnt-responsive *Axin2*⁺ cells in the mouse mammary gland can switch fate based on the to the developmental stage. Tracing cells expressing *Axin2* during the embryonic and puberty stages, suggested a switch in Wnt/ β -catenin signaling activity that takes place around birth. *Axin2* expression marked the prospective luminal lineage at the embryonic stage between E12.5 and E17.5, but it was exclusively expressed in the myoepithelial lineage in the prepubescent mammary gland at P14 and P16.

Furthermore, the basal-restricted Axin2⁺ cells defined in-vivo behaved as bi-potent stem cells when sorted and transplanted, suggesting that the transplantation unmasked a regenerative potential of those cells that was not evident in-situ (van Amerongen et al., 2012).

Another study that used high-resolution 3D imaging together with single cell labeling reported that the mammary epithelium is maintained by multi-lineage basal stem cell that play fundamental roles in morphogenesis and homeostasis. High-resolution fate mapping using CK5 and CK14 genes in the adult mammary gland revealed clonal epithelial patches that comprised both luminal and myoepithelial cells, hence came from a common basal precursor (Rios et al., 2014). The luminal layer was also shown to contain luminal-restricted progenitors that are long-lived and contribute to tissue development during different stages of puberty and pregnancy. Thus, it was suggested the lineage-restricted progenitors coexist with multipotent stem cell in the mammary gland and react depending on the regenerative and developmental needs (Rios et al., 2014).

Lineage tracing studies identified luminal restricted progenitors when traced using CK8, CK18, and Elf5 promoters. Cells expressing produce only luminal lineages (Rios et al., 2014; Van Keymeulen et al., 2011) . Using the Notch1 promoter to trace cells in the mammary gland, the existence of ER α ^{neg} luminal progenitors was demonstrated (Rodilla et al., 2015). Although these cells were multipotent during embryonic development, they became uni-potent after birth (as they never generate myoepithelial nor ER α ^{pos} cells in adult mice). They were also able to repopulate the entire mammary gland in transplantation assays, especially when they were stimulated by pregnancy-induced hormones. Notch 1 marked only ER^{neg} cells and this suggested that within the luminal population there are separate progenitors for ER^{neg} and ER^{pos} cells. Interestingly, when luminal cells that express Notch1 where transplanted alone they were able to regenerate a mammary gland particularly from pregnant MECs. When they were transplanted in a 1:1 ratio with myoepithelial cells, they only gave rise to luminal cells only. This proved again that transplant assays might force uni-potent cells to differentiate into lineages they don't differentiate into under normal conditions (Rodilla et al., 2015).

All these studies helped in answering many question regarding the cellular hierarchy of the mammary gland. However, they brought also controversy into the literature because of the contradicting results from the lineage tracing experiments using the CK5 and CK14 promoter (Rios et al., 2014; Van Keymeulen et al., 2011). There might be multiple explanations for the controversy in observations between different lineage tracing studies regarding the existence of a multipotent stem cell in the basal layer (Prater et al., 2014; Rios et al., 2014; Van Keymeulen et al., 2011; van Amerongen et al., 2012).

First, insertion-site effects might occur between different knock-in and knock-out mouse strains. This might affect the timing and level of gene expression and ultimately determine whether Cre-recombinase is expressed in the stem or progenitor subset. Another reason might be that 2D florescent imaging might not be accurate enough and cannot provide quantitative data for the number of cells that express the reporter gene compared to the high resolution 3D imaging (Rios et al., 2014).

One of the important reasons might also be due to the fact that different studies used different concentrations of 4-hydroxytamoxifen (4OHT) to induce Cre activity and gene expression in MECs. 4OHT is toxic at high doses and might induce apoptosis in the mammary gland cells (Shehata et al., 2014). It is an oestrogen receptor antagonist and has a direct effect on mammary gland development (Asselin-Labat et al., 2010; Rios et al., 2014). Using 4OHT at doses ≥ 1 mg/25 g body weight showed to decrease the basal populations when analysed by flowcytometry. 4OHT at doses ≥ 5 mg/25 g body weight induced a transient increase in caspase-3-mediated apoptotic cell death within the mammary epithelium (Rios et al., 2014; Shehata et al., 2014). Thus caution must be taken when interpreting results from lineage tracing experiments as the use of different concentrations of tamoxifen might skew the stem cell frequency.

Taken all these findings together, lineage-tracing experiments have helped in answering many question regarding the cellular hierarchy of the mammary gland. However, it has also highlighted the complexity of that hierarchy and the possibility of high plasticity, where stem cells can switch their fate depending on the context of development (van Amerongen et al., 2012). There is now accumulating evidence for a heterogeneous

MaSC compartment comprising fetal MaSCs, slow cycling cells, and both long-term and short-term repopulating cells (Visvader and Stingl, 2014). Lineage tracing has also identified diverse luminal- restricted progenitor subtypes in the mouse mammary gland types. Bi-potent stem cells and luminal progenitors are responsible to drive morphogenesis and homeostasis of the ductal tree (Rios et al., 2014; Sale and Pavelic, 2015; Van Keymeulen et al., 2011).

Elucidation of the normal cellular hierarchy is an important step toward understanding the “cells of origin” that is responsible for driving breast cancer initiation and tumor formation. Future studies might utilize lineage tracing in understanding the MaSCs-microenvironment interaction and the determination of stem cell fate. This will be practically useful in studying the role adhesion receptors in stem cells, and how they facilitate the interaction with the microenvironment and pass signals into the cell to determine its fate.

In the following section I will discuss in depth how the microenvironment can influence stem cell behavior, how these signals are transduced by adhesion receptors such as integrins, and how this interaction might be deregulated in diseases such as cancer.

1.3 Identification of the location of mammary stem cells

The identification of the location of stem cells is critical for studying their interaction with their microenvironment. The lack of specific markers that clearly label and identify MaSCs, made it difficult to identify their exact location.

Fluorescent label retention techniques have been used to identify mammary stem cell position in-vivo. This technique is based on labeling the mammary gland cells in-vivo with a fluorescent dye such as Bromodeoxyuridine (BrdU) before the animal reaches puberty. This dye incorporates in the newly synthesized DNA and dilutes out after cell division (Larouche et al., 2010). Stem cells are slow dividing long-lived cells and therefore can retain the label throughout the mammary gland development (Booth et al., 2008). Immunostaining of the label-retaining cells showed that stem cells are distributed asymmetrically across the tissue and are enriched in the large primary ducts that are closer to the nipple. Stem cells decreased in frequency as the primary ducts further

branched into secondary and tertiary structures after puberty (Fernandez-Gonzalez et al., 2009).

Within the mammary duct, activated stem cells reside at the forefront of rapidly developing ductal terminal end buds (TEB) during puberty and in alveolar buds during pregnancy (Bai and Rohrschneider, 2010). To date two locations of the MaSCs were identified within the mammary duct. These include a basal position in the myoepithelial layer, and within the luminal epithelial compartments (Tiede et al., 2009). However it is still not clear whether those two populations are functionally distinct from one another.

1.4 Mammary stem cells microenvironment

The mammary stem cell needs to go through different stages during the gland's development. It needs to make a decision on whether to maintain the quiescence state, to become a progenitor or to fully differentiate into myoepithelial and luminal lineages. The stem cell niche is the local tissue microenvironment that surrounds the stem cells. It contributes to stem cell regulation and driving its different developmental stages. This is achieved by a tight control of systemic hormones and local growth factors as well as physical signals. An important character of the stem cell is to be able to respond and differentiate into different types of cells in response to particular tissue components (LaBarge et al., 2009; Muschler and Streuli, 2010).

The normal mammary gland microenvironment can direct the differentiation of cells with regenerative potential into mammary epithelial lineages, even if they are isolated from non-mammary origins. For example, when neural stem cells (NSCs) cells were mixed with mammary epithelial cells, NSCs and their progeny contributed to mammary epithelial growth during ductal morphogenesis, where some of them acted like luminal cells and produced milk proteins and others adopted the myoepithelial phenotypes (Booth et al., 2008b). This phenomenon was also observed with adult mouse testicular cells transplanted into the mouse mammary fat pad (Boulanger et al., 2007).

Interestingly, even tumor cells from human origin, when incorporated into the mouse mammary gland microenvironment, lose their tumorigenicity and fully differentiate into

human-specific mammary epithelial cells *in vivo*. This suggested that mammary microenvironment plays also a major role in cancer suppression. (Bussard et al., 2010).

The ECM plays an important role in directing the complex events of mammary gland's morphogenesis during different developmental stages. Mammary gland ECM changes its composition during the different stages of nulliparous, pregnancy, lactation, involution, and regression. Matrix isolated from nulliparous animals promoted the formation of epithelial ducts and their branching, matrix isolated from mid-involuting mammary glands induced cell death, matrix isolated from late-stage involuting glands restored glandular development, while matrix isolated from nulliparous animals restricted glandular morphogenesis (Schedin et al., 2004).

These observations indicate that the mammary ECM has a central role in directing the fate of stem cells within the tissue. However, virtually nothing is known about the type of ECM proteins involved. Fully differentiated cells of the alveolar epithelial lineage require specific interactions with laminin basement membrane in order to coordinate signals from soluble ligands (eg. prolactin and IGF) to control differentiation and survival respectively but so far nothing is known about these environmental requirements for MaSCs (Du et al., 2012; Jeanes et al., 2011; Lee et al., 2009b).

1.5 Adhesion receptors for MaSCs –ECM interaction

Cells in multicellular organisms cannot function individually. When epithelial cells lose contact with their surrounding ECM they undergo apoptosis (Gilmore et al., 2000; Prince et al., 2002; Wang et al., 2004). An interaction between the cell and its microenvironment including the ECM and other types of cells in the tissue is essential for the organs' appropriate functioning. Interestingly, stem cells can also produce their own ECM proteins, which maybe a cell-autonomous mechanism for their maintenance. For example, epithelial stem cells synthesize laminin, a major component of the basement membrane, thereby providing the necessary adhesion-ligands necessary for the maintenance of their niches (O'Reilly et al., 2008).

Mammary stem cells are thought to reside in the basal compartment of the mammary gland, and therefore to interact directly with ECM proteins. The adhesion receptors on

the cells' surface are the cells' first interaction tools with the ECM and the adjacent cells. Indeed, many of the markers used for isolating stem cells are adhesion receptors. However, it is essential to have a deeper understanding of the signaling pathways downstream of the stem cell-ECM adhesion in order to understand how it influences the self-renewal and differentiation potential of stem cells. The adhesion molecules on mammary stem and progenitor cell are therefore no longer looked at as just sorting markers, but I would also argue that they are functional markers with a major role in stem cell maintenance.

1.6 Integrins and their down-stream signaling

1.6.1 Integrin structure:

Integrins are a class of surface receptors that play a major role in the cell-matrix interaction through the binding of glycoproteins such as laminin, fibronectin and collagen. Integrins are also involved in cell-cell interactions (Humphries et al., 2006). They are heterodimeric molecules that contain α and β subunits that non-covalently link and can form 24 different $\alpha\beta$ heterodimeric receptors. The α and β subunits are constructed from several domains with flexible linkers between them. A typical size of the α - and β -subunits are around 1000 and 750 amino acids, respectively. Each subunit has a large extracellular domain, a single transmembrane domain and a short unstructured cytoplasmic domain.

The extracellular domain directly binds to ECM components, while the intracellular domain connects to large assembled complexes with cytoskeleton proteins (such as α -actinin and talin), scaffolding proteins (such as ILK, PINCH and parvin), cell-signaling proteins (such as FAK, Rac1, calreticulin, and cytohesin) (Arnaout et al., 2007; Campbell and Humphries, 2011; Luo et al., 2007). The cytoplasmic domains differ considerably between individual integrin subunits allowing for integrin-specific signaling responses, although some motifs are common (Legate and Fässler, 2009).

Integrins can modulate a large panel of intracellular signaling pathways that determine the adhesion, migration, differentiation, survival, polarity, proliferation or apoptosis of the cell (Arnaout et al., 2007; Delon and Brown, 2007; Streuli, 2009; Zaidel-Bar et al.,

2007). Integrins are also important in controlling stem cell fate. It might be through binding to specific components of the ECM matrix, cell-cell interaction, responding to mechanical stiffness, and cross talking with other important stem cell pathways (Prowse et al., 2011; Streuli, 2009).

1.6.2 Integrin adhesion to ECM :

The binding between integrins and ECM proteins is not specific. One integrin homo or heterodimer might recognise different ECM ligands. The most common integrin ligands are collagen, laminin, fibronectin and vitronectin (Chen et al., 2012). (Figure 1.4) illustrates integrin receptors interactions with specific extracellular ligands (Luo et al., 2007; Xiao et al., 2004; Xiong et al., 2001).

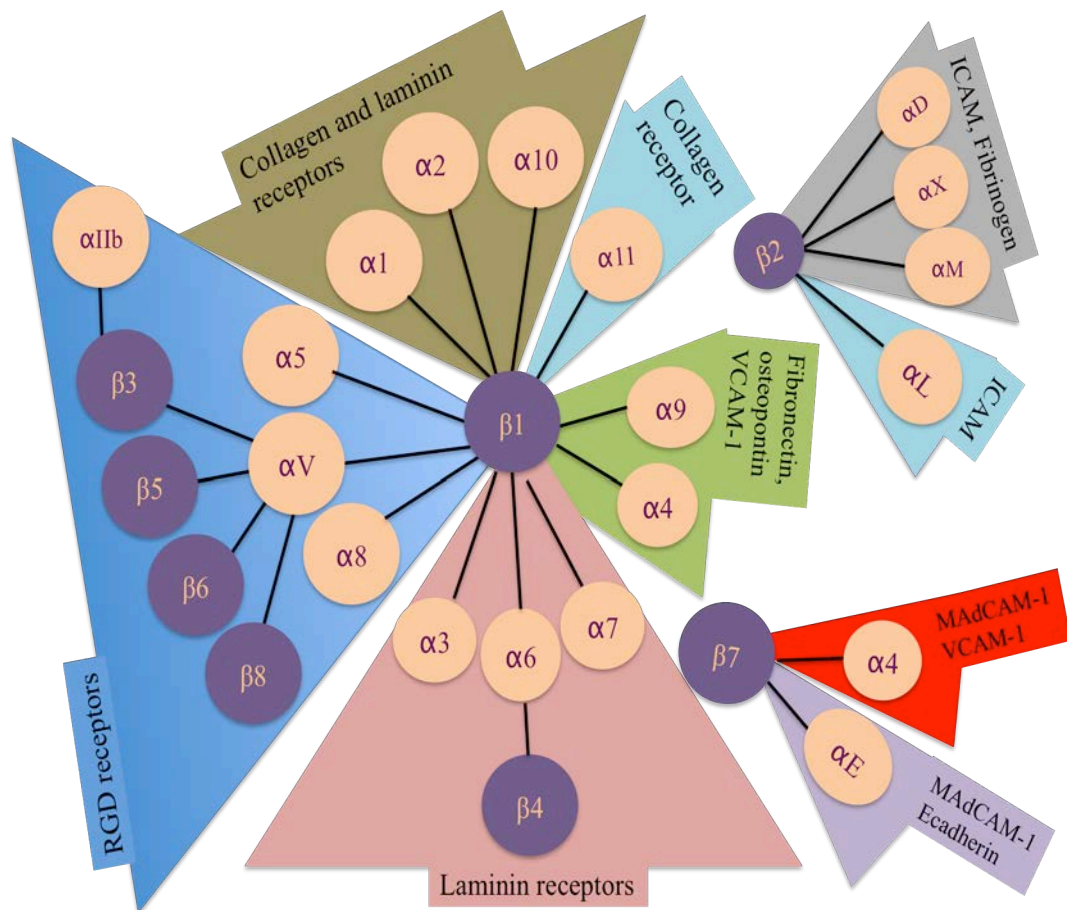


Figure 1.4 Integrin receptors and their ligands

Diagram showing the different binding combinations of integrin receptors and their ECM ligands. One integrin might bind to one or more type of ligands in the ECM.

The activation of integrins adhesion to the ECM can be either through an inside-out or an outside-in fashion. During inside-out activation, the cytoplasmic domain of the integrin binds to various signaling proteins within the cytoplasm. This process can control integrin-mediated cells adhesion to the ECM and the integrin-mediated cell migration through the ECM. The outside-in activation mode involves first the binding of integrin to insoluble ligands of the ECM followed by the activation of integrin-dependent intracellular signaling pathways. Both types of activation require conformational changes of the integrin receptors (Streuli and Akhtar, 2009).

Integrin activation requires the presence of the divalent cations Mg^{2+} or Ca^{2+} in the ECM. When ECM ligand binds between the two integrin subunits, it induces conformational changes. Integrins change their conformation from a latent state, in which they adopt a bent confirmation, into an extended confirmation that is characterised by a high ligand affinity. This conformational change physically pushes the two subunits apart and initiates downstream signaling. Scaffold proteins like talin and kindlins bind to the cytoplasmic domain of the β -integrin subunit, allowing the cytoplasmic tails to separate and the 18 extracellular domains to straighten.

Structural proteins such as talin and vinculin serve also serve as bridges between the integrin cytoplasmic tail and the actin cytoskeleton. The integrin cytoplasmic domain lacks intrinsic enzymatic activity, therefore, the structural alteration assumed following integrin ligation recruit mediators to their cytoplasmic. These recruited proteins interact and transmit signals by which integrins can modulate functions such as proliferation, differentiation, and migration.

Integrin-dependent adhesion induces phosphorylation events that trigger activation of numerous signalling intermediates. Ligated integrins recruit several nonreceptor tyrosine kinases, including focal adhesion kinase (FAK), integrin-linked kinase (ILK), and Src-family kinases. Integrin signalling also regulates Rho-family GTPases which play a central role in controlling actin cytoskeleton organization and cell shape changes (Barczyk et al., 2010; Jeanes et al., 2012a), Rac1 which a member of the Rho GTPases was shown to be important in skin and lymphocytes stem cells (Benitah et al., 2005; Jamieson et al., 2015). In addition, integrin signalling activates multiple signalling pathways that affect gene expression patterns, such as the MAP kinases (ERK, JNK,

and p38) and the transcription factors c-fos, c-jun, and NF- κ B. Some of the downstream integrin signalling molecules were shown to be important in adult stem cells such as FAK and Rac1 (Luo et al., 2013; Yoon et al., 2011). Different crosstalk between integrins and receptor tyrosine kinase-mediated signalling have been reported. These crosstalk allow the control of the growth factor-induced intracellular events by the adhesion machinery (Streuli, 2009; Wei et al., 2015) (Figure 1.5)

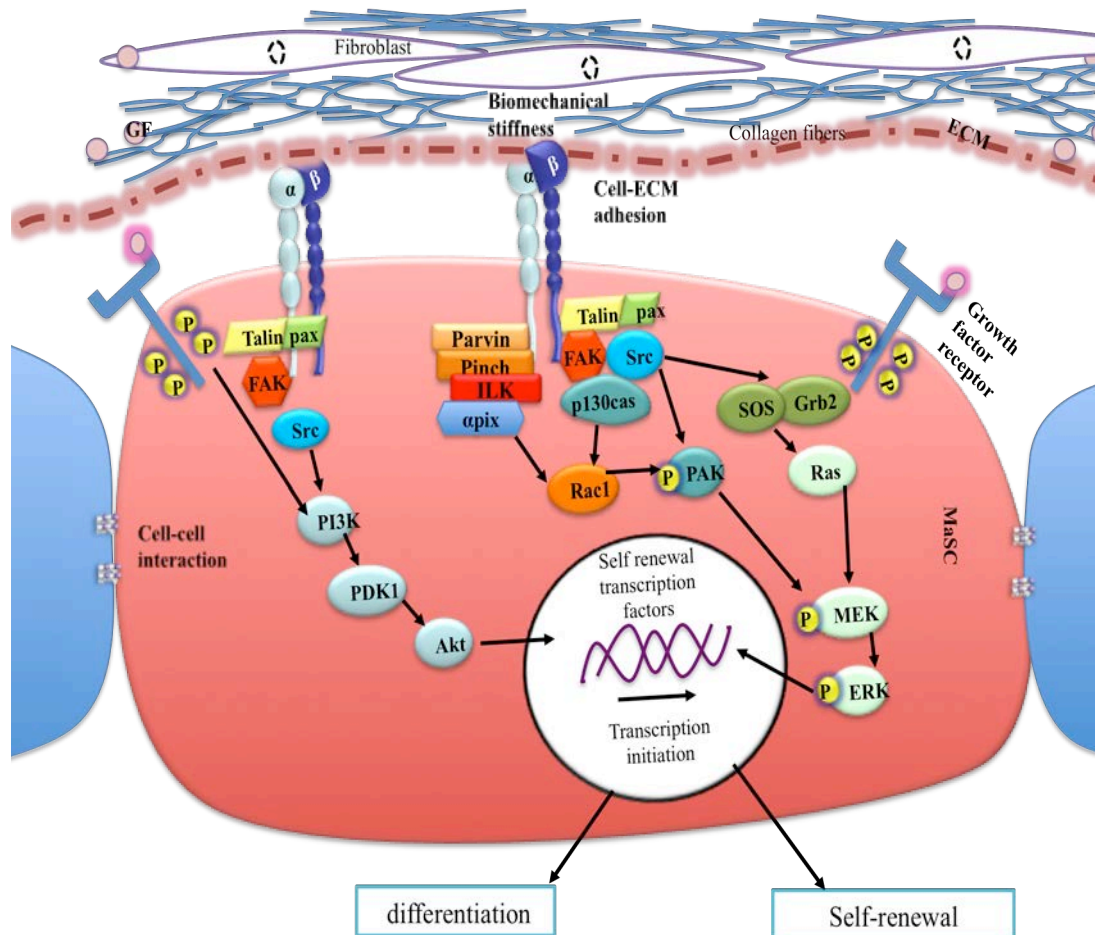


Figure 1.5 Integrin signaling in MaSCs. MaSCs express high levels of integrins. When integrins bind to the ECM they are able to detect mechanical force and the biochemical components of the ECM and activate their downstream signaling. Many signaling intermediates are recruited to the cytoplasmic tail of the integrins. Talin binds to the integrin cytoplasmic domains, promoting conformational changes that lead to the separation of the cytoplasmic domains. Also, the extracellular domains straighten. In the active conformation, integrins can form clusters with other integrins, and recruit proteins such as FAK, paxillin, ILK and Src to form the adhesion complex or adhesome. The recruited proteins are able to transmit signals within the cell and in this way; integrins are able to participate in stem cell decisions. Integrin can also co-operate with growth factor signaling pathways through the activation of the PI3K/AKT pathway or the activation of the MEK/ERK pathways. Those signals might be transmitted to the nucleus and activate stem cell pathways that promote self-renewal or differentiation. Abbreviations: ECM: Extracellular Matrix; GF: growth factors; tal: talin; pax: paxillin; FAK: Focal Adhesion Kinase; ILK: Integrin-Linked Kinase.

1.6.3 integrins cross talk with stem cell pathways in adult and embryonic stem cells

Integrins are highly expressed in many adult and embryonic stem cells. Integrin promoters are under the control of key transcription factors that are essential for stem cell pluripotency, such as OCT4 and SOX2. This suggests that stem cells highly express them as part of maintaining their stemness. This may be because they are key regulators of stem cell interaction with their niches (Yu et al., 2012).

Integrins enable stem cells to sense their microenvironment, adhere in the correct position and respond appropriately to the ECM physical and biochemical properties (Larsen et al., 2006; Marthiens et al., 2010). They also contribute to the homing of the stem cells to their niches (Ellis and Tanentzapf, 2010).

Integrins are also necessary for the fundamental property of the stem cell, which is asymmetric division (Betschinger and Knoblich, 2004). Asymmetric division results in two daughter cells, one of which is similar to the original, and a second daughter that differentiates. In contrast, symmetric division results in the formation of two daughter stem cells. Switching between the asymmetric and symmetric division is a key property of the stem cell (Betschinger and Knoblich, 2004). This switch is at least partially mediated through integrin signaling. Differential activity between Integrin-ECM mediated adhesion and cadherin cell-cell adhesion is essential for the regulation of centrosome positioning and spindle angle during cell division. Thus, stem cells loss of integrin adhesion resulted in the altered orientation of the stem cell division axis and loss of control in the switch between symmetric and asymmetric division (Lechler and Fuchs, 2005; Lu et al., 2001; Taddei et al., 2008a).

In the mammary epithelium, the integrin receptors expressed are all from β 1-integrin and β 4-integrin heterodimer types. They are more highly expressed in the basal cells than the luminal cells (Prince et al., 2002; Taddei et al., 2003). β 3-integrin was reported in luminal progenitors (Asselin-Labat et al., 2006a) although we have not been able to detect it in-vivo but only it in tissue cultured primary cells (data unpublished).

Integrins that have been used to identify stem cells and progenitors include (β 1, β 3, α 6 and β 4)-subunits (Shackleton et al., 2006; Stingl et al., 2006a). However, very little is

known about their role in stem and progenitor cell biology. β 1-integrin is likely to have a key role because deleting β 1-integrin in K5-expressing basal cells in the gland abolished the gland's regenerative potential although it has not been definitively confirmed that β 1-integrins are essential for stem cell function (Li et al., 2005; Taddei et al., 2008).

Although the deletion of β 1-integrin severely affected the mammary gland development, deleting the α 6-integrin, α 3-integrin and β 4-integrin genes in the mammary gland did not affect normal ductal morphogenesis and branching, or the normal luminal and myoepithelial cell distribution. It also had no effect on the ability of the mammary cells to respond during pregnancy with normal alveogenesis and synthesis of β -casein (Klinowska et al., 2001). What compensates for these integrin subunits is still not understood and a remaining question whether the knockdown of these integrin subunits can affect the basal stem cell population and the formation of mammary glands in secondary transplants.

1.7 Integrin signaling in different types of adult stem cells and the possibility of similar signaling in mammary stem cells

Integrins regulate and co-operate with many signaling pathways involved in a variety of adult stem cells. In neural stem cells, β 1-integrin affects the Notch signaling pathway through a direct interaction between the β 1-integrin and the Notch receptor and affecting its internalization via covalin-dependent mechanism (Campos et al., 2006a). In chick embryos, signaling of β 1-integrin via ILK directs phosphorylation of GSK3 β to permit Wnt pathway activation and, thereby, Notch signaling in the anterior presomitic mesoderm. These two signaling pathways then cooperate to promote somite formation (Rallis et al., 2010). Another example of co-operation between the integrins and the Notch pathway is in endothelial cells. Cells adhesion to laminin-111 adhesion triggers the Dll4 expression, leading to subsequent Notch pathway activation. Moreover, knockdown of α 2 β 1 and α 6 β 1 integrins abolished Dll4 induction, which revealed a selective integrin signaling acting upstream of Notch pathway (Estrach et al., 2011).

β 1-integrin regulates hedgehog-signaling pathway in prostate epithelium. β 1-integrin knockdown reduced the proliferation of these cells and affected the expression of GLI1

protein. GLI is a transcription factor known to be regulated through the sonic hedgehog pathway. Down-regulation of β 1-integrin inhibited IGF-IR (insulin-like growth factor type 1 receptor) and AKT activation. The proliferation of the β 1-integrin knockdowns was rescued with the repression of GLI1. This showed that β 1-integrin controls the proliferation of the prostate epithelium cells in a GLI1-dependant manner (Goel et al., 2010).

α 6-integrin maintains the self-renewal potential in human embryonic stem cells via prolonged activation of the PI3K/AKT signaling pathway and suppression of P53 gene expression. The knockdown of the α 6-integrin resulted in the upregulation of the p53 and the differentiation of the stem cells into 3 different germ layers (Yu et al., 2012).

These studies on how integrins control stem cells in adult tissue can provide interesting potential pathways through which integrins can influence the self-renewal and differentiation in MaSCs.

1.8 Non-integrin adhesion receptors in stem and progenitor cells

In addition to integrins, mammary cells have other receptors that bind to the ECM including dystroglycan, collagen receptor, and syndecans. Each of these binds specific ECM components and can affect the mammary development (McCave et al., 2010). Non-integrin adhesion receptors that were used for isolating MaSCs include CD44, CD24 and EPCAM.

CD44 is a type I trans-membrane glycoprotein receptor for the glycosaminoglycan hyaluronan (HA), a major component of ECM (Aruffo et al., 1990; Naor et al., 1997). Other CD44 ligands include osteopontin, serglycin, collagens, fibronectin, and laminin (Goodison et al., 1999). Following the ligand binding, CD44 interacts with various cytoskeletal proteins and GTPases (e.g., RhoA, Rac1 and Cdc42) and induces reorganization of the actin cytoskeleton and regulation of cellular migration and morphology (Bourguignon, 2008).

CD44 has a differential expression in the normal mammary gland compartment. It is highly expressed in MaSCs and its expression in differentiated cells is influenced in part

by hormones and growth factors such as IGF-1 and EGF which regulate the growth and differentiation of the mammary epithelium (Hebbard et al., 2000). Similar to $\beta 1$ -integrins receptors, CD44 signals through FAK which then associates with PI3Kinase and activates mitogen-activated protein kinase (MAPK) at its downstream. This results in the cells obtaining stem cell properties although the mechanism remain unknown (Fujita et al., 2002).

CD24 is used to distinguish different cell populations in the mammary gland. Based on CD24 staining, mammary gland cells were divided into 3 populations that were CD24⁻, CD24^{low/medium} and CD24^{high} and that represented the non-epithelial, basal and luminal populations respectively (Sleeman et al., 2006). CD24 is a negative regulator mammary gland development as the genomic knockout of CD24 adhesion molecules from breast epithelial cells resulted in an accelerated mammary gland ductal extension during puberty and an enhanced branching morphogenesis, resulting in increased furcation in the ductal structure. (Cremers N et al., 2010).

1.9 Adhesion receptors as markers for isolating mammary stem cells

Recently MaSCs have been isolated from human and mouse mammary gland based on their specific expression of extracellular markers (LaBarge et al., 2009; Stingl, 2009). This section will summarize what has been published on markers for sorting stem cells and lineage restricted progenitors.

1.9.1 Stem cells reside in the EPCAM^{medium} / CD24^{medium}, $\beta 1$ / $\alpha 6$ -integrin^{high} population in mouse and in EPCAM^{medium}, $\beta 1$ / $\alpha 6$ -integrin^{high} in human

Mouse mammary epithelial negative sorting for lin markers (to exclude hematopoietic cells) followed by sorting using CD24 and $\beta 1$ -integrin, or CD24 and $\alpha 6$ -integrin, generates 4 distinct cell populations (Figure 1.6). Stem cells are enriched in the population sorted for lin⁻, CD24^{medium} and $\beta 1$ -integrin^{high} or within the population of lin⁻, CD24^{medium} and $\alpha 6$ -integrin^{high}. The MRU frequency within this population is 1 in every 60–90 cells (Stingl et al., 2006a). Epithelial Cell Adhesion Molecule (EPCAM) can be used instead of CD24 for sorting mouse cells and will give similar sorting profiles (Shehata et al., 2012).

For sorting human MaSCs, EPCAM was used instead of the CD24 marker. The epithelial human mammary cells sorted using EPCAM and $\alpha 6$ -integrin had a similar distribution to the one generated from the mouse mammary gland; the stem/basal cells had a lin^{-} , EPCAM^{medium}, $\alpha 6$ -integrin^{high}. This population was further sorted based on the expression of Mucin1, cell surface associated protein (MUC1) into 1) biopotent progenitors in lin^{-} , EPCAM^{medium}, $\alpha 6$ -integrin^{high} and MUC1⁻ population and 2) luminal restricted progenitors in the lin^{-} , EPCAM^{medium}, $\alpha 6$ -integrin^{high} and MUC1⁺ populations (Eirew et al., 2008).

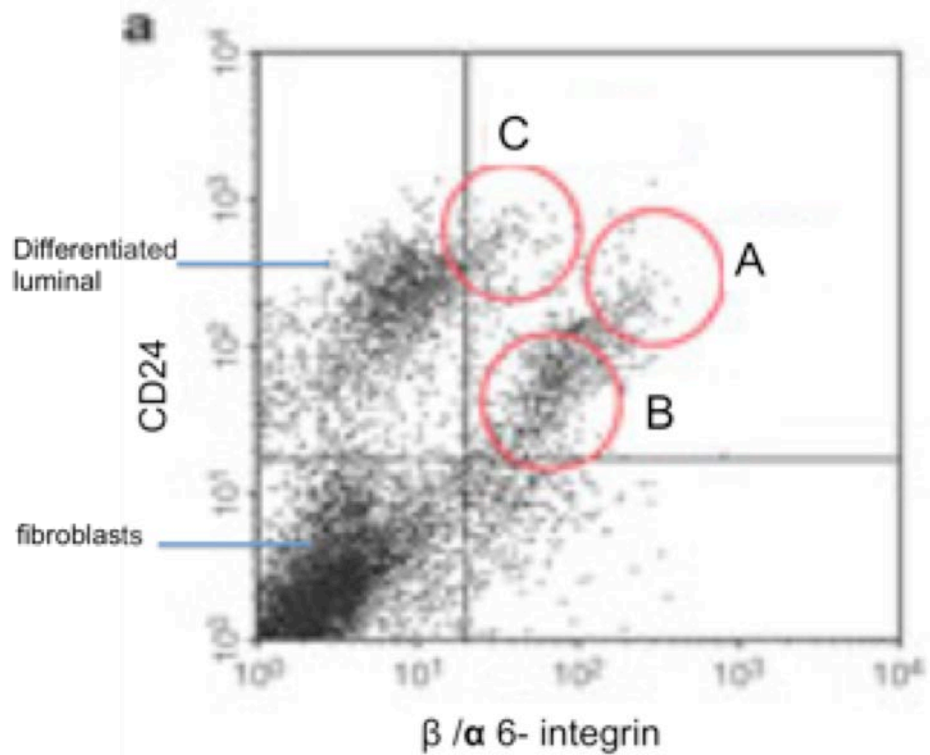


Figure 1.6 FACS sorting of mammary epithelium based on CD24, $\beta 1 / \alpha 6$ -integrin markers. A. Population represents MaSCs ($CD24^{\text{medium}}$, $\beta 1 / \alpha 6$ -integrin^{high}) B. myoepithelial population ($CD24^{\text{low}}$, $\beta 1 / \alpha 6$ -integrin^{high/med}) C. luminal progenitor population ($CD24^{\text{high}}$, $\beta 1 / \alpha 6$ -integrin^{low}). Figure adapted from (Stingl et al., 2006).

1.9.2 β 3-integrin and α 2-integrin for identifying luminal progenitors

β 3-integrin (CD61) and α 2-integrin (CD49b) are expressed in the luminal restricted progenitors (Asselin-Labat et al., 2006a; Shehata et al., 2012). The luminal-restricted progenitors are enriched in the (CD24^{high}, β 1-integrin^{medium}, α 6-integrin^{medium}, and β 3/ α 2-integrin^{high}) phenotype, whereas the bi-potent stem cells that generate myoepithelial cell progeny have a phenotype that is similar to the MRUs (CD24^{med} β 1-integrin^{high} α 6-integrin^{high}). Differentiated luminal cells have a (CD24^{high} β 1-integrin^{low} α 6-integrin^{low} β 3-integrin⁻) phenotype (Figure 1.6 and Figure1.7) (Asselin-Labat et al., 2006a; Sleeman et al., 2007a; Stingl, 2009; Stingl et al., 2006b). Figure1.7 summarizes the classification of mammary epithelial hierarchy based on the expression of adhesion receptors (Figure1.7).

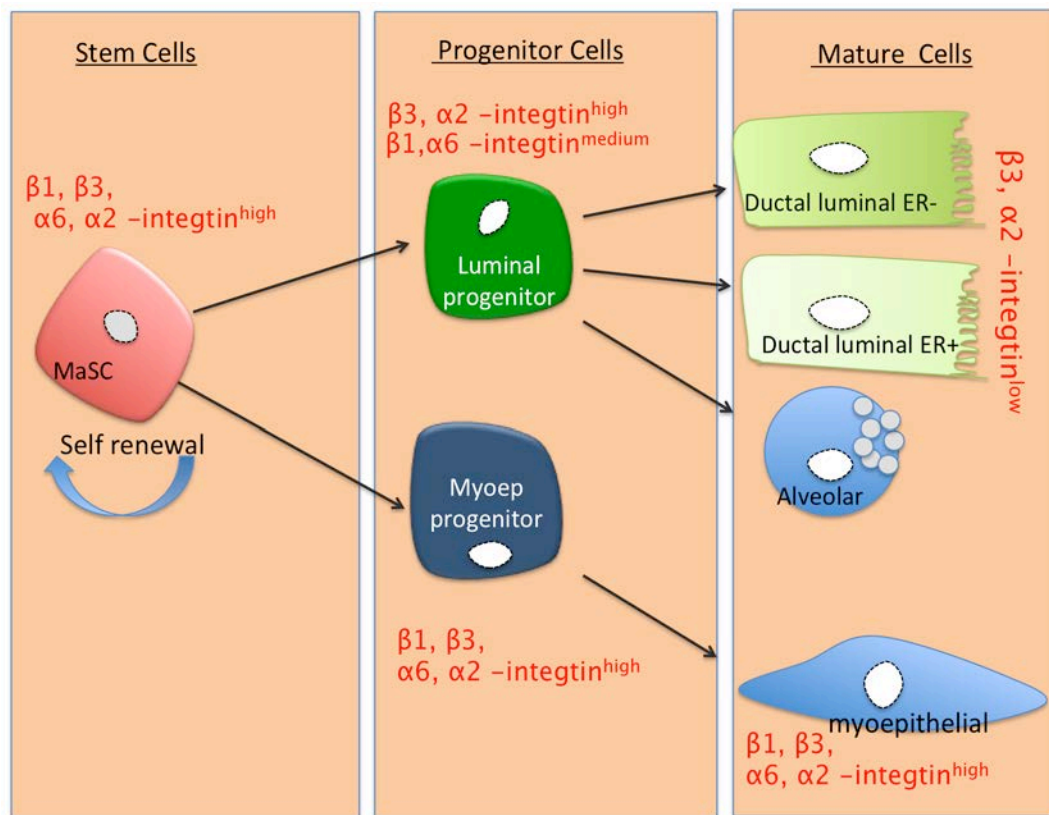


Figure1.7 Expression of integrins in mammary stem cells, lineage-restricted progenitors and differentiated mammary cells. Bi-potent stem cells (MaSCs) express high levels $\beta 1$, $\beta 3$, $\alpha 2$, and $\alpha 6$ -integrin. As stem cells become luminal-restricted progenitors, they maintain high expression of $\beta 3$ and $\alpha 2$ but become $\beta 1$ and $\alpha 6$ - medium. Once they fully differentiate into ductal luminal or alveoli cells they become $\beta 1$, $\beta 3$, $\alpha 2$, and $\alpha 6$ - integrin low. Myoepithelial progenitors and differentiated myoepithelial cells maintain their high expression of $\beta 1$, $\beta 3$, $\alpha 2$, and $\alpha 6$ -integrins. Therefore, it is not possible to distinguish stem cells from differentiated myoepithelial cells based on the expression of integrins

1.9.3 Cell-Cell interaction between stem cells and differentiated cells in the stem cell niche.

Stem cells reside in the basal compartment of the mammary gland and therefore are adjacent to the myoepithelial cells. Myoepithelial compartments seem to contribute the stem cell niche through the production of necessary growth factors, cell-cell interaction and production of ECM components such as laminins, collagens, fibronectin, heparan sulphate proteoglycans and SPARC (Faraldo et al., 2005).

Stem cells have higher expression in $\alpha 6$ -integrin than the differentiated myoepithelial cells. However, there was no significant difference in gene expression between the two basal compartments (Stingl J et al., 2006).

Cell-Cell interaction is essential for the differentiation of stem cells into luminal cells but not myoepithelial lineages. When cells were only interacting with the ECM substrate they tend to differentiate only into myoepithelial cells, however, when they started touching each other, the luminal phenotypes emerged. E-cadherin junction formation between cells is the driving force for MaSCs differentiation into luminal cells (LaBarge et al., 2009).

1.9.4 Hormone and growth factor receptors in mammary stem cells

Stem cells sorted for $CD24^{\text{medium}} \beta 1\text{-integrin}^{\text{high}} / \alpha 6\text{-integrin}^{\text{high}}$ from mouse mammary gland were also shown to be negative for estrogen receptor ($ER\alpha$), progesterone receptor (PR), and erbB2 but are positive cytokeratin14 (CK14) and Epidermal Growth Factor Receptor (EGFR) which confirmed their basal origin (Asselin-Labat et al., 2006b). The fact that stem cells were shown to be estrogen receptor (ER) and progesterone receptor (PR) negative made it likely that the effects of estrogen on mammary stem cells are mediated indirectly (Sleeman et al., 2007b).

1.9.5 Hormone receptors and milk proteins in luminal progenitors

Luminal cells which are $CD24^{\text{high}}$ were further sorted using the Stem Cell antigen (Sca-1) and revealed that within the luminal population there is a luminal restricted progenitor population which is $Sca-1^{-}$ which expresses milk proteins such *Csn β* , *Ltf*, *Mfge8*, and

Wap as and a fully differentiated luminal population which is Sca-1⁺ and is positive for genes involved in responding to systemic hormones. These include *Estrogen receptor α* (ER α), progesterone receptor (PR), prolactin receptor (*Prlr*), *Cited1*, and *S100A6* genes (Sleeman et al., 2007). c-kit which is a highly expressed gene in embryonic stem cells was shown to be a sensitive marker for luminal progenitor cells that were ER⁻ and Sca⁻ (Regan et al., 2012). Taken all these classifications together, it is more likely that 3 types of lineages exists in the mammary gland: secretory, steroid hormone receptor-expressing and myoepithelial (Stingl, 2009)

1.10 Breast cancer stem cells

Many strong similarities have been found between stem cells and cancer cells, specifically in one of the most important and useful property of stem cells, which is the capability of self-renewal. Studies of neoplastic tissues have provided evidence of self-renewing, stem-like cells within tumors, which have been called cancer stem cells (CSCs). Those have been defined as rare cells within the tumor population that possess an indefinite potential for self-renewal, which drives tumorigenesis and seed new tumors. They can also differentiate into different types of cells and result in the heterogeneity of the tumor tissue. (Ailles and Weissman, 2007; Reya et al., 2001).

Other similarities have been observed between normal and cancer stem cells. These include the ability to differentiate, increased number of membrane transporter proteins, anti-apoptotic pathways, telomerase activity, anchorage independence as well as the ability to migrate and metastasize (Liu et al., 2005). These accumulating evidence provided support for the cancer stem cell hypothesis. This hypothesis made it widely believed that the presence of at least one cancer cell with a stem cell property is essential for the tumor initiation, disseminating, and metastasis.

Breast tumours are highly heterogeneous with several distinct sub-types categorized based on their molecular and histological characteristics (Weigelt et al., 2010). The biological basis for this heterogeneity is still poorly understood, although there are some distinct phenotypic and genotypic correlations. Breast cancers can be categorized in a number of different ways including clinical parameters, by histology, or gene

expression profiles (Molyneux and Smalley, 2011). On a molecular level there are three categories of breast cancer molecular phenotype described. These include the ‘basal-like’, HER2 and luminal breast cancers (Weigelt et al., 2010). A key issue in breast cancer is the relationship between the normal cellular developmental hierarchy and different subtypes of breast cancer biology and the effect of genomic mutations in specific mammary cell lineages on tumours heterogeneity and progression.

Stem cells and luminal progenitors are more susceptible than differentiated cells for becoming cancer cells (Polyak, 2011). They live longer than differentiated cells and therefore, they are more prone to accumulating mutations. They also share many self-renewal pathways with the cancer cells (Charafe-Jauffret et al., 2009; Liu et al., 2005). Understanding the concept of the ‘cell of origin’ in breast cancer will help in finding better treatment of the disease and overcome the problem of heterogeneity that causes different responses to cancer treatment (Polyak, 2011). Many studies are therefore focusing on expressing common breast cancer mutations in different subtypes of the normal mammary gland epithelium and then identify the molecular profiles of these cells and compare them to common tumors (Koren et al., 2015; Molyneux et al., 2010).

One example is the *BRCA1* mutation, the majority of breast tumors arising in carriers of germline mutations in *BRCA1* have a distinctive basal-like phenotype (Lakhani et al., 2005; Palacios et al., 2005). It has therefore been reported that loss of *BRCA1* function in basal stem cells results in tumor formation and is associated with a uncontrolled luminal differentiation (Liu et al., 2008; Vassilopoulos et al., 2008). Interestingly, when deleting *Brcal* in mouse mammary luminal progenitors, the luminal cells produced tumors that phenotypically similar to the human *BRCA1* breast cancers. They also resembled the majority of sporadic basal-like breast tumors. However, when deleting *Brc1* in basal cells, the deletion generated tumors that express molecular markers of basal breast cancers but do not histologically resemble either human *BRCA1* or the majority of sporadic basal-like breast tumors. This suggested that the molecular profile of cancer might not necessary reflect the origin on the tumor and some cells might acquire plasticity and multipotent characteristics with the expression of specific mutation (Molyneux et al., 2010).

PIK3CA^{H1047R}, is another example of one of the most frequent mutations occurring in human breast cancer (Cancer Genome Atlas Network, 2012). When *PIK3CA*^{H1047R} was overexpressed in lineage-committed basal Lgr5-positive and luminal CK8-positive cells of the adult mouse mammary gland, it caused cells to acquire multipotent stem-like characteristics. The mutation increased the regenerative potential of these cells in transplant assays. It also gave rise to different types of tumors depending on the population where the mutation was expressed. The tumour cell of origin influenced the frequency of malignant mammary tumours. This suggested a mechanism involved in the formation of heterogeneous, multi-lineage mammary tumours expressing this mutation (Koren et al., 2015).

Studying stem cell biology is also very beneficial in understanding breast cancer metastasis. The metastasis process was recently shown to be an early event in primary tumor formation, where cells carrying stem cell markers can disseminate into secondary sites and become responsible for secondary tumor formation later on in the patient's life (Weng et al., 2012). There are specific organs to where the breast cancer stem cells prefer to metastasize. These include lymph node, bone, brain, lung, and liver (Weng et al., 2012). The reason why cancer stem cells prefer to metastasize to these organs could be because they contain the correct ECM complexes that adhesion molecules on cancer stem cells can bind to and seed the new tumor. Finding the correct therapy that disrupts this cancer-cell ECM interaction can provide a great improvement in the treatment of those who suffer from the metastatic disease.

1.11 Adhesion molecules as markers for isolating cancer stem cells.

Adhesion molecules that were used to isolate stem cells from normal human breast epithelial cells were also used to isolate cancer stem cells from BRCA1 mutated tumors. The (CD24^{low}, β 1 and α 6-integrin^{high}) populations contain cells that have a significant higher capacity to generate tumors in-vivo and show stem cell properties in-vitro in comparison to the (CD24⁻, β 1/ α 6-integrin⁻) population (Vassilopoulos et al., 2008).

Breast cancer stem cells also reside in the CD44⁺CD24^{-/low}. This population is responsible for the tumor cell initiation when transplanted in mice even at a very low frequency of 100 cells. Those cells can produce differentiated types of cancer cells that

were found to have a CD44⁻CD24^{high} phenotypes (Al-Hajj et al., 2003). CD44⁺CD24^{low} population also expresses high levels of pro-invasive genes and highly invasive properties. However, this phenotype is not sufficient to predict capacity for pulmonary metastasis (Sheridan et al., 2006).

1.12 Adhesion receptors and tumor initiation mechanisms

Wrong adhesion events in the mammary gland can initiate breast cancer. The loss of polarity markers is believed to be an early indicator of malignant transformation (Lelièvre, 2010; Namba et al., 2004).

Integrins act like mechanotransducers that regulate cell fate. Increasing tissue stiffness in tumor tissue has resulted in an abnormal up regulation of integrins downstream signaling that resulted in enhanced ERK activation, increased ROCK-generated contractility and focal adhesions. This caused disruption of acini formation in 3D culture a typical feature of cancer formation (Paszek et al., 2005)

Higher matrix density can increase adhesion clustering at the cell-matrix interface and chronically elevated activation of a Focal Adhesion-RhoGTPase-MAPK network. This activation resulted in up regulation of many proliferation-associated genes that have clinical prognostic relevance (Provenzano et al., 2009). Deletion and targeting of cell β 1-integrin and its downstream signaling pathways in cancer cells had a major effect on tumor initiation and invasive growth (Guo et al., 2006; White et al., 2004).

β 1-integrin co-operates with important signaling pathways that are involved in the self-renewal and metastasis potential of tumor cells that overexpress the ErbB2 (HER2 in human) oncogene. The deletion was associated with decreased levels of the phosphorylated forms of c-Src, p130Cas, paxillin, Akt, and Stat3 molecules. Loss of β 1-integrin was associated with a significant reduction in the Epidermal Growth Factor Receptor (EGFR) phosphorylation levels (Huck L et al., 2010)

Down stream signaling of integrin pathways in cancer has an influence on CSCs. Focal Adhesion Kinase (FAK) is one of the most prominent components of integrin signaling. FAK is a non-receptor tyrosine kinase predominantly localized in focal adhesions of

adherent cells (Parsons, 2003). Knockout of FAK in primary mouse breast cancer resulted in reducing the pool of CSCs. This was characterized by a decrease in the Lin⁻ CD24⁺ β 1-integrin⁺ β 3-integrin⁺ population and reduction in ALDH activity. In-vivo tumor transplantation experiment showed that knocking out FAK resulted in a decreased ability of tumor formation (Luo et al., 2009).

1.13 Targeting the ECM-Cell interaction for preventing adhesion-mediated cancer therapy resistance

Cancer stem cells have a known property for their resistance to chemotherapy and radiation therapy and therefore are responsible for the cancer reoccurrence. In fact, non-adjuvant chemotherapy enriched for the cancer stem cell population (CD24^{low}, CD44^{high}) and to target mainly the differentiated cells (Li et al., 2008). It has been shown that targeting breast cancer adhesion molecules in combination with doxorubicin can achieve a higher sensitivity of breast cancer stem cells to chemotherapy treatment (Van Phuc et al., 2011). In HER-2 positive cancer cells, β 1- integrin mediates an alternative resistance pathway after using HER2-targeting therapies such as trastuzumab and lapatinib. Combining those treatments with targeting of β 1 integrin by siRNA or FAK by FAK-inhibiting compounds increased apoptosis in the cancer cells after the HER-2 directed treatment (Huang et al., 2011).

Inhibition of β 1 integrin by specific antibodies also increased the sensitivity of the breast cancer cell-line to radiation therapy (Park et al., 2008). Laminin B5 chain peptide A5G27 inhibited the binding between CD44v3-heparan sulfate and FGF2, thus decreasing FGF2-induced activity and in-trun inhibit metastasis- and angiogenesis (Hibino et al., 2005). Since adhesion receptors are important in cancer resistance, more investigation in finding therapies that target CSCs-ECM interaction can provide a promising approach in targeting CSCs mediated therapy resistance.

1.14 Conclusion

Currently, very little is known about the extracellular signaling molecules controlling stem cells and their progenitor fate decisions. Evidence is emerging that local microenvironmental cues may have a role and there is a possibility that communication between stem cells and their niche may alter in cancer. I therefore hypothesise that the integrin adhesion receptors currently used to sort stem cells might play an important role in the stem cell-microenvironment interaction. In my research project, I plan to investigate this hypothesis because it may provide a better understanding of how deregulated cell-matrix interactions may contribute to the formation of cancers.

1.15 Aims and objectives of the project

The above discussion has revealed that very little is known about the stem cell niche or about the receptors on stem cells that interact with niche components and their downstream signaling. Although integrins are frequently used as markers to isolate stem cells and luminal progenitors, their role in self-renewal and the formation of progenitors is unknown. The aim of my project is to conduct functional studies to identify whether or not stem cells ECM receptors are essential their maintenance and lineage selection.

The overall strategy is to use genetic deletion of integrins using transgenic mice and lentivirus shRNA technology to knockdown ECM integrins in stem cells, and then test for their stem cell behavior using different stem cell assays.

The specific aims of my project are:

- 1) Establish stem cell and progenitor cell sorting by flow cytometry.
- 2) Establish in-vitro methods for testing the stem cell property of stem cells. These include mammosphere and organoid forming assays.
- 3) Optimise lenti-virus infection of primary cells and knockdown of different adhesion receptors in-vitro.
- 4) Test the effect of deleting β 1-integrin and its downstream targets such as Rac1 and ILK in mammary stem cells and progenitor cells self-renewal and differentiation.
- 5) Perform rescue experiments using downstream β 1-integrin components in β 1-integrin null cells.

6) Link integrin signaling with other pathways that were known to be involved in stem cell maintenance such as Wnt/ β -catenin and Notch signaling. Also, identify key stem cell transcription factors that might be regulated by β 1-integrin and result in stem cell fate decision.

During my first year, aims 1-3 were completed. These provided the necessary tools to genetically manipulate the expression of integrins then test the down regulation of β 1-integrin pathway on stem cells. In the second year aims 4 and 5 were completed, where β 1-integrin downstream targets that were important in β 1-integrin control of stem cells and luminal progenitors were identified. For the third year, β 1-integrin was linked with Wnt/ β -catenin signaling and key molecules involved in the cross-talk between these two signaling pathways were identified (aim 6).

In summary, this project has provided new mechanisms by which β 1-integrin signaling controls stem cells and progenitor fate decision in primary MECs. This will hopefully provide an insight on how abnormal signaling of adhesion receptors in stem cells can result in the transformation of these cells into cancer stem cells.

Chapter 2: Materials and Methods

Chemicals used to make up solutions were of Analar grade or better and purchased from Merck Ltd. (Dorset, UK) otherwise stated

2.1 Molecular Biology

2.1.1. Plasmids and oligonucleotides

pLVTHM plasmid was purchased from Addgene (12247). The lentiviral viral envelope plasmid CMV-VSVg (PMD2G) packaging plasmid, psPAX2 and were obtained from TronoLab (Lausanne, Switzerland). pLV-Venus lentiviral gene delivery system and pLV-Venus-H1-sh β miR were kindly gifted by Dr. Pengbo Wang. Dr. Paulina Moreno-Layseca provided the pLV-Venus-H1-sh β 1miR-activeRac1. All oligonucleotides for sequencing, PCR, and mutagenesis were synthesized by Sigma-Aldrich (Cambridge, UK).

2.1.2 Bacterial transformation and plasmid DNA extraction

Luria Broth (LB) containing, 5g Yeast extract, 10g Tryptone 10g NaCl and LB agar (LB supplemented with 15g/l agar) were obtained from University of Manchester internal Stores. Super Optimal broth with Catabolite repression (SOC) media was purchased from Invitrogen (Paisley, UK). To amplify the plasmids, one aliquot of 100 μ l JM109 strain *E.coli* bacteria was obtained from at -80°C. Bacteria were thawed on ice for approximately 10 minutes. Then, 100ng-1 μ g of DNA was added to 50 μ l of bacteria we and incubated for 15 minutes. This was followed by the bacteria undergoing heat-shocked at 42°C for 30 seconds and put back on ice for additional 5 minutes. 500 μ l of SOC media was added to each transformation tube before incubating them for 1 hour at 37°C. After incubation, 200 μ l of bacteria were spread onto LB agar plates containing the relevant antibiotic: Ampicillin (50 μ g/ml, Sigma Aldrich® Cat no. A0166-5G) or Kanamycin (50 μ g/ml, Sigma Aldrich® Cat no. A0166-5G). The plates were left overnight at 37°C and Colonies were picked next days and cultured overnight in 10 ml of LB medium containing 100 μ g/ml Ampicillin. Plasmid DNA was extracted from

grown colonies using QIAprep spin miniprep kit© (Qiagene- 27106) as per manufacture's instruction. For Maxi preps 1 colony per plasmid was picked from Agar plate and grown in 200 ml of LB medium and plasmid were purified using Maxi-prep purification kit (NucleoBond® Xtra Max Plus Kit) (ThermoFisher -NZ74041650).

2.1.3 DNA extraction from ear clips of transgenic mice

For genotyping transgenic mice, for the presence of $\beta 1$ -integrin-flox gene or the presence of CReESR genes a PCR reaction must be performed for the gene of interest. DNA was first extracted from ear clips of mice when they reached weaning age (3-weeks). Briefly, ear punches were digested in 50 μ l of Direct PCR Lysis solution (Viagen Biotech) supplemented with 10 μ l/ml of Protease K (NEB -P8107S). The lysis was done at 55°C in a shaking incubator overnight. The lysates were then heated at 85°C for 15 minutes to inactivate the reaction. The tubes containing lysates were then centrifuged at maximum speed. 1 μ l of DNA was used for each PCR reaction.

2.1.4 Polymerase Chain Reaction (PCR)

PCR reaction was done for CreESR or for $\beta 1$ -integrin fxfx genes to check for their presence in the transgenic mice genome. The PCR Mix contained 1 μ l of DNA, 12.5 μ l of 2X MyTaq Master Mix (Bioline – 25041), 1 μ l of 10 μ M forward primer, 1 μ l of 10 μ M reverse primer and the reaction was made up to 25 μ l by adding 9.5 μ l of dH₂O.

The PCR conditions for CreESR and $\beta 1$ fxfx were as follow:

- 95°C for 5 minutes.
 - 95°C for 30 seconds
 - 56°C for 30 seconds
 - 72°C for 1 minute
 - 72°C for 10 minutes
 - 4°C for ever
- } 30X

Genotyping primers sequences were as follow

- CReESR:
 - Forward: 5'-AACCTGGATAGTGAACAGGGGC-3'

- Reverse: 5'-GGAACCGACTTGACGTAGCCAGC-3'

Expected size of PCR product =540bp

• β 1-integrin-fx/fx:

- Forward: GCCGCCACAGCTTTCTGCTGTAGG

- Reverse: CTGATCATTCCAATCCAGGAAACC

Expected product size of wild type allele is 300 bp .

Expected product size for fx/fx allele is 335 bp.

The PCR products were loaded on 2% agarose gel and resolved for 30 minutes at 120V.

Only mice that were homozygous for β 1-integrin fx/fx gene were used in my experiments.

2.1.5 RNA extraction

Cells from organoid cultures were collected by centrifugation at 100g for 5 minutes. The supernatant was then removed and the cells were resuspended in 1 ml peqGOLD Trifast reagent (PEQ LAB- 30-2010) and incubated for 5 minutes at room temperature. After incubation, 200 μ l chloroform was added and tubes were shaken vigorously for 30 seconds. They were then incubated for 3 minutes at room temperature. The suspension was centrifuged at 14000g for 15 minutes, and the top aqueous phase was removed into a fresh 1.5 ml tube. 0.5 ml isopropanol was added to the aqueous layer and the suspension was incubated for 10 minutes at room temperature. This was followed by a centrifugation of the samples at 14000g for 10 minutes. The supernatant was then removed and the pellet was washed with 1ml of 75% ethanol. The supernatant was then removed and the pellet was allowed to dry for 10 minutes. The pellet containing the RNA was then resuspended in 50 μ l of RNase-free water and stored at -80°C. The RNA concentration was determined using Nano-drop NSpectrophotometer ND-1000 from Thermo-scientific.

2.1.6 cDNA synthesis

Complementary DNA (cDNA) was synthesised using the RNA-to-cDNA kit purchased from Applied Biosystems (Product number 4387406) according to the manufacturer's instructions. Briefly, a mix was done in 200 μ l PCR tube. The mix

contained: 10 μ l of 2X Reverse Transcriptase buffer, 1 μ l of Reverse Transcriptase enzyme, 100 ng of RNA, The reaction was made up to a final volume of 20 μ l by adding dH₂O to the mix. Reactions were then placed into a thermal cycler for incubation at 37°C for 60 minutes, followed by an enzyme inactivation at 95°C for 5 minutes. cDNA was stored at -20°C.

2.1.7 Real time quantitative PCR (qPCR)

To measure the mRNA expression of genes of interest, primers were first designed to anneal only to cDNA and not to genomic DNA. To do so, primers were designed to anneal at the junction between two exons and amplify a final product not greater in size than 200bp. The qPCR reaction made up using the following. 1X Fast SYBR Green Master Mix (Applied Biosystems 4472908), 200 nM final of each Forward and Reverse primers, and MilliQ water to make each reaction to 20 μ l. To a 96-well optical qPCR plate, by 2 μ l of each cDNA sample +18 μ l of the reaction master mix was added to each experimental well. The qPCR reaction was performed on a StepOnePlus qPCR instrument using the following protocol. Uracil DNA-glycosylase was activated by incubating at 50°C for 2 minutes, followed by AmpliTaq DNA polymerase activation by incubating at 95°C for 2 minutes. PCR cycles were performed by 40 repeated cycles of DNA denaturation at 95°C for 15 seconds, followed by DNA extension at 60°C for 1 minute.

2.2 Cell biology methods

2.2.1 Isolation of primary cells from virgin mice

8 to 12 week-old wild type female (ICR) mice or β 1-integrin, Rac1, ILK^{fx/fx}:CreTM knock out mice (Itg β 1^{fx/fx}, Cre-ERTm) were culled by asphyxiation with CO₂ and the mammary glands were dissected and chopped into small pieces. The tissue was then enzymatically digested with collagenase/trypsin mix [195 ml of H₂O+ 9.8 mg F10 medium (Sigma), 120 mg NaHCO₃ HEPES-Na (Sigma), 150 mg Trypsin (Gibco 840-7250), 300 mg Collagenase A (Roche), 5 ml FBS (Lonza)] for one hour at 37°C in a shaker. Cells were then spun for 1 minute at 300 rpm. The pellet was re-digested with collagenase/trypsin mix for 30 minutes while the supernatant was spun for 3 minutes at

800 rpm. The pellet was kept on ice and labeled as pellet 1 and the supernatant was spun at 1500 rpm for 10 minutes. Pellet from this wash was saved on ice and called pellet A. After the second digestion was completed, cells were spun at 800 rpm for 3 minutes. The pellet obtained was called pellet 2. The supernatant was spun for 10 minutes at 1500 rpm. Supernatant from this wash was then discarded and pellet was called pellet B. pellet A and B were combined and washed with Ham's F12 (Lonza) media by spinning at 800 rpm for 3 minutes. Pellet was called pellet 3 and the supernatant was discarded. Pellet 1, 2 and 3 were combined together and washed with 15 ml of Ham's F12 by spinning at 800 rpm for 3 minutes. The washing step was repeated 3 times. This method enriched for organoids that contain epithelial cells, while the washing steps removed other types of cells such as fibroblasts, red blood cells and hematopoietic cells.

To culture cells on 2D collagen, plastic plates were coated with collagen I extracted from rat tails at a density of $100 \mu\text{g}/\text{cm}^2$. Collagen plates were incubated at 37°C for 1 hour then washed three times with 1X PBS. For laminin-rich reconstituted basement membrane coating, growth factor-reduced matrigel (EHS) (BD Biosciences) was thawed overnight at 4°C and was spread on plastic dishes at a density of $20 \mu\text{l}/\text{cm}^2$. The EHS-coated dishes were incubated at 37°C for 1 hour.

Collagen and EHS plates were conditioned for 1 hour at 37°C with 2X serum-fetuin mixture (Ham's F12 media supplemented with 20% FBS, 1mg/ml fetuin (Sigma), 200 units/ml penicillin, $200 \mu\text{g}/\text{ml}$ streptomycin, $100 \mu\text{g}/\text{ml}$ gentamicin, $0.5 \mu\text{g}/\text{ml}$ fungizone, $10 \mu\text{g}/\text{ml}$ insulin, $2 \mu\text{g}/\text{ml}$ hydrocortisone, $20 \text{ ng}/\text{ml}$ EGF (Sigman). Cells were resuspended in equal volume in Ham's F12 media and plated on the top of the conditioned plates. Cells were seeded at a density of $2.5 \times 10^5/\text{cm}^2$ on collagen or at $5 \times 10^5/\text{cm}^2$ on EHS plates and were incubated at 37°C with 5% CO_2 . Cells were fed on alternate days with Ham's F12 media supplemented with 10% FBS, 100 units/ml penicillin, $100 \mu\text{g}/\text{ml}$ streptomycin, $50 \mu\text{g}/\text{ml}$ gentamicin, $0.25 \mu\text{g}/\text{ml}$ fungizone, $5 \mu\text{g}/\text{ml}$ insulin, $1 \mu\text{g}/\text{ml}$ hydrocortisone, $10 \text{ ng}/\text{ml}$ EGF). To induce knockout of $\beta 1$ -integrin genes in cells isolated from $\beta 1$ -integrin^{fx/fx}, Cre-ERTm mice, 4hydroxytamoxifen was added at a final concentration of 100 nM to the culture media at time of plating and after the first media change.

2.2.2 Culturing of 293T cell-lines

293T cell-line were cultured in DMEM-F12 media, supplemented with 5% FBS and 1% penicillin/streptomycin. Cells were fed with fresh media every 2 days. To passage 293T cell-line, complete media was removed and cells were washed 2 times with 1XPBS. Trypsin was added to the plate and cells were incubated with at 37°C for 5 minutes till 90% of the cells came of the dish. Media containing FBS was added to stop the effect of trypsin. Cells were then collected in falcon tubes and washed with complete media, then replated at the optimal density.

2.2.3 Lentivirus production

To produce the lentivirus, 293T cells were transfected at a confluence of 50-70% with 6 µg of PLVTHM control vector, 3 µg of psPAX2 and 4.5 µg of PMDG.2 plasmids using 1XPEI transfection reagent. 6 hours following transfection the media was changed into complete media and the cells were left overnight. Next day the cells were treated for 6 hours with sodium butyrate and then changed into fresh complete media. 24 hours post sodium butyrate addition; the supernatant was collected and centrifuged at a speed of 21,000Xg for 4 hours. The pellet was resuspended in 500 µl of blank media.

2.2.4 Lentivirus infection

Primary MECs were transduced with one vial of virus in 6-well plates under low attachment conditions in organoid forming media containing 1µg/ml polybrene. Next day, the media was changed and another infection was performed. Next day, the media was changed again and the cells were left for additional 48 hours before being sorted for GFP expression.

2.2.5 FACS staining and sorting

Organoids obtained from primary preps were spun at 1500 g for 5 minutes and supernatant was discarded. To obtain single cells from organoids, 2 ml of trypsin-Versene (Lonza) was added to the pellet and incubated at 37°C for 2 minutes. Pellet was broken up with rapid pipetting. DNase (New England BioLabs) was then added in a

final concentration of 1 μ g/ml to the pellet and incubated at 37°C for 5 minutes. Complete media was then added to stop the action of trypsin. Cells were washed with complete media and spun down at 1500 rpm for 5 minutes, then strained through a 0.45 μ m cell strainer to obtain single cells. Cells were washed with 1X PBS and resuspended in 400 μ l sorting buffer (2.5% FBS in PBS). To stain cells, 3 μ l of each directly labeled antibody was added per 10 million cells and incubated on ice for 1 hour. Cells were then washed with sorting buffer and resuspended in 400 μ l of sorting buffer.

Cells were sorted using BD FACS Aria (Beckton Dickinson) at low pressure using 488, 685 and UV laser. Antibodies used for sorting experiments are; EPCAM- APC (e-bioscience 175791), CD24-APC (e-bioscience 170242), β 1-integrin-efluor 450 (e-bioscience 48-0291), α 6-integrin- efluor 450 (e-bioscience 48-0495) and β 3-integrin-488 (biolegend 104311).

2.2.6 Mammosphere assay

24-well plates were coated with 1.2% polyhema to prevent attachment of epithelial cells. Plates were left to dry for 1 hour at room temperature and then washed with 1X PBS. Primary cells were seeded at a density of 2000 cells/cm² in MEBM media (Lonza) supplemented with 1X B27 (invitrogen), 20ng/ μ l EGF, 5 μ g/ml insulin, 0.5 μ g/ml hydrocortisone (sigma). Mammospheres obtained from primary cells were left for 7 days then counted. To generate mammospheres from EPH4 cell-lines, single cells were seeded at 5000cells/cm² and mammospheres were counted at day 4. Mammospheres from primary and cell-lines were counted at a 10X magnification.

2.2.7 Organoid forming assay

Cells were grown at a clonal density of 2X10³/ cm² in 24-well plates. The plates were coated with 1.2% polyHEMA to prevent adhesion and growth of the primary MECs on plastic. The cells were grown media containing (EPiCULT-B media (stem cell technologies) supplemented with 5% Matrigel, 5% FBS, 10ng/ml EGF, 20ng/ml bFGF, 4 mg/ml heparin, and 10 μ M Y-27632). Cells were left for 10 days to form organoids then counted at 4X magnification.

3. Statistical analysis:

Statistical analysis was done using Microsoft Excel or GraphPad PRISM Data Analysis software. Statistical significance was determined by Student's t test for paired samples when comparing two groups. One-way ANOVA statistical test was used when comparing more than two groups. Differences between samples were considered to be significantly different when $p = <0.05$. For all graphs shown, error bars represent \pm standard error of the mean. For two groups, the means may have 1-4 asterisks centred over the error bar to indicate the relative level of the p-value. In general, "*" means $p < 0.05$, "**" means $p < 0.01$, "***" means $p < 0.001$, and "****" means $p < 0.0001$. The p value will also be stated in the figure legend.

Chapter 3-Results: Finding suitable methods for quantifying stem cells and luminal progenitor frequency in mouse mammary gland

3.1 Introduction

The lack of specific stem and progenitor cell markers in the mammary gland made it difficult to quantify stem cell frequency within a given population. The gold-standard method for determining the number of mammary stem cells in a specific cell population is through transplantation assays in the clear mammary fat pads of immune-compromised mice (NOD-SCID) or in mice from the same inbred colonies. Those mice will have to be surgically “cleared” of their endogenous epithelium. A single stem cell is able to generate a complete mammary gland. The mammary fat pad transplantation method involves the removal of endogenous mammary epithelium when the mice are between 3-4 weeks old. At this stage the endogenous mammary epithelium is not developed and can be removed by excising the area between the lymph node and the nipple. Experimental cells can then be injected into the “cleared” fat pad, and tissue is subsequently examined after the mouse undergoes puberty and pregnancy. The percentage of cells engrafted (cells containing stem cell properties) can be determined by injecting a variety of cell dilutions into different groups of mice. Statistical tests used to determine the significance in the differences between the control and the test groups include the Extreme Limiting Dilution Analysis <http://bioinf.wehi.edu.au/software/elda/>. A cell that has the ability to generate a functional gland in a transplantation experiment is referred to as an MRU (Eirew et al., 2008; Ginestier et al., 2007; Kuperwasser et al., 2004). (Figure 3.1)

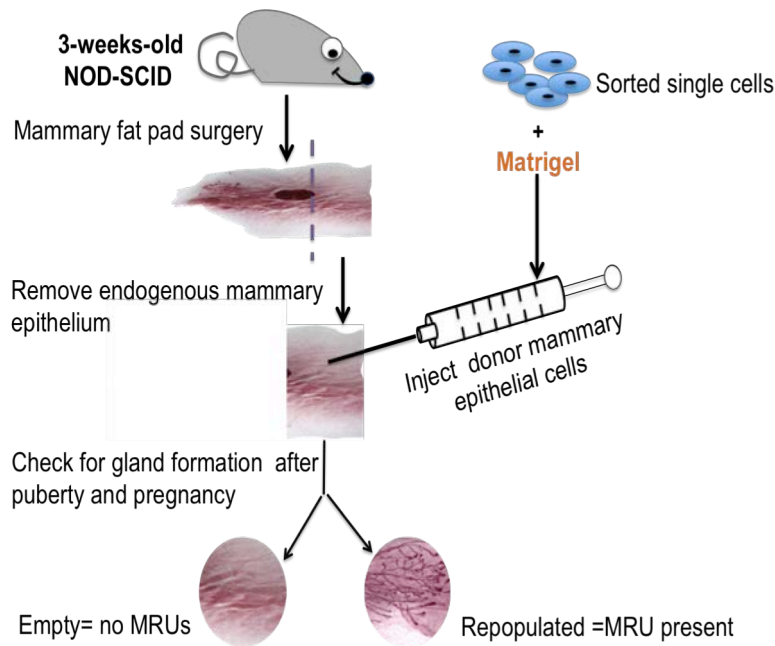


Figure 3. 1 Steps for testing the presence and frequency of stem cells in a given mammary epithelial population.

The cells are isolated from donor mice then injected into a cleared mammary fat pad of a 3 week-old recipient immune-compromised mouse that had undergone mammary fat pad clearing surgery. Mice are then allowed to reach puberty and pregnancy and the mammary fat pad is then stained and tested for the presence of newly developed mammary glands originated from the donor cells (Kuperwasser et al., 2004). The frequency of MRUs can be determined by injecting different dilutions of donor cells into different groups of recipient mice. A single MRU has the ability to repopulate the fat pad with fully

Although in-vivo assays are highly recommended to measure stem cell frequency, they are not cost and time efficient. Alternative in-vitro assays were therefore developed to measure stem and progenitor frequency. Studies that aim to compare prevalence of stem cells between different populations in-vitro use functional characteristics of stem cells such as resistance to apoptosis, clonal proliferation under anchorage-independent condition and ability to differentiate into luminal and myoepithelial lineage (Dontu et al., 2003; Smalley et al., 2012; Stingl, 2009). Those cultivation systems are used for the propagation of mouse mammary stem and progenitor cells. This allows the study of pathways involved in stem/progenitor cells self-renewal and differentiation (Liu et al., 2006). However, those methods are not fully standardised in the literature and different groups use different methods for quantifying the percentage of stem cells in a specific population. It was therefore important for my project to characterise and establish the best method for measuring stem cell frequency in-vitro. To achieve that, different published methods were compared (Dontu et al., 2003; Guo et al., 2012) and the best

method for studying integrin signaling in stem cells was chosen. The best approach to genetically manipulate stem cells and luminal progenitors using lentivirus as gene delivery vectors was then optimised. The results in this chapter therefore provided me with the appropriate tools for studying integrin signaling in mammary stem cells.

3.2 Results

3.2.1. Separation of mammary gland epithelial cells using flow cytometry

This project is based on understanding how adhesion receptors, used for sorting stem cells from different mammary epithelial populations, contribute their stem cell behavior. Those adhesion receptors such $\beta 1$ and $\alpha 6$ - integrin are present in other differentiated population such as myoepithelial and luminal populations, but at different expression levels. From previous studies, sorting mammary epithelial cells requires the use of at least 3 different markers simultaneously. The best method for such a separation is FACS sorting, as it will give an accurate measurement of protein expression level relative to a control sample (Shackleton et al., 2006; Smalley et al., 2012; Stingl et al., 2006a). It is therefore important to find the best FACS sorting conditions and antibody colour combinations that enable a clear separation of the different populations within the mammary gland.

To optimise the best sorting conditions, mammary glands from nulliparous female mice (age 8-12 weeks) were enzymatically dissociated into single cells. Fibroblasts and epithelial cells were isolated from contaminating lymphocytes and endothelial cells by negative sorting of CD45, CD31 -PEcy7 marker (Figure 3.2).

To obtain a good separation of different mammary epithelial cell populations, Allophycocyanin (APC) (650nm/660nm) colour was used in combination with e-Fluor 450 (407nm/450nm). The reason for using those two colours is that they have distant excitation/emission peaks; therefore less laser compensation is required for flow cytometry sorting. Cells were successfully sorted using combinations of different markers, these include CD24 with $\alpha 6$ -integrin and EPCAM with $\alpha 6$ -integrin. The best separation of epithelial population was obtained from the sort of $\alpha 6$ -integrin (eFluor 450) with EPCAM (APC). From these combinations, 3 different populations were

resolved. These include luminal, basal and fibroblasts (Figure 3.2). In order to make sure that the separation of the different population is pure, sorted cells were cyto-spun on slides and staining for basal and luminal cytokeratin markers were done. These markers included CK5 for Basal and CK8/18 for luminal cells respectively. The staining showed that the protocol for FACS separation resulted in pure luminal and basal populations (Figure 3.3).

The α 2-integrin conjugated with R-phycoerythrin (PE) (496/578) was also added to the combination of antibodies. This was done in order to distinguish the luminal progenitor cells, which express high levels of α 2-integrin, from the differentiated luminal cells which express low levels of α 2-integrin (Shehata et al., 2012) (Figure 3.2). The FACS analysis and sorting technique seemed to be very accurate. Therefore, the FACS sorting technique was used in order to separate and measure stem cell frequency after knocking down integrins and their downstream targets.

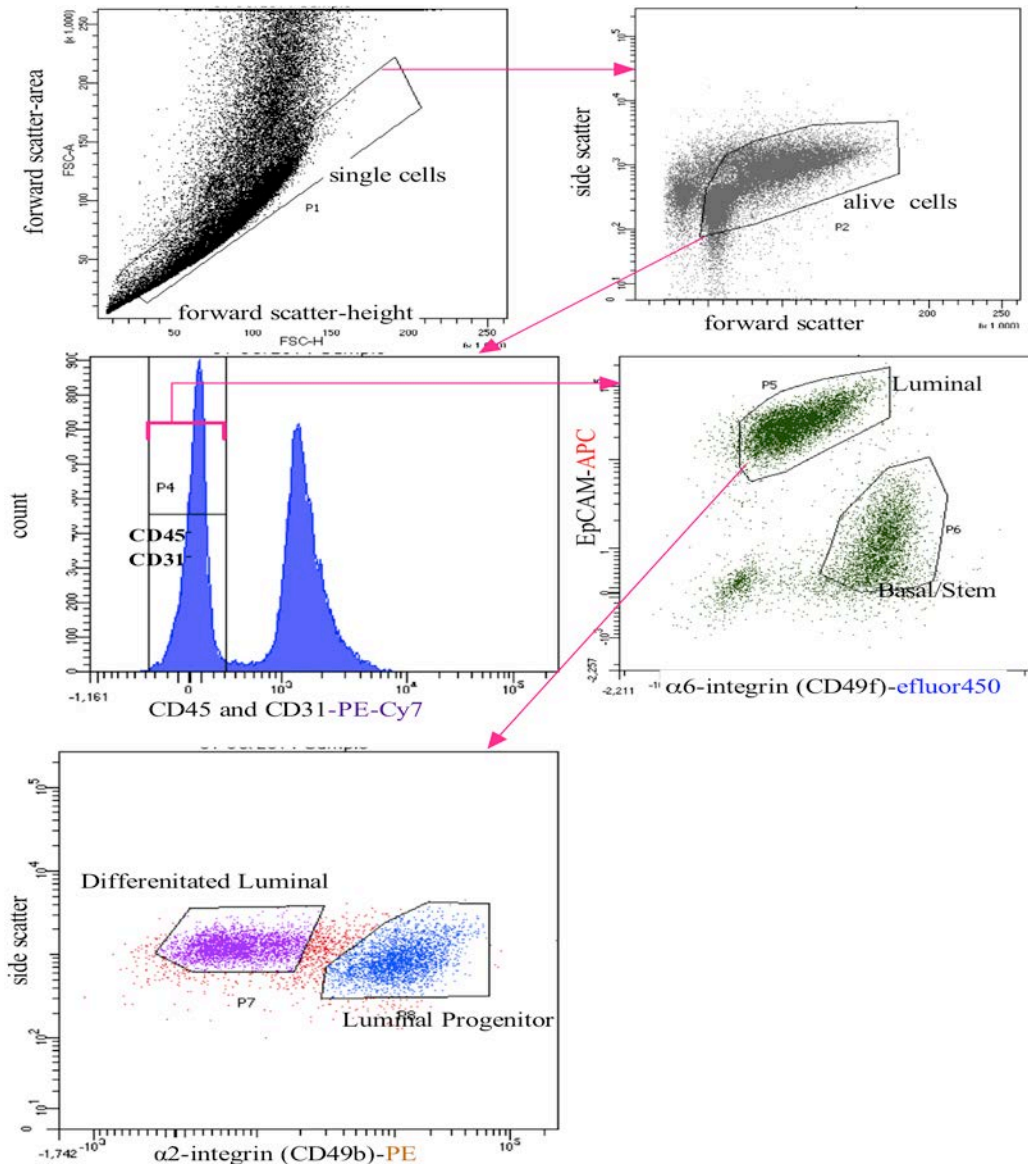


Figure 3.2 Sorting of luminal and basal population from mammary epithelial cells. Primary MECs were enzymatically isolated from nulliparous adult female mice. MECs were made into single cells using trypsin and were passed through a 40-micron cell strainer. They were then washed with blank HamsF12 media and incubated with primary sorting antibodies. $1 \mu\text{l} / 5 \times 10^6$ cells of each antibody was used for staining. The staining was done in $500 \mu\text{l}$ FACS buffer (2%FBS in PBS). Sorting antibodies included CD31-PE-Cy71 (e-bioscience-170242), CD45-PE-Cy7 (e-bioscience25-0451-81), EpCAM-APC (e-bioscience 175791), $\alpha 6$ -integrin-fluor450 (ebioscience-170242) and $\alpha 2$ -integrinPE (e-bioscience 12-0495). Cells were incubated for 1 hour on ice, washed, and sorted using BD FACSaria™ III cell sorting machine at low pressure. Gating was done on single viable cells using forward and side scatters. Cells were then negatively sorted for CD45 and CD31 markers to remove lymphocytes and endothelial cells (P4). The P4 population contained epithelial cells and fibroblasts. Epithelial cells were further fractionated into Basal and luminal cells. Basal/ stem cells were in the $\alpha 6$ -integrin^{high}, EPCAM^{low-medium} population (P6). Luminal cells were in the $\alpha 6$ -integrin^{low}, EPCAM^{high} (P5). The luminal cells were further divided into $\alpha 2$ -integrin^{high} (P8) population that contained luminal progenitors, and $\alpha 2$ -integrin^{low} (P7) that contained differentiated luminal. Cells were then collected in blank Hams12 media for further experiments.

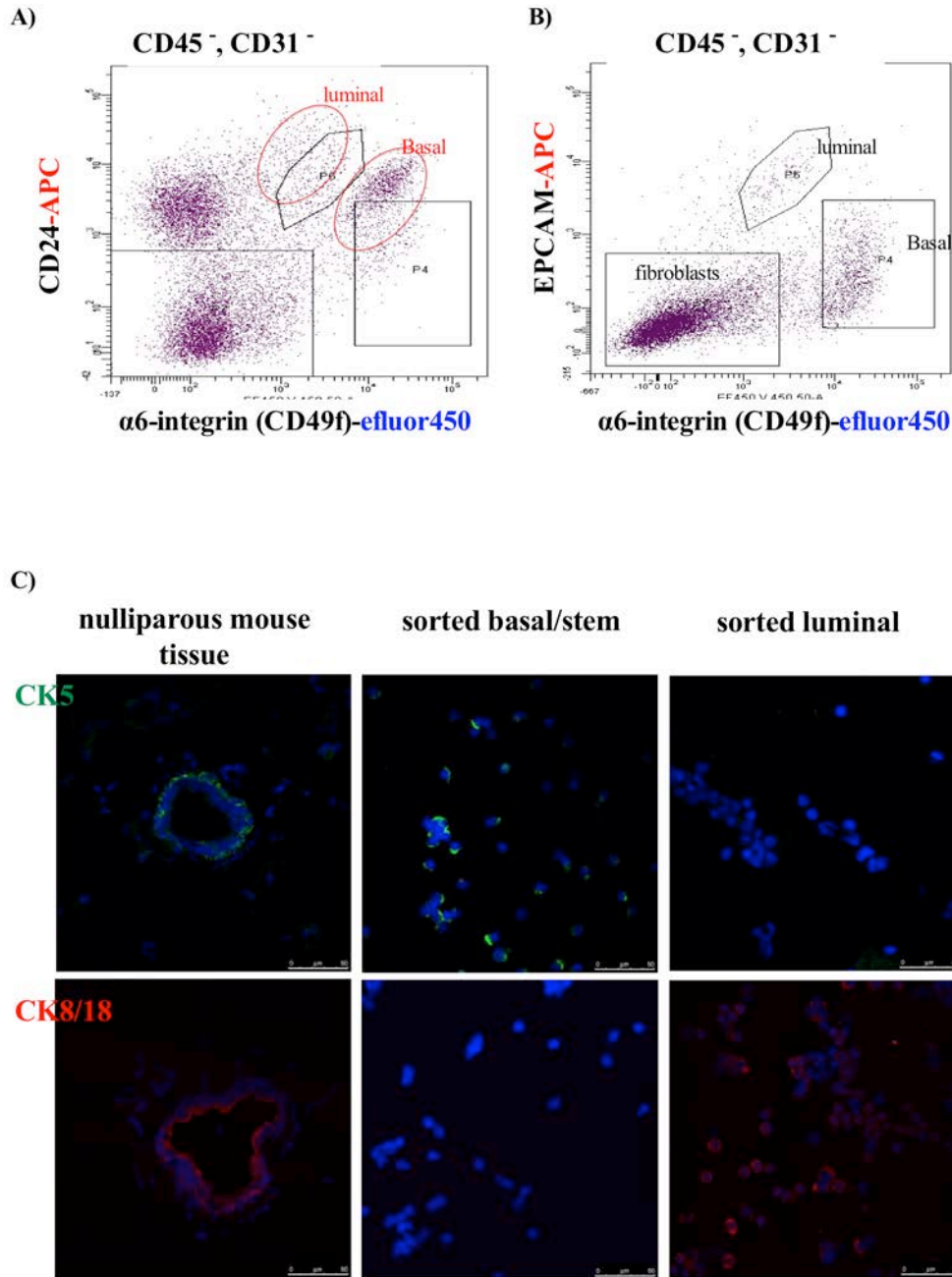


Figure 3.3: Combining EPCAM and $\alpha 6$ -integrin for sorting results in a better separation of basal/ luminal populations compared to the CD24/ $\alpha 6$ -integrin combination. A) Sorting epithelial cells that are $CD45^{-}, CD31^{-}$ with CD24-APC and (e-bioscience-170242) $\alpha 6$ -integrin-efluor450 B) sorting epithelial cells that are $CD45^{-}, CD31^{-}$ using EPCAM-APC and $\alpha 6$ -integrin-fluor 450 (ebioscience-170242). This sorting shows clear separation of basal and luminal cells. C) Cells were collected from sorted luminal and basal populations that were separated using EPCAM and $\alpha 6$ -integrin combination. They were then fixed using 4% Formaldehyde and cyto-spun on glass slides. The cells were stained for luminal marker CK8/18 (PROGEN-GP1) and basal marker CK5 (Abcam-ab53121). DAPI was used to counter stain nucleus. Nulliparous mouse mammary tissue was stained in parallel to insure specificity of the antibody.

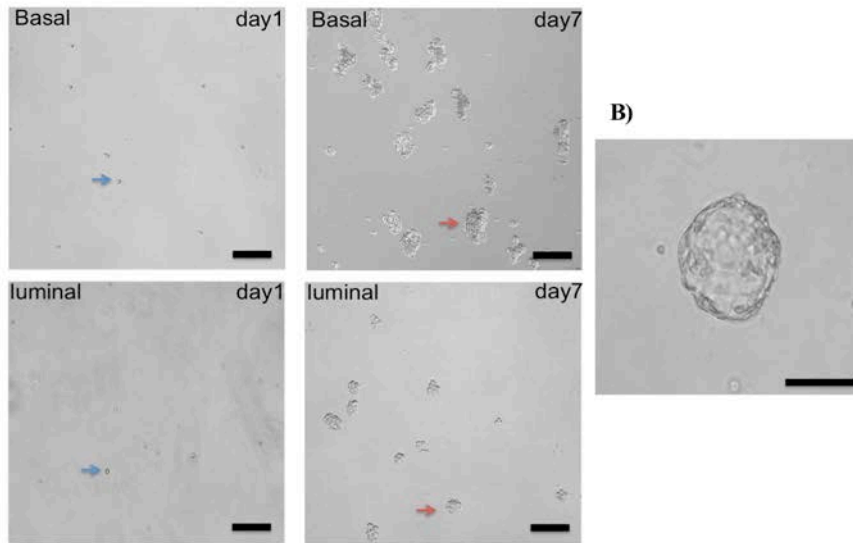
3.2.2 The mammosphere assay is not ideal for measuring stem cell frequency in primary mouse MECs.

The mammosphere stem cell-enrichment system is based on the fact that only stem/progenitor cells possess the ability to proliferate in serum-free suspensions under ultra-low attachment conditions, and thereby, form non-adherent spheres called mammospheres. The majority of differentiated MECs will undergo apoptosis under these conditions except for those that have stem cell property can propagate. Therefore, mammospheres were shown to be enriched with early stem/progenitor cells that were able to differentiate along all three mammary epithelial lineages as well as to form clonally generated complex functional structures in reconstituted 3D culture systems (Dontu et al., 2003). Mammosphere formation efficiency was used by some groups to measure stem/progenitor cell frequency from human mammary stem cells and from tumor cells of human origin (Dontu et al., 2003). However, the use of this assay to measure stem cell frequency from normal mouse mammary gland is controversial in the literature. Some groups use this assay for cells derived from nulliparous mouse MECs (Dong et al., 2013), while others criticise the use of this assay for mouse cells. The latter argument being that mouse MECs tend to clump together in mammospheres culture conditions (Deleyrolle et al., 2008; Louis et al., 2008; Stingl, 2009).

I tested the mammosphere assay on primary MECs. It is known from in-vivo studies that the majority of cells with regenerative capacity in the mammary gland are within the basal population (Stingl, 2009). I wanted to test whether results from the mammosphere assay will provide a similar conclusion on stem/progenitor cell frequency in basal and luminal populations. This will make the mammosphere assay a reliable quantitative method to test for the stem/progenitor cell frequency within the virgin mouse mammary gland. To test for the frequency of cells with regenerative capacity in luminal and basal populations, single sorted cells were cultured in 6-well plate that were coated with 1.2% polyHEMA to make the plate non-adhesive and prevent cell-attachment. The cells were cultured in serum-free mammosphere media which was made of: MEBM basal media supplemented with: 1X B27, 20ng/ml EGF, 5µg/ml Insulin, 0.5 µg/ml Hydrocortisone and 50 µg/ml Gentamicin. Cells were cultured at clonal density (2000/cm²) and allowed to form mammospheres for 7-10

days. Mammospheres formed were counted under 4X magnification to determine the stem cell frequency. In my studies, less than 1% of the cells were able to survive and form mammospheres after 7 days. The basal population contained higher number of cells with sphere forming ability and formed larger mammospheres than the luminal population (Figure 3.4). These results provide further evidence that the majority of stem cells reside within the basal population and are in direct contact with the ECM proteins; thereby they express high levels of integrins.

A)



C)

Mammosphere Forming Efficiency in Sorted Cells

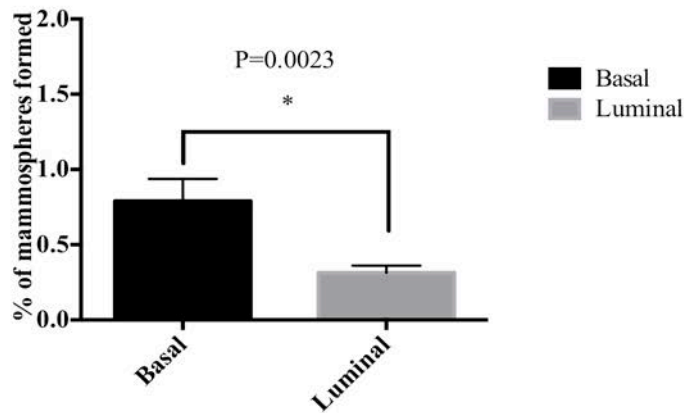


Figure 3.4 Mammosphere forming assay in luminal and basal populations. A) Single cells were seeded from sorted luminal and basal population (from MECs isolated from ICR mice) under low attachment condition in mammosphere media at a density of 2×10^3 cells/cm² in a 6-well plate. Cells were left for 7 days to form mammospheres. Blue arrows are pointing to single cells, red arrows are pointing to mammospheres. Images were taken at 10X magnification. Scale bar = 1mm. B) Enlarged image of a single mammosphere. Image was taken at 20X magnification. Scale bar = 1mm. C) Percentage of mammospheres formed from sorted basal and luminal populations, n=3. Error bars are representative of +/- standard error of the mean. Student t-test was used to determine the statistical significance.

Although mammospheres were successfully obtained from sorted mouse cells, I found however, that the mammosphere assay have major limitations. These limitations might cause inconsistency with my results. First, it is known from the literature that mammospheres can be passaged and re-plated to form secondary mammospheres at a greater efficiency (Dong et al., 2013; Lee et al., 2009a). I managed to dissociate primary mammospheres into single cells by trypsinizing and then passing the cells through a 40- μm strainer. However, when re-plating those single cells in mammosphere media, they were not able to form secondary mammospheres. Obtaining secondary mammospheres is essential to prove that those spheres originated from single stem cells with regenerative capacity.

Another limitation was that mouse MECs clumped together when isolated from mice and plated directly into mammosphere assay without FACS sorting (Figure 3.5). It was therefore important to check the percentage of mammospheres that are forming from cell clumping and the percentage of mammospheres that are originating from single cells in primary MECs that were not sorted. To do so, a mouse line that expresses GFP protein in all its cells was used. MECs were extracted from these mice and a mixing mammosphere experiment was performed, where cells from GFP mice were mixed at a 1:1 ratio with cells obtained from wild type ICR mice. The cells were then plated in an ultra-low attachment at a density of $2 \times 10^3 / \text{cm}^2$ and allowed to form mammospheres between 7 to 10 days. The mammospheres were then collected by low-speed centrifugation (800 rpm), fixed, stained with DAPI, and counted under a fluorescent microscope. Around 70% of the mammospheres were pure WT or pure GFP mammospheres. 30% of the mammospheres had mixed WT and GFP cells in them (Figure 3.5). This indicates that some of the mammospheres formed could be originating from cells clumping or mammosphere fusion rather than originating from single cells.

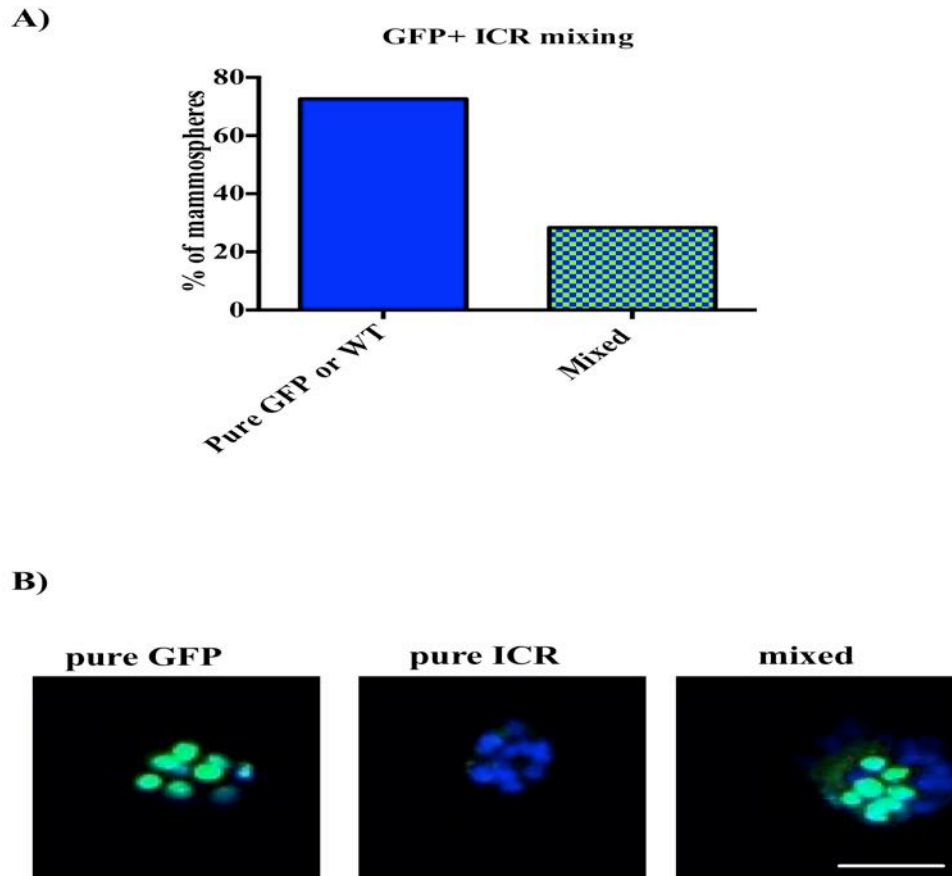


Figure 3.5: GFP and ICR mammosphere mixing experiment. Primary MECs were enzymatically dissociated from the mammary gland of GFP-expressing mice and wild type ICR mice. They were then strained through a 40-micron filter in order to obtain single cells. The cells were mixed at 1:1 of GFP:ICR ratio and plated in mammosphere media at a density of 2000/cm² in 24-well low attachment plates. The cells were allowed to grow and form mammospheres for 7-10 days. The mammospheres were then collected by gentle centrifugation, fixed, and stained with DAPI to distinguish ICR mice from GFP. Total number of mammospheres was counted under 4X magnification. A) Number of pure spheres (i.e. contain either ICR or GFP cells) and number of mixed spheres (i.e. contain cells of both ICR and GFP) were counted and divided by the total number of spheres formed and multiplied by 100 (to obtain a percentage of each group). n= 1 B) fluorescent image showing mammospheres formed from pure ICR, pure GFP and mixed ICR/GFP spheres. Nucleuses were counterstained with DAPI. Scale bar=1mm .

Another limitation with the mammosphere assay was that it was not possible to distinguish those mammospheres that originated from bi-potent stem cells from those originated from luminal- restricted progenitors. As I am anticipating that integrin signaling might be involved in different stem and progenitor pathways, it would be more useful to use an assay that can distinguish those two stem cell types from one another. This will help in performing further experiments in order to dissect β 1-integrin pathway and obtain mechanistic data.

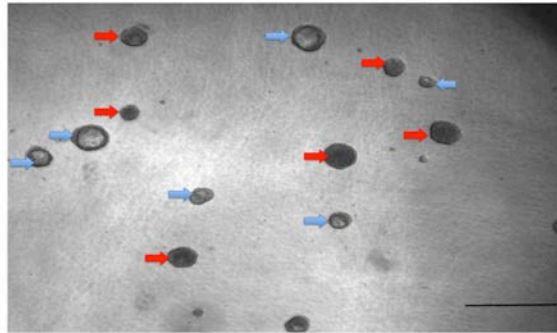
3.2.3 mammary bi-lineage cells and luminal progenitors produce 3D organoids in matrigel

Mammary organoid- forming cells refer to those stem cells/progenitors that produce discrete 3D colonies when plated in low concentration matrigel at clonal density (i.e. $2 \times 10^3 / \text{cm}^2$) (Guo et al., 2012). Similar to the mammosphere assay, the plates must be coated with 1.2% polyHEMA to prevent adhesion and growth of the primary MECs on plastic. There is a correlation between the organoid forming and the mammary repopulating capability (Guo et al., 2012). The protocol for this involves growing cells in media containing (EPiCULT-B media supplemented with 5% Matrigel, 5% FBS, 10ng/mlEGF, 20ng/ml bFGF, 4 mg/ml heparin, and 10 μ M Y-27632). This media is similar to the mammosphere media but with addition of serum, matrigel and ROCK inhibitor. It is known that adding matrigel increases organoid-forming activity. Also the addition of ROCK inhibitor increases mammary organoid-forming activity in-vitro (Guo et al., 2012; Prater et al., 2014; Sato et al., 2009; Watanabe et al., 2007).

Before using this assay in any of my experiments, I first wanted to see whether I can use the published protocol in (Guo et al., 2012) or if it will require modifications. Therefore, the ability of MECs obtained from adult ICR wild type mice to produce organoids was tested. Single cells were obtained from primary preps by treating them with trypsin and straining them through a 40-micron cell strainer. Cells were then seeded in organoid media at a density of $2 \times 10^3 / \text{cm}^2$ in low attachment 24-well plates and left for 7-10 days to form organoids. The structures were then counted under phase contrast microscope at 4X magnification. The number of organoids produced from an organoid-forming assay (OFA) is an indication of the number of cells that have bi-lineage differentiation potential properties, a property of stem cells (Guo et al., 2012). I managed to produce

both bi-lineage and luminal progenitor organoids (Figure 3.6). Organoids produced from bi-lineage cells can be distinguished from those produced from luminal progenitors by their morphology. Those that originate from bi-lineage cells will produce solid (filled) organoids while those produced from luminal progenitors will be hollow organoid (Guo et al., 2012).

A)



B)

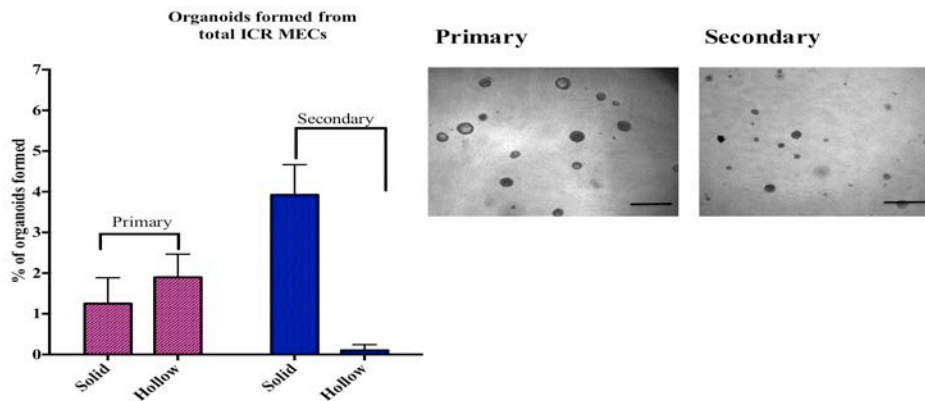


Figure 3.6 bi-lineage and luminal progenitors form solid and hollow structures in organoid forming assays. Primary MECs were enzymatically dissociated from ICR mice using collagenase. They were treated with trypsin and dissociated into single cells. The cells were strained through a 40-micron cell strainer to obtain single cells. They were then cultured in low attachment 24-well plates at a density of $2 \times 10^3/\text{cm}^2$. The cells were cultured in mouse EpiCULT-B media containing: 5% Matrigel, 5% FBS, 10ng/ml EGF, 20ng/ml bFGF, 4 mg/ml heparin, and 10 μM Y-27632 (Rock inhibitor). Cells were left for 7-10 days to form organoids. Secondary organoids were obtained from trypsinising primary organoids at day7. To trypsinise organoids, they were first collected by centrifugation at 800 rpm for 3 minutes. Organoids were then washed by 1 ml of PBS containing 5M EDTA. 1 ml of primary trypsin was added to the organoids and gentle up and down pipetting was applied in order to dissociate organoids into single cells. Cells were then passed through a 40-micron strainer and re-seeded in organoid assay to form secondary organoids. A) Phase contrast microscope image of organoids was taken on day 7 of the assay using 4X magnifications. Red arrows are pointing at solid organoids that originated from stem cells. Blue arrows are pointing at hollow organoids that originated from luminal progenitors. Scale bar= 1mm. B) Quantification of percentage of primary and secondary organoids formed from wild type ICR mice. The number of solid and hollow organoids was counted for primary and secondary organoids and divided by the original number of seeded cells. Left is bar graph representing these percentages. Right, phase contrast images taken at 4X

In order to check whether the organoids obtained came from a clonal origin, a label retention experiment was performed. Primary MECs were labeled with a lipid dye known as PKH26 (Li et al., 2013) . This dye is fluorescent with an excitation/emission peak of 551/567. PKH26 binds to the phospholipids on the cell membrane of living cells and can be used as a cell tracker (Ude et al., 2012). Each time the cell undergoes a division the dye becomes more diluted. Stem cells are known to be slow dividing cells and therefore are able to retain the PKH26 label (Tosoni et al., 2012). It was therefore expected that the solid organoids would have at least one cell that retains the PKH26 label. This will be extra evidence that those organoids originated from single cells rather than cells clumping together.

To label cells, primary cells were isolated from nulliparous mice and strained through a 40- μm filter to obtain single cells. 10^7 cells were then washed 3 times with blank media and then pulsed for 1 minute with $2 \times 10^{-6}\text{M}$ of PKH26 in 1ml of blank media. Adding 1 ml of serum stopped the reaction, and cells were washed 3 times with serum-free media. The cells were then counted again (to check for viability as the dye might be toxic) and $4 \times 10^3/\text{cm}^2$ cell were plated in organoid media in a 24-well plate. The percentage of cells that were successfully stained with the dye was checked and 100% of the cells were found to have taken up the dye. This was shown by FACS analysis and by looking under fluorescent microscope (Figure 3.7 A). The cells were then left for 7-10 days to form organoids. Stem organoids contained one or two cells with label retention while the other cells in the organoid were either weakly stained or negative (Figure 3.7 B). This provided evidence that those organoids contained a slow-dividing cell with label retention property, a characteristic of stem cells.

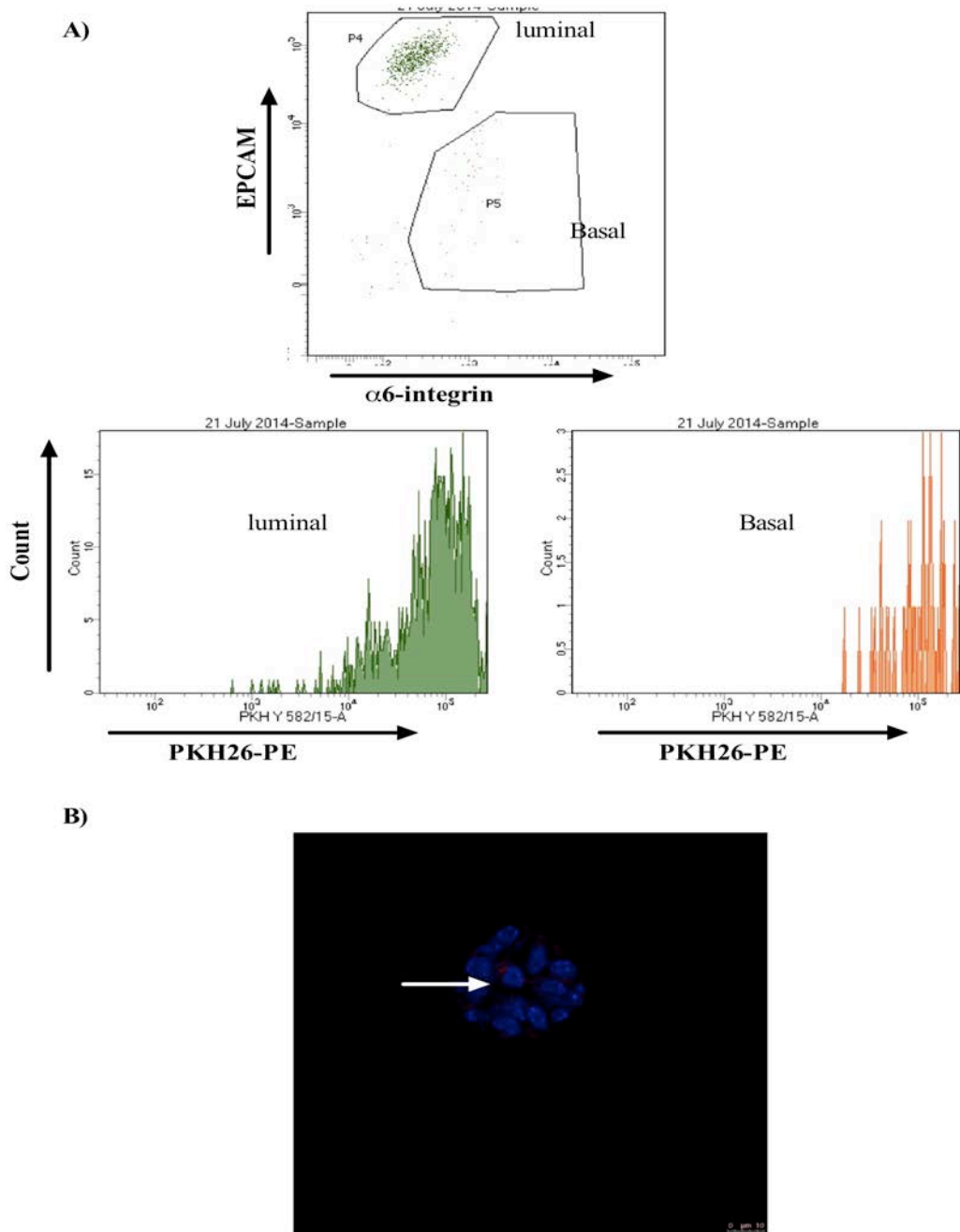


Figure 3.7 Solid organoids contain a slow dividing label-retaining cell. Primary cells were isolated from nulliparous mice and strained through a 40-micron filter to obtain single cells. 10^7 cells were counted and washed 3 times with blank media and then pulsed for 1 minute with 2×10^{-6} M of PKH26 in 1ml of blank media. Adding 1 ml of serum stopped the reaction and cells were then washed 3 times with serum-free media. 4×10^3 /cm² cell then were plated in organoid media in a 24-well plate. A) Flow cytometry analysis of cells after staining with PKH26 was done in association with $\alpha 6$ - integrin-fluor 450 and EPCAM- APC markers to determine the percentage of basal and luminal cells stained. Samples were analysed using BD FACSARIA™ III cell sorting machine. The cells were then left for 7-10 days to form organoids. B) Fluorescent image of a stem organoids. White arrow pointing to cells with label retention property stained in red, image was taken at 60X magnification.

I then sorted basal /stem cells (CD45/CD31^{neg}, EpCAM^{low}, and α 6-integrin^{high}), luminal progenitors (CD45/CD31^{neg}, EpCAM^{low}, α 6-integrin^{low}, and α 2-integrin^{high}), and differentiated luminal (CD45/CD31^{neg}, EpCAM^{low}, α 6-integrin^{low}, and α 2-integrin^{low}) cells. As expected, basal cells formed filled mainly organoids, with a percentage of 3-4 % efficiency (Figure 3.8). Less than 1% of the basal cells formed hollow organoids. Sorted luminal progenitors formed mainly hollow organoids with a high percentage of 10-12 % efficiency. The differentiated luminal population did not form any organoid.

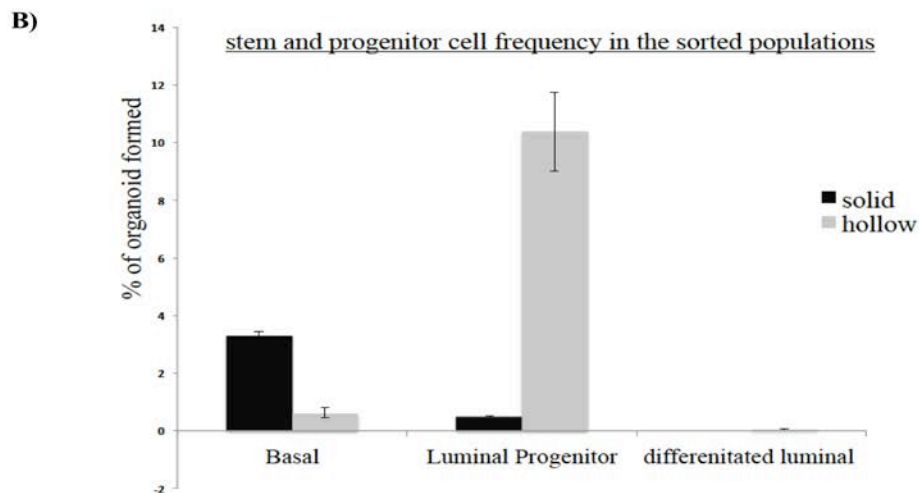
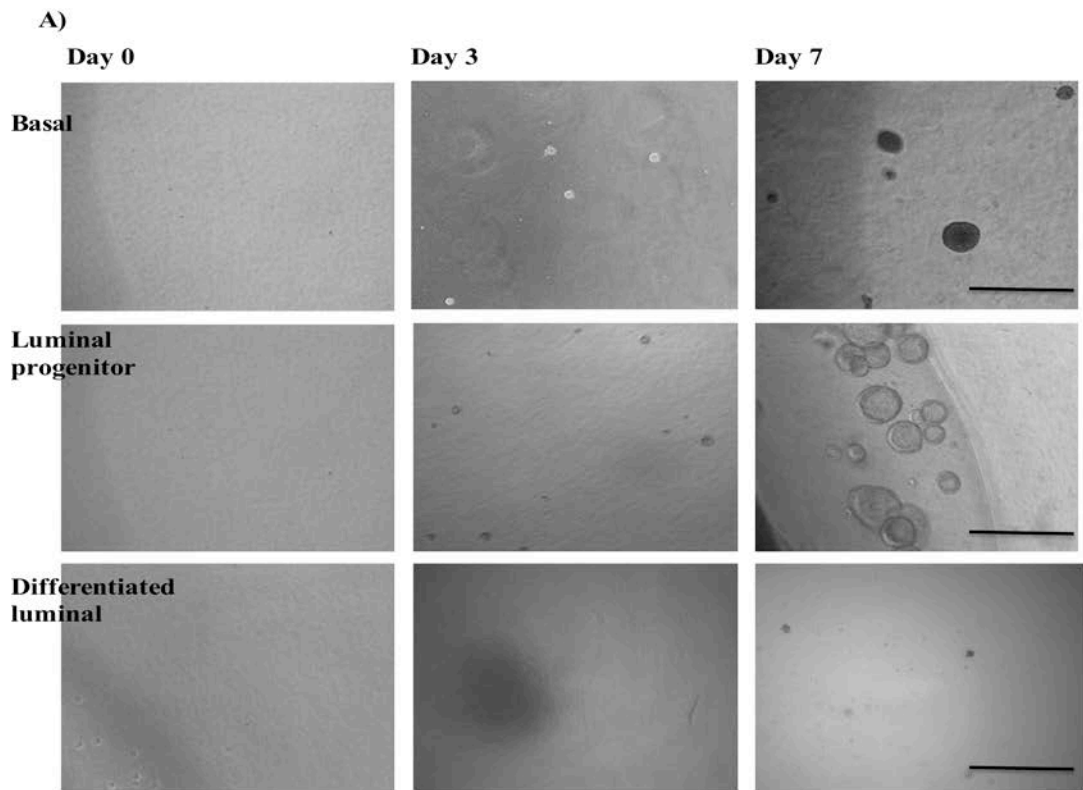


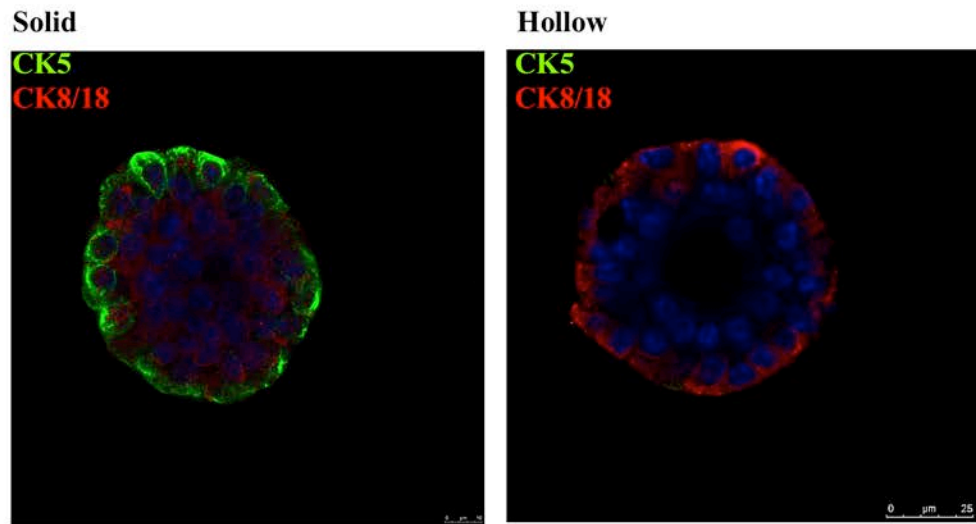
Figure 3.8 Sorted basal and luminal cells produced solid and hollow organoids respectively. Primary cells were isolated from nulliparous mice and passed through a 40-micron filter to obtain single cells. They were then sorted for basal /stem cells (CD45/CD31^{neg}, EpCAM^{low}, and α 6-integrin^{high}), luminal progenitors (CD45/CD31^{neg}, EpCAM^{low}, α 6-integrin^{low}, and α 2-integrin^{high}), and differentiated luminal (CD45/CD31^{neg}, EpCAM^{low}, α 6-integrin^{low}, and α 2-integrin^{low}) cells. The cells were seeded in organoid forming assay at a density of 2×10^3 /cm² A) phase contrast images (taken at 4X magnification) of days 0,3 and 7 of organoids formed from basal/stem, luminal progenitor, and differentiated luminal populations. Scale bar=1mm. B) quantification of solid and hollow organoids formed from basal, luminal progenitor and differentiated luminal cells at day 7-10.

In order to prove that solid organoids came from a bi-lineage cell that can differentiate into luminal and myoepithelial cells, lineage-specific markers: CK8/18 for luminal and CK5 for myoepithelial (Stingl, 2009; Stingl et al., 2005) were used in order to stain these organoids and identify the lineages of the cells produced in solid and hollow organoids. The dual staining of CK5 and CK8/18 staining showed that solid organoids were bi-layered: they contained luminal cells in the middle and myoepithelial cells were at the periphery (surrounding the luminal cells). The hollow colonies were made of only luminal cell, indicating that they came from a uni-potent progenitor (Figure 3.9).

Organoids coming from basal/stem cultures and from luminal progenitors were dissociated into single cells using trypsin. Those organoids were stained for α 6- integrin and EPCAM using FACS antibodies. The results showed that solid organoids (originated from the basal population) repopulated both basal and luminal cells. However, the cells obtained from the hollow organoid cultures contained only luminal cells (Figure 3.9).

These results confirms that bi-lineage cells were mainly in the basal population and that luminal progenitors can be distinguished from differentiated luminal cells based on their high expression α 2-integrin. These results were similar to the findings obtained in (Shehata et al., 2012).

A)



B)

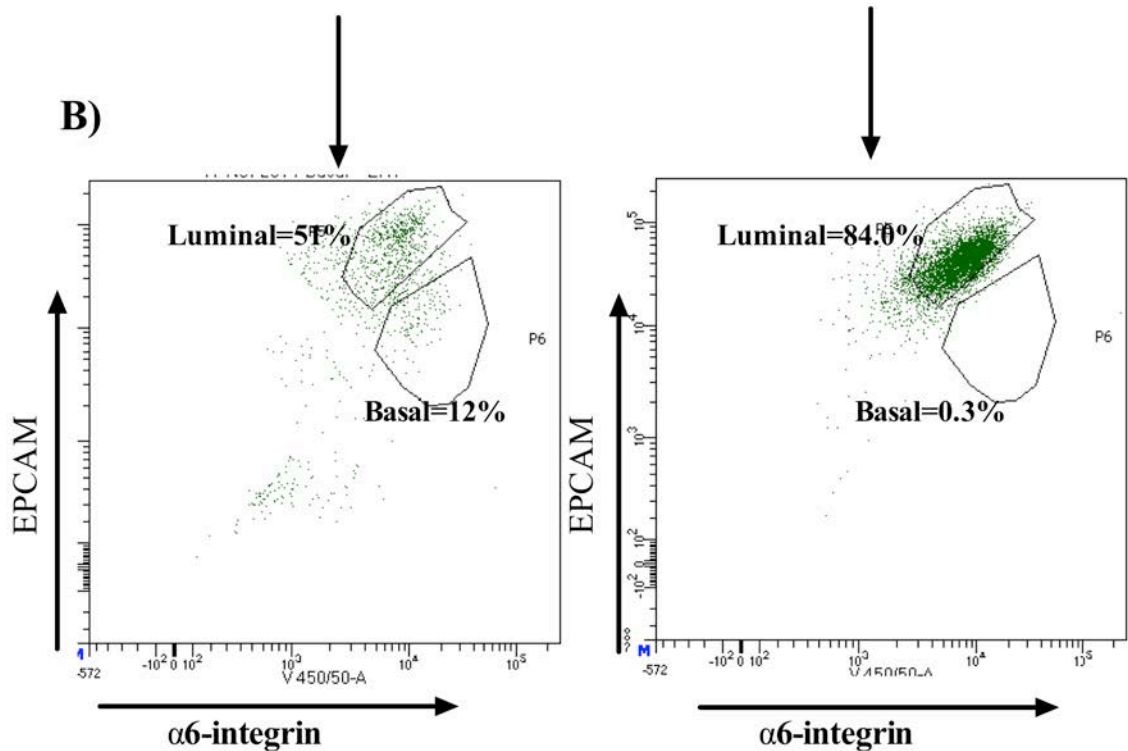


Figure 3.9 Analysis of cell types in solid and hollow organoids A) Solid and hollow organoids were collected by gentle centrifugation at 800 rpm. They were then fixed with 4% formaldehyde and stained using luminal marker CK8/18 (PROGEN-GP1)(Red) and basal marker CK5 (Abcam-ab53121)(Green). B) Solid organoids (left) and hollow organoids (right) from basal/stem and luminal progenitor cultures were dissociated and stained with EPCAM and $\alpha 6$ -integrin. This was done to check for the type of populations present in these organoids. The samples were analysed using BD FACSaria™ III cell sorting machine.

The organoid assay involves using a (Rho-associated protein kinase) Rock inhibitor. Rock protein is downstream β 1-integrin signaling (Lu et al., 2008), the pathway which will be studied in this project. I therefore wanted to check whether I could eliminate the Rock inhibitor from my organoid culture conditions in order not to block a known downstream target of β 1-integrin. To achieve that an experiment was performed where sorted basal, luminal progenitor, and differentiated luminal cells were grown in organoid media in an ultra low attachment 24-well plate at a density of $2 \times 10^3/\text{cm}^2$. This was done for each sorted population with and without the addition of 10 μM Rock inhibitor. Without the Rock inhibitor, cells were not able to form organoids from the basal, luminal progenitors, or differentiated luminal populations. This suggested that the Rock protein and its downstream targets in the β 1-integrin pathway must be inhibited in order for stem and progenitor cells to become active. Therefore, it can be concluded that the Rock inhibitor must be included in future organoid forming assays.

Based on these results, it was concluded that the organoid formation assay has more advantages over the mammosphere formation. The benefits of this assay were that this assay can distinguish between spheres that are originating from bi-lineage cells and those that came from luminal progenitors. Also, the organoid culture condition promoted sphere formation from single cells and the presence of matrigel-prevented cells aggregating together. It was also possible to obtain secondary organoids from this assay, which was important to show that bi-lineage cells within these organoids have self-renewal capacity, an important property of stem cells.

3.2.4 Optimising the use of lentivirus as a gene delivery method in primary MECs:

This project involves manipulating gene expression of β 1-integrins and its downstream targets in stem cells and then test for stem cell behavior in MECs. The organoid assay was chosen as the main assay for quantifying stem and progenitor cell frequency in a given MEC populatio. The time required to obtain readout for this assay is 7-10 days. Therefore, it important to find a stable system to overexpress or knockdown the gene of interest in those stem and luminal progenitor cells.

The lentivirus system is ideal to overexpress or knockdown integrins and then test for their effect on stem cell behavior. Lentiviral vectors are efficient vehicles for transgene delivery. They can infect both fast dividing and slow dividing cells (Mátrai et al., 2010).

This strategy will also allow the performance of rescue studies and will allow a better tracking of the infected cells in-vivo and in-vitro as they will be GFP positive. However, the challenge is to obtain high infection efficiency in primary MECs. Infecting primary cells is difficult and many previous students in our lab had to switch from doing their studies in primary MECs to cell-line; due to problems with the infection efficiency (Moreno Layseca, 2015; Rooney, 2014).

For my project it is essential that experiments are performed in primary cells. This is mainly because unlike cell-lines, primary MECs are better representatives of what is happening in-vivo. These cells are not transformed in any way and thus; studying the mechanisms controlling stem cells in these cells is more reliable. It was therefore highly important to optimise the infection conditions of primary MECs by lentivirus.

In order to investigate the mechanism controlling stem cells pathways in primary MECs downstream of β 1-integrin, gain and loss of function studies will be performed. This strengthens the need for an efficient gene transfer method to use on MECs. Therefore, the best methods by which lentiviral vectors can deliver the gene of interest into primary MECs was optimised.

3.2.4.1 Production of Lentiviral particles:

A critical part of the lenti-virus transduction is producing high titers of lentivirus, especially that primary MECs are difficult to transduce. This protocol has been optimised in our lab (Moreno Layseca, 2015; Rooney, 2014). Producing lentiviruses that express the shRNA for the gene of interest requires that transfection of 3 different plasmids. These include the lentiviral backbone HIV-1 transfer plasmid that contains the shRNA or the cDNA for the gene of interest, the packaging plasmid, and the envelope protein-expressing plasmid.

The lentiviral backbone plasmid is the PLVTHM plasmid (Du et al., 2009) . It contains the H1 promoter followed by shRNA cloning sites and a GFP sequence under the over-expression of Elf α promoter (Cárcamo-Orive et al., 2008; DelaRosa et al., 2009). The vector lacks encoding 70 proteins involved in virus replication and infection, making the virus only able to infect once. It contains however important sequences known as the long terminal repeats (LTR) to insure integration of the shRNA sequence into the target cell's genome. The plasmid also contains a virus self-inactivating sequence (SIN) as well as a Ψ sequence that allows packaging of the viral particle (Mátrai et al., 2010a).

The second plasmid is envelope plasmid (PMDG.2). This vector expresses a G protein of a vesicular stomatitis virus (VSV-G). The receptor for this envelope protein is ubiquitously expressed on mouse cells. This increases the transduction efficiency of the viruses produced. The VSV-G also confers stability of the viral particles, which enables concentrating the virus via ultracentrifugation. Taken these factors together the VSV-G envelope would be suitable for infecting primary MECs (Farley et al., 2007). The packaging plasmid (psPAX2) encodes the viral genes gag, pol, tat and rev; which are responsible for the production of the reverse transcriptase, integrate and the capsid protein (Pereira et al., 2015).

To produce the lentivirus, cells were transfected at a confluence of 50-70% with 6 μ g of PLVTHM control vector, 3 μ g of psPAX2 and 4.5 μ g of PMDG.2 plasmids using 1XPEI transfection reagent. 6 hours following transfection the media was changed into complete media and the cells were left overnight. Next day the cells were treated for 6 hours with sodium butyrate and then changed into fresh complete media. 24 hours post sodium

butyrate addition, the supernatant was collected and centrifuged at a speed of 21,000Xg for 4 hours. The pellet was resuspended in 500 µl of blank media

To check whether high titer of lentivirus was obtained, 3T3 cells (that were 70% confluent) were infected in a 6-well plate with 500 µl of lentivirus (derived from the supernatant of one T75 flask). 48 hours post infection, 100% of the 3T3 cells were infected with the virus as they were all expressing GFP. This indicated that high titer of lentivirus was achieved (Figure 3.10).

3.2.4.2 Infection primary MECs by PLVTHM virus

The next step after producing high titre of lentivirus, was to optimise the best culture condition for infecting primary MECs. I tried infecting cells in 3D matrigel, 2D collagen, on plastic, in suspension, and in organoid media. 1 vial of concentrated virus was used for 1 well of a 6-well plate containing one of each culture condition. The infection was done overnight. Next day media was changed into fresh complete media and cells were left for 72 hours post infection. The cells were then trypsinised into single cells and analysed for GFP expression using flow cytometry to obtain an accurate percentage of cells transduced by the lenti-virus. I found that on 3D matrigel and 2D collagen the infection was not better than 5 % as shown by FACS analysis (Figure 3.10). I then tried infecting on plastic as shown in (Welm et al., 2005) but the cells could not survive being on plastic for 3 days. Infecting in suspension overnight was also not ideal and the majority of cells did not survive these conditions.

However, when cells were infected in organoid media 15% infection efficiency was obtained (Figure 3.10). This infection rate was better than other conditions but it was still not efficient enough for obtaining sufficient number of cells to perform my experiments. A double infection was then performed, where the first infection was done on day 0 overnight. The media was changed next morning, cells were re-infected again with another vial of virus, and media was changed next day. The cells were analysed for GFP expression on day 3-post first infection. The infection efficiency was found to have increased from 10% to around 50% Figure (3.10). Cells were then sorted for GFP positive cells and seeded in

organoid formation assays. The viral transduction process did not have an effect the organoid formation efficiency (Figure 3.11). Also, both bi-lineage cells and luminal progenitor were infected as both solid and hollow organoids that express GFP were obtained (Figure 3.10). The GFP expression was stable throughout the 7-10 days of the organoid assay, showing that the expression of the gene of interest was stable. I therefore decided to follow this double infection protocol for the rest of my project as I found that it is resulting in high and stable infection of bi-lineage and progenitor cells. Protocol is summarised in (Figure 3.11).

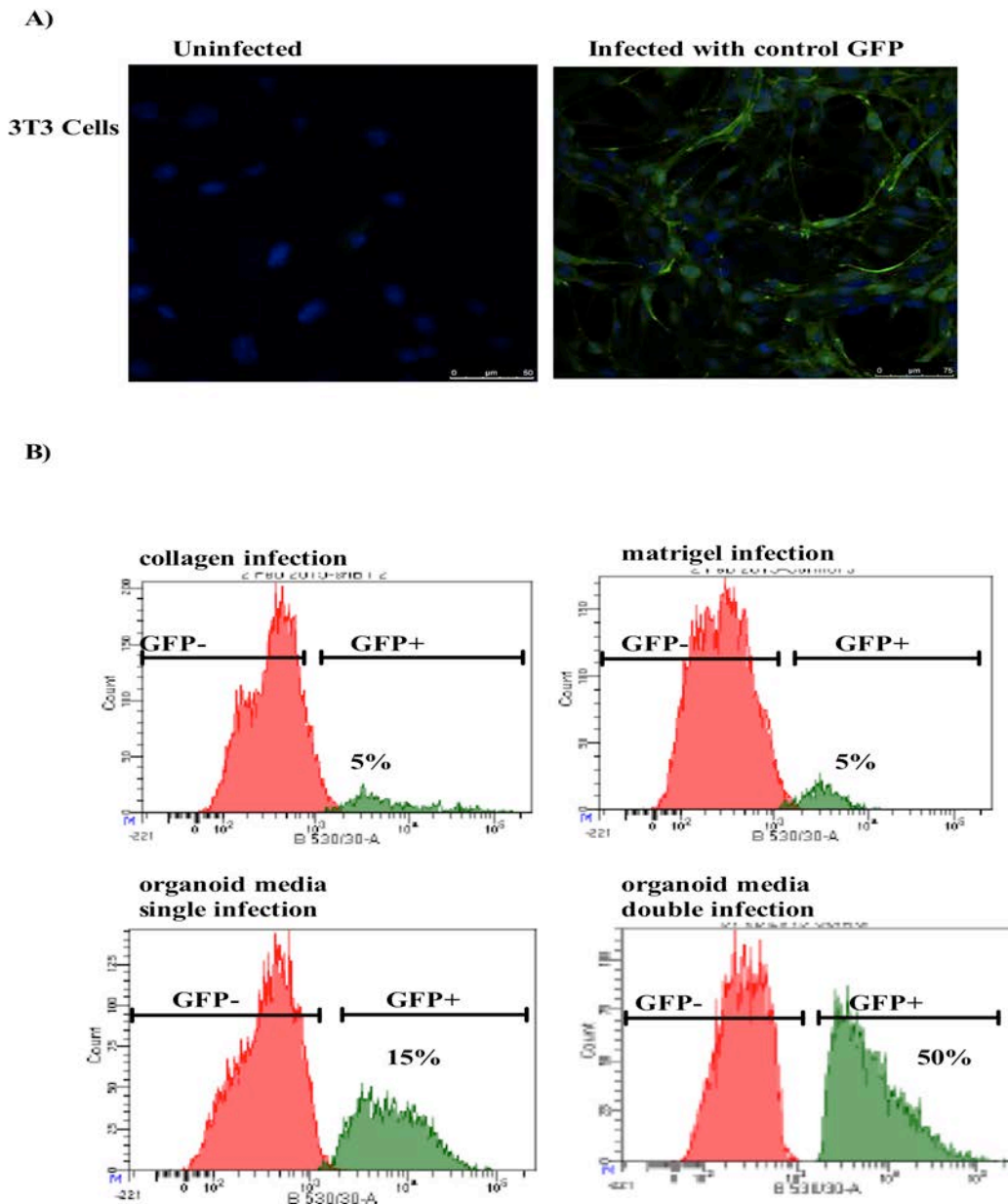


Figure 3.10 Infection of primary MECs in different culture conditions. A) Infection of 3T3 cells: 3T3 cells were grown coverslips in a 6-well plate until they were 70% confluent. They were then infected with one vial of concentrated lentivirus (obtained from one T75 flask) expressing GFP. 48 hours post infection, coverslips were removed from wells and cells were fixed and stained with DAPI to stain nucleus. B) Infecting cells in different culture conditions: MECs were isolated from virgin adult mice and trypsinised into single cells. For single infections, cells were cultured either on 2D collagen, 3D matrigel or in organoid media (in low attachment plates). Next day, the cells were infected with lentivirus expressing GFP overnight. The media was changed next morning into fresh media and cells were left for 48 hours before analysing them for GFP expression using flow cytometry. For double infection in organoid media, single cells were infected on day 0, then media was changed next morning and cells were re-infected with another vial of virus. Next day the cells were washed again and left for 48 hours before analyzing them with FACS for GFP expression.

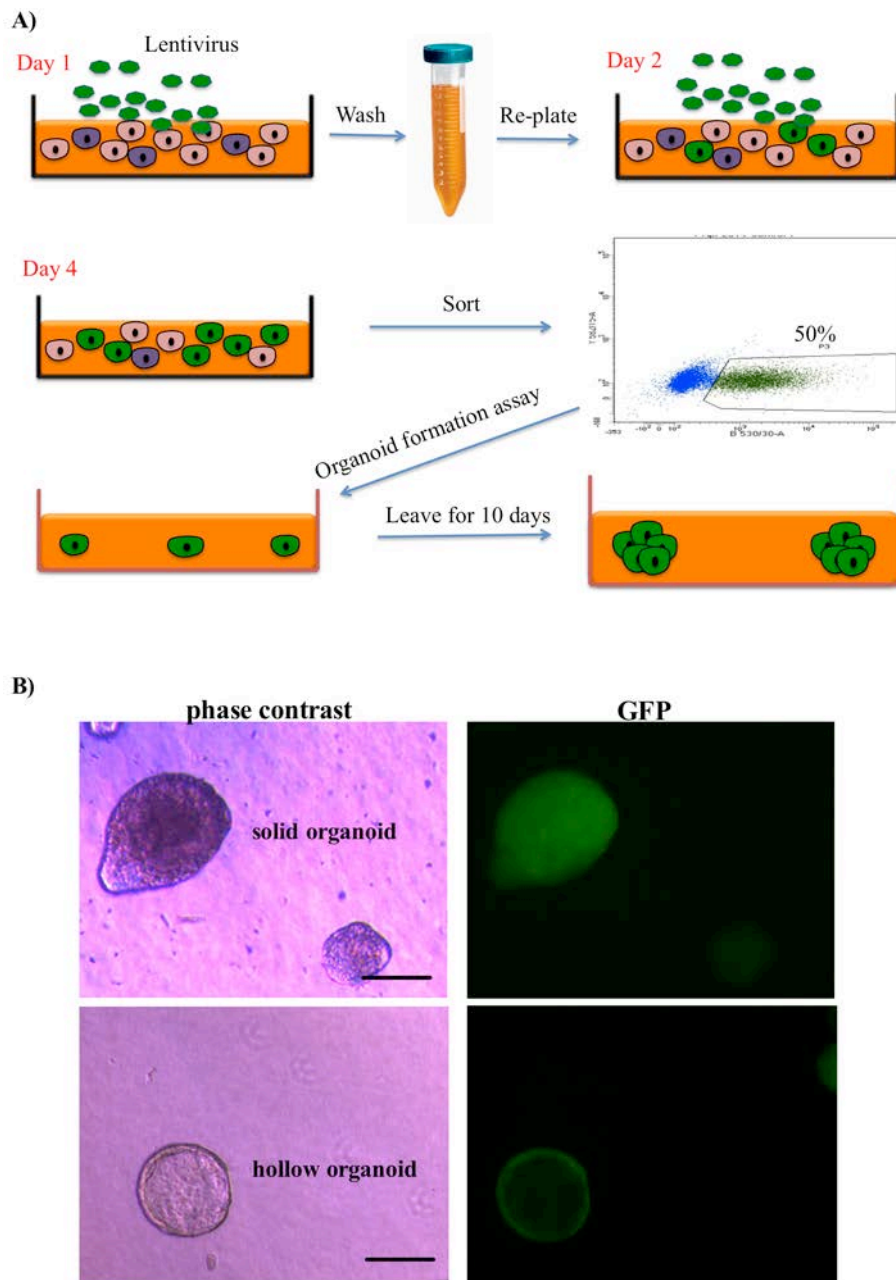


Figure 3.11 Infecting bi-lineage and luminal progenitors with lentivirus A) Summary of lentivirus infection protocol: primary MECs were infected twice with concentrated lentivirus on day 0 overnight, cells were then washed on day 1 and re-infected with another vial of concentrated virus overnight. The cells were washed next day, resuspended in fresh organoid media and then left for an additional 48 hours to allow GFP expression. The cells were then sorted for GFP expression using FACS Aria III cell sorter. B) Following GFP sorting, the cells were plated in organoid media and were allowed to form organoids for 10 days. Right - phase contrast of organoids, left- fluorescent image of organoids infected with lentivirus.

3.3 Discussion:

3.3.1 Flow cytometry cell sorting for isolating stem cells

Cells isolated from primary mammary preps contain a mixture of cell types. These include lymphocytes, fibroblasts, luminal, and basal cells. Flow cytometry sorting is a powerful and sensitive tool for separating those different populations based on the expression specific extracellular markers. It is essential when sorting for different markers to choose the correct colour combination that will enable this separation and will decrease any potential overlapping between different populations. CD45,CD31-PECy7 enabled a good negative selection of epithelial cells from contaminating lymphocytes and endothelial cells. I found that using $\alpha 6$ -integrin-efluor450 and EPCAM-APC antibodies enabled the separation of basal, luminal and fibroblasts populations. Moreover, this colour combination will allow cells expressing GFP or YFP shRNA lentivirus infection experiments to be sorted effectively.

Stem cells are sorted based on their expression of specific adhesion receptor. These include $\beta 1$ -integrin, $\alpha 6$ -integrin, EPCAM and CD24. Antibodies to EPCAM/ $\alpha 6$ -integrin or CD24/ $\alpha 6$ -integrin result in a similar profile of sorted cells from mammary gland. My results confirmed that use of EPCAM marker instead of CD24 gives a better separation between luminal and myoepithelial cells (Shehata et al., 2012; Smalley et al., 2012). The profile of the luminal population is EPCAM^{high}/ $\alpha 6$ -integrin^{low}, basal cells are within the EPCAM^{med/low}/ $\alpha 6$ -integrin^{high} population, and fibroblasts are negative for both EPCAM and $\alpha 6$ -integrin expression. Stem cells reside within the basal population and have a phenotype of EPCAM^{med}/ $\alpha 6$ -integrin^{high} (Shehata et al., 2012; Stingl, 2009). Immunostaining experiments confirmed that these sorted cell population were indeed representative of luminal, basal epithelial cells. This indicated the high purity of the populations sorted. The luminal population was further divided into luminal progenitor with a profile of (EPCAM^{high}, $\alpha 6$ -integrin^{low} and $\alpha 2$ -integrin^{high}) and the differentiated luminal had a profile of (EPCAM^{high}, $\alpha 6$ -integrin^{low} and $\alpha 2$ -integrin^{low}) (Shehata et al.,

2012). The use of flow cytometry is therefore a powerful tool that will be used in this project to determine stem and progenitor cell frequency.

3.3.2 Mammosphere and organoid formation assays in determining stem cell frequency

Mammary epithelial cells under non-adherent conditions undergo apoptosis after a few hours (Gilmore et al., 2000). However a low percentage of cells are able to survive and proliferate to form mammosphere (Dontu et al., 2003). Those cells possess stem/progenitor properties such as self-renewal and differentiation. It is known that basal cell population contains the highest percentage of cells that can regenerate a fully functional mammary gland in-vivo and can give rise to both luminal and myoepithelial cells. The percentage of mammosphere forming cells within the luminal cell population is much lower than that within the basal population. The majority of cells with a stem cell property within the luminal population are luminal-restricted progenitors (Asselin-Labat et al., 2006a; Stingl et al., 2006a).

Nothing was previously known about the mammosphere forming ability within the luminal and the basal population obtained from virgin mouse mammary gland. From my mammosphere assays results, it can be concluded that basal population contains more stem/progenitor cells than the luminal population. This fits with previous in-vivo studies that showed that basal population contain higher stem the luminal population (Asselin-Labat et al., 2006a; Stingl et al., 2006a). The mammosphere assay however will not be used during my project as an in-vitro method to assess for stem/progenitor cell frequency; as it was not suitable for non-sorted cells due to high cell clumping. The method also could not distinguish between spheres that were formed from bi-lineage cells and those that were formed from luminal progenitors. I therefore decided to use the organoid assay as the main assay for determining stem cell frequency in-vitro.

My results showed that the organoid assay is more reliable than the mammosphere assay for the following reasons: First, the organoids form from single cells and chances of cells clumping are very low due to the presence of matrigel. This was shown with the PKH26 label retention experiment. Second, one can distinguish from the morphology of the

organoids of those that come from luminal progenitors and those that originate from bi-lineage cells. The bi-lineage cells produce solid organoids with bi-layered structure of myoepithelial and luminal cells. While the luminal progenitors, produce hollow organoids with only luminal cells. Finally, the organoid assay produces spheres at a much higher efficiency than the mammosphere assay. This might be due to the addition of matrigel to the culture. Matrigel contains necessary laminins (such as laminin 511) that are important extracellular ligands for the maintenance of stem cells. They are also the ligands for $\beta 1 / \alpha 6$ -integrin adhesion receptors. It might be that adding laminins to the culture activates the integrin pathway and thereby activates stem cells and luminal progenitors (Domogatskaya et al., 2008; Hongisto et al., 2012).

3.3.3 Infecting stem cells with lentivirus

My project will involve manipulating the expression of adhesion receptors in stem cells using. Our lab has a genetically modified $\beta 1$ -integrin^{fx/fx}-CreTM mouse line. This line contains mice that have their $\beta 1$ -integrin gene flanked by two loxp sequences and also expresses Cre recombinase that is activated when adding 4-hydroxytamoxifen (4-OHT) to the cell culture (Akhtar and Streuli, 2013a). The active Cre causes deletion of the $\beta 1$ -integrin gene. This system is very helpful in studying $\beta 1$ -integrin signaling in stem cells. However, it is important to confirm results obtained from deleting $\beta 1$ -integrin knockout mice using another gene manipulation method like knocking down using shRNA-expressing lentivirus.

The lentivirus gene knockdown system will also enable tagging cells that express the shRNA sequence with a fluorescent marker, in this project it will be the GFP protein. Lentiviruses enable stable expression of the shRNA which makes them suitable for long stem cell cultures (Mátrai et al., 2010b). It also enables performing rescue experiments with downstream targets of $\beta 1$ -integrin. Those targets will be stably expressed with the shRNA sequence, providing a powerful tool for studying mechanisms involved in stem cell self-renewal and differentiation. The lentivirus system is also time and cost efficient. It can be used to delete genes in wild type mice that are more easily available than the genetically modified mice.

For this project, lentivirus infection of primary MECs was optimized and the lentivirus was able to infect both bi-lineage cells and luminal progenitors. An infection of 50% efficiency was obtained. This was the best infection efficiency obtained in our lab for primary cells. I also performed the infection in the stem cell-promoting organoid culture, which is better than infecting on 2D plastic, collagen, or in Matrigel; as infecting on those substrates did not give high infection efficiency and the culture conditions might cause the stem cells to differentiate. In the next chapter, these tools will be used in studying the β 1-integrin signaling in mammary stem cells and luminal progenitors.

Chapter4-Results: β 1-integrin-Rac1 Signaling Pathway is Important in Mammary Stem cells

4.1 Introduction:

Integrins that have been used to identify mammary stem cells and progenitors include (β 1, β 3, α 6 and β 4)-subunits (Shackleton et al., 2006; Stingl et al., 2006a). However, very little is known about their role in mammary stem cell biology. β 1-integrin is likely to have a key role because deleting β 1-integrin in K5-expressing basal cells in the gland abolished the gland's regenerative potential although it has not been definitely confirmed that β 1-integrins are essential for stem cell function (Taddei et al., 2008b). This project aims to investigate if β 1-integrin and its downstream targets have an effect on mammary stem and progenitor frequency. This was examined using in-vitro and in-vivo stem cell assays optimised in the previous chapter. I also aimed to show mechanisms involved in connecting β 1 integrin receptor with the stem cell phenotype, by examining the involvement of two β 1-integrin signaling intermediates, Small GTP Binding Protein (Rac1) and Integrin-Linked Kinase (ILK).

Rac1 is a Rho GTPases, implicated in cellular processes involving intracellular adhesion, cytoskeletal reorganization, cell motility, proliferation, and gene transcription. Rac1 also plays a role in many types of adult stem cells (Benitah et al., 2005; Guo et al., 2008; Maddala et al., 2011; Stappenbeck and Gordon, 2000). This is achieved by regulating a variety of stem cell signaling pathways in different adult tissues such as; nuclear factor kappa B (NF κ B), wnt/ β -catenin signaling and phosphatidylinositol 3-kinase (PI3K).(Genot et al., 2000; Myant et al., 2013; Wu et al., 2008a)

Rac1 is also involved in the tumourigenic process of different kinds of cancers including breast cancer (Myant et al., 2013; Rosenblatt et al., 2011; Yan and Ouellette, 2015). Our lab showed that in the mammary gland, Rac1 is downstream β 1-integrin in both pregnant and nulliparous MECs (Akhtar et al., 2009; Jeanes et al., 2012b).

Another downstream target of β 1-integrin is Integrin-linked kinase (ILK). ILK is a β 1-integrin binding adaptor protein and a key transducer of β 1-integrin signaling required for prolactin-induced differentiation of MECs from pregnant mice. In these cells, ILK is required for β 1-integrin activation of Rac1. This Rac1 activation was necessary for signaling transduction to control Stat5 expression and production of beta-casein in response to the activation of prolactin receptor (PrlR) (Akhtar et al., 2009). However, in the nulliparous mammary gland β 1-integrin activation of Rac1 was not dependent on ILK and was independent of adhesion complex signaling (Moreno Layseca, 2015). Therefore, β 1-integrin activates these two proteins through two independent pathways in the nulliparous gland and they might have different downstream effectors (Moreno Layseca, 2015).

Due to their importance in transducing β 1-integrin signaling, ILK and Rac1 are two attractive downstream targets of β 1-integrin. Therefore, I chose to test whether they have different effects on stem cells and luminal progenitor phenotypes.

In this chapter, unlike in the study of (Taddei et al., 2008b), where β 1-integrin was only deleted in CK5 expressing cells, β 1-integrin, Rac1 and ILK were deleted in total MECs. Then, I investigated whether deleting those proteins had an effect on stem cells and luminal progenitor phenotypes using stem cell assays. This allowed me to determine whether β 1-integrin signaling has a role in stem as well as progenitor cells rather than just in CK5-expressing cells.

I also made use of my optimised lenti-virus system to infect primary cells and thereby to knockdown or overexpress different components of β 1-integrin signaling. Figure 4.1 shows my strategy in manipulating gene expression of β 1-integrin and its downstream targets then checking for stem cell frequency.

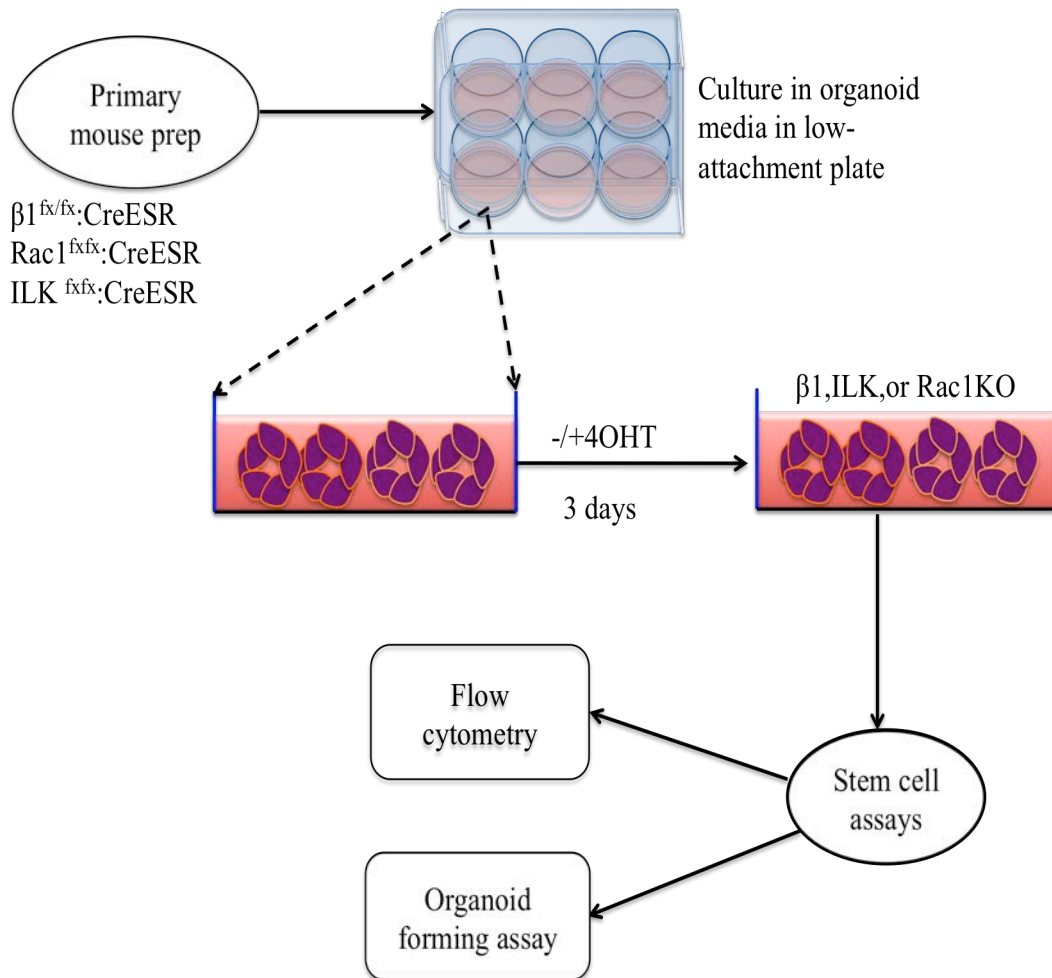


Figure 4.1 Knockout strategy for $\beta 1$ -integrin, ILK and Rac1 genes and testing for stem/progenitor frequency. Primary MECs were isolated from female nulliparous transgenic, age 8-12 weeks using enzymatic digestion of mammary gland tissue. MECs were cultured in 3 ml of organoid media under low attachment conditions. They were then treated with either 3 μ l ethanol (control) or 3 μ l of 100 μ M 4-hydroxytamoxifen (4OHT) (to achieve a final concentration of 100 nM 4OHT and to knockout the gene of interest). After 3 days, MECs were collected and dissociated into single cells using trypsin, counted, analysed for flow cytometry and put in organoid forming assay in order to measure stem/progenitor frequency.

4.2 Results

4.2.1 β 1-integrin knockout abolishes solid and hollow organoid-forming cells in-vitro.

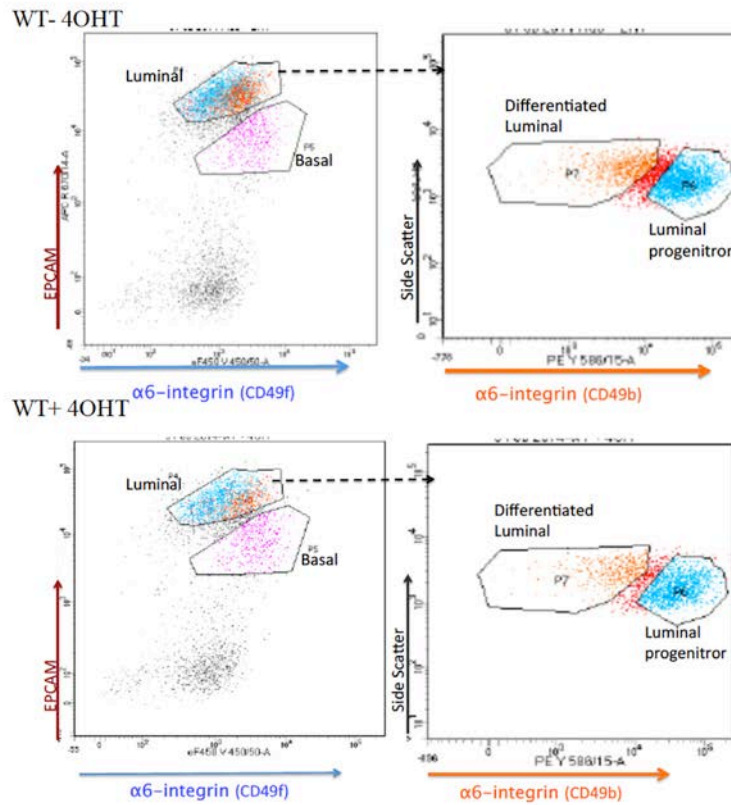
To determine the role of β 1-integrin in mammary stem cells in primary cells, our established β 1-integrin^{fx/fx};CreERTM mouse lines was used. This line has the β 1-integrin-gene flanked by two loxP sites and expresses CreESRTM. CreESRTM is a recombinase, which is activated when 4-hydroxytamoxifen (4OHT) is added to the culture media, resulting in β 1-integrin gene deletion. Sufficient protein depletion is achieved at day 3-4 (Akhtar and Streuli, 2013).

4OHT is used in treating breast cancer, as it is an oestrogen receptor antagonist. It is therefore essential to use a very low dose of 4OHT for a short period of time that achieves gene deletion but does not prevent the estrogen receptor signaling axis in both luminal and stem cells (Asselin-Labat et al., 2010). Our lab used a concentration of 100 nM to achieve gene deletion in treated cells (Akhtar and Streuli, 2013a; Jeanes et al., 2012). In order to rule out that the addition of 4OHT at a concentration of 100 nM to primary MECs will have an effect on solid and hollow organoid frequency, the effect of adding 4OHT on cells obtained from mice with β 1-integrin^{fx/fx} gene but do not express CreESRTM was tested. Those mice will be referred to as wild type (WT) mice.

Total MECs from nulliparous WT mice were isolated and plated in low-attachment 6-well plates at a density of 5×10^5 in organoid media for 3 days with and without 100 nM of 4OHT. MECs were then dissociated into single cells and some of the cells were analysed using flow cytometry for stem cells and luminal progenitor markers. The rest of the single cells were counted and plated in organoid media at a density of $2 \times 10^3/\text{cm}^2$ in a 24-well plate for 10 days. On day 10, solid and hollow organoids were counted under 4X magnification. The addition of 4OHT at the concentration of 100nM did not have an effect on either stem or luminal progenitor population frequency as shown by FACS analysis (Figure 4.2). The organoid-formation assay also showed that there was no effect on solid or hollow organoids (Figure 4.3).

Thus, any phenotype that will be observed from deleting β 1-integrin or its downstream targets will be due to the gene deletion and not to the addition of 4OHT.

A)



B)

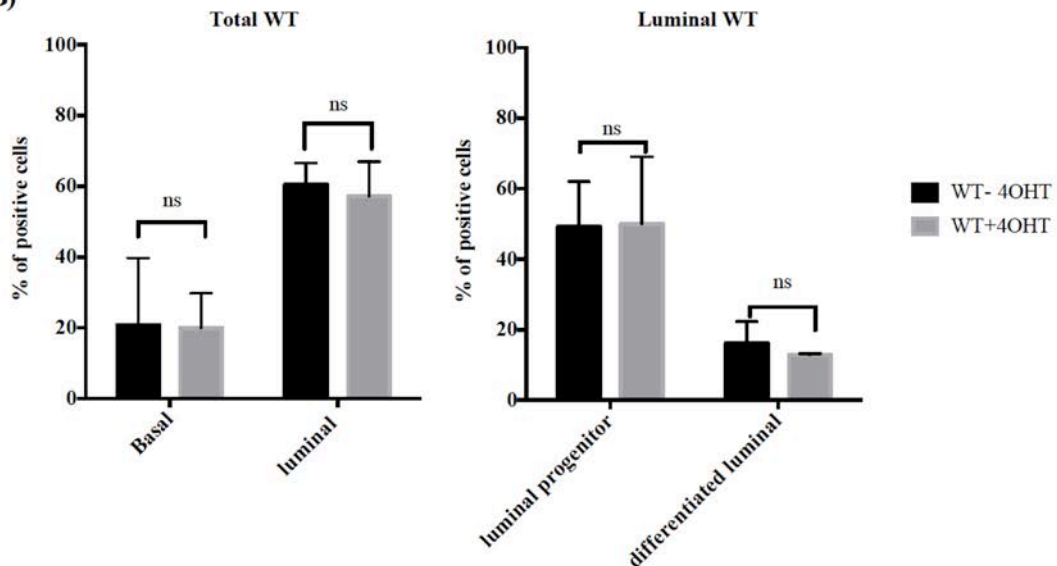
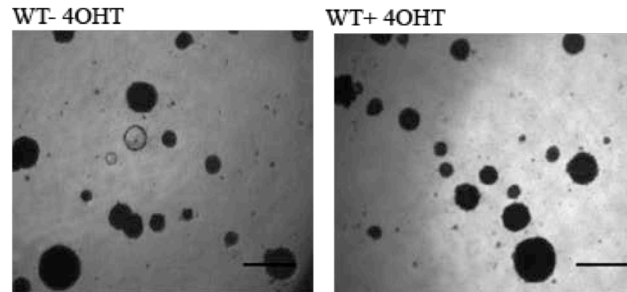


Figure 4.2 Addition of 100nM of 4OHT does not affect basal cells or luminal progenitor frequency when analysed by FACS. Cells from primary MECs from WT mice were cultured at a density of 5×10^4 /well in a 6-well plate +/-4OHT. Cells were then dissociated using trypsin/EDTA at day 3, passed through a 40 micron filter and stained for the following markers CD45, CD31, EpCAM, $\alpha 6$ -integrin, and $\alpha 2$ -integrin. This was done in order to distinguish the populations of basal/stem, total luminal, luminal progenitor, and differentiated luminal. A) FACS diagram showing the four populations in WT-/+ 4OHT. B) Quantitation of the four populations in WT-/+4OHT (n=3). Statistical significance was determined by Student's t test for paired samples. Error bars in the graph represent standard error of the mean, ns= non significant.

A)



B)

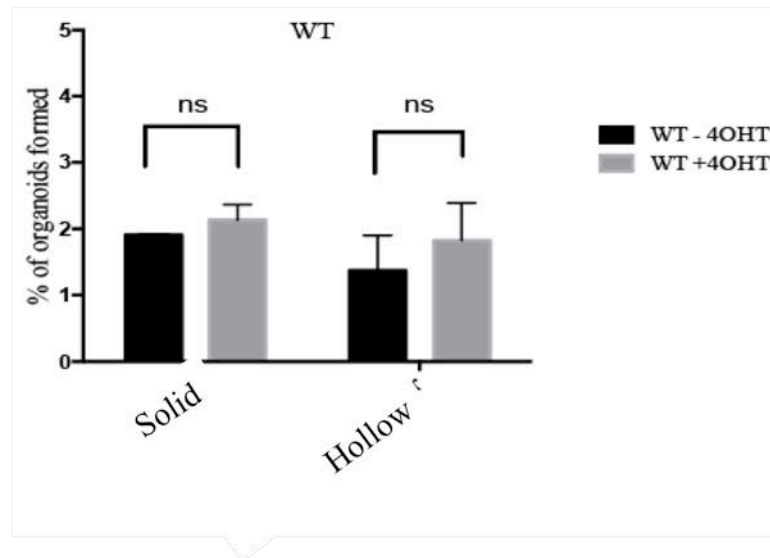


Figure 4.3 Addition of 100nM of 4OHT does not affect solid or hollow organoid-forming efficiency.

Primary MECs were isolated from WT mice (i.e. They have $\beta 1$ -integrin gene floxed but do not express CreESRTM) and cultured as single cells in organoid media at a density of 5×10^5 in an ultra-low attachment 6-well plate for 3 days, with and without 4OHT. At day 3, MECs were collected, dissociated into single cells, and cultured in organoid-forming media in an ultra-low attachment 24-well plate at a density of $2 \times 10^3/\text{cm}^2$. Stem and luminal progenitor organoids were counted at day 10 at a 4X magnification a) phase contrast image of organoids formed in WT-4OHT and WT+4OHT. Scale bar = $500\mu\text{m}$ b) number of organoids formed was counted at day 10 and divided by the number of cells seeded at day 0 in order to calculate the percentage of organoids formed from WT cells +/- 4OHT (n=3). Statistical significance was determined by Student's t test for paired samples. Error bars in the graph represent standard error of the mean. ns= non significant.

The effect of knocking out β 1-integrin on solid and hollow organoid frequency was tested. Total MECs from nulliparous β 1-integrin^{fx/fx};CreERTM mice were isolated and plated in low-attachment 6-well plate at a density of 5×10^5 in organoid media for 3 days with and without 4OHT to delete β 1-integrin gene. The deletion of the gene was confirmed by qRT-PCR at day 3 (Figure 4.4.A). Protein depletion was checked by plating cells on coverslips overnight post 4OHT treatment and stained by an immunofluorescent antibody for β 1-integrin protein. Treatment with 4OHT successfully depleted the β 1-integrin protein (Figure 4.4.b).

Cells were then dissociated into single cells and plated in organoid media at a density of $2 \times 10^3/\text{cm}^2$ in a 24-well low-attachment plate for 10 days. Solid and hollow organoids were counted under 4X magnification. Knocking out β 1-integrin significantly abolished the formation of both stem and luminal progenitor organoids (Figure 4.4. C and D). This indicates that β 1-integrin is involved in stem cells as well as luminal progenitor maintenance.

FACS analysis of single cells obtained from β 1-integrin knockout cells showed that there was a significant decrease in stem/basal cell population, but no significant changes in the luminal population. However within the latter, there was a significant decrease in luminal progenitors and a significant increase in differentiated luminal cells in the absence of β 1-integrin (Figure 4.5). These results provide, for the first time, evidence that β 1-integrin is required for stem cell and luminal progenitor activity in the mammary gland. They also suggest that the depletion of β 1-integrin might cause a change in the population of luminal cells away from progenitors into a more differentiated population.

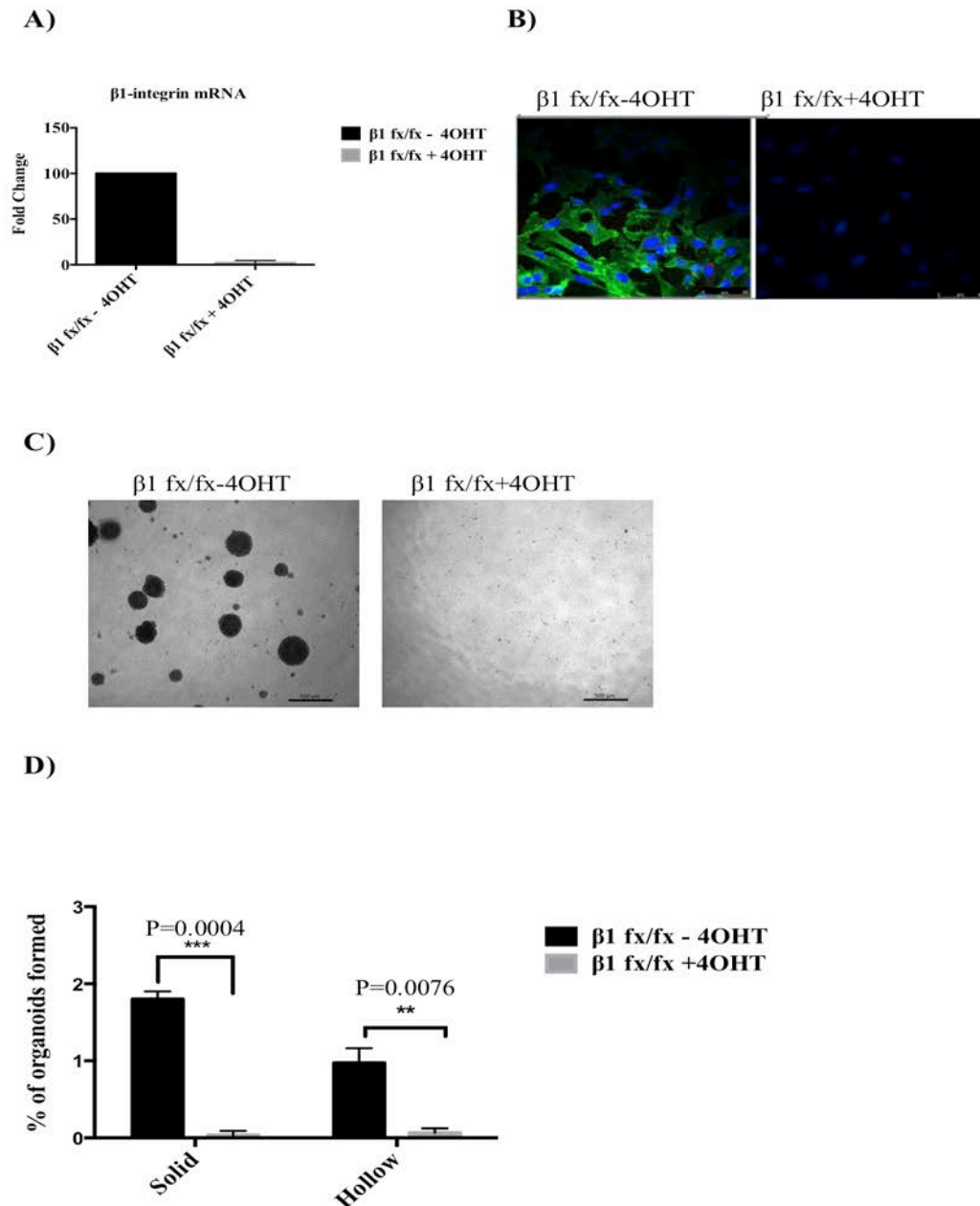


Figure 4.4 Knocking out $\alpha 1$ -integrin in primary cells abolishes solid and hollow organoid-forming populations. Primary MECs were isolated from $\beta 1$ -integrin^{fx/fx};CreESR mice and cultured as single cells in organoid media at a density of 5×10^5 /well in an ultra-low attachment 6-well plate for 3 days with and without 4OHT. Cells were then dissociated into single cells and cultured in organoid forming media in an ultra-low attachment 24-well plate at a density of 2×10^3 /cm². a) Gene expression level measured by qRT PCR b) Immunofluorescence staining of $\beta 1$ -integrin^{fx/fx} cells on day 3 were allowed to attach on collagen coated coverslips for 24h and immunofluorescent staining was performed to check for the depletion of $\beta 1$ -integrin protein. Left: $\beta 1$ -integrin^{fx/fx} - 4OHT right: $\beta 1$ -integrin^{fx/fx}+4OHT. C) Phase contrast image of organoids formed on day10, scale bar= 500 μ m. D) Number of organoids formed was counted on day 10 and divided by the number of cells seeded at day 0 in order to calculate the percentage of organoids formed from $\beta 1$ -integrin^{fx/fx} cells +/- 4OHT (n=4). Statistical significance was determined by Student's t test for paired samples. Error bars in the graph represent standard error of the mean.

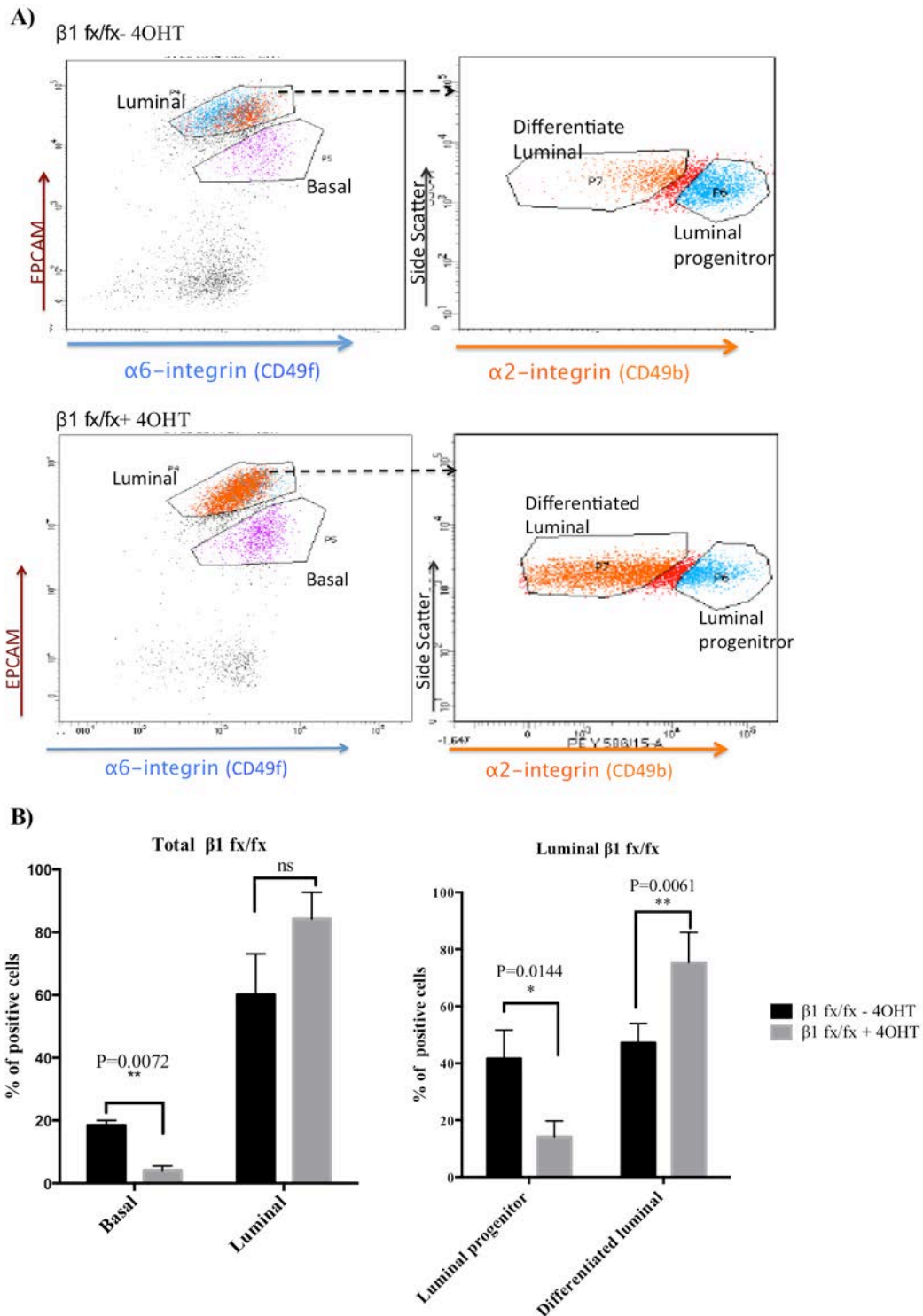


Figure 4.5 $\beta 1$ -integrin knockout reduced basal/stem and luminal progenitor population when analysed by FACS.

Cells from primary MECs of $\beta 1$ -integrin^{*fx/fx*}.CRreSR mice were cultured at a density of 5×10^4 /well in a 6-well plate +/-4OHT. Cells were then dissociated using trypsin/EDTA at day 3, passed through a 40 micron filter and stained for the following markers CD45, CD31, EpCAM, alpha6-integrin, and alpha2-integrin. This was done in order to distinguish the populations of basal/stem, total luminal, luminal progenitor, and differentiated luminal. A) FACS diagram showing the four populations in $\beta 1$ -integrin^{*fx/fx*} +/- 4OHT. B) Quantitation of the four populations in $\beta 1$ -integrin^{*fx/fx*} +/- 4OHT (n=3). Statistical significance was determined by Student's t test for paired samples. Error bars in the graph represent standard error of the mean.

4.2.2 Knocking out ILK as a downstream target of β 1-integrin had no effect on solid or hollow organoid-formation efficiency.

After showing that knocking out β 1-integrin causes a dramatic effect on stem and Luminal progenitor frequency, I wanted to explore the mechanism by which β 1-integrin signaling regulates stem cells. An important downstream target of β 1-integrin is ILK protein, which is a major part of the IPP complex. In the absence of ILK, the other components of the IPP complex are not recruited to adhesion complexes (Rooney, 2014). ILK inhibition and knockdown was also recently reported to reduce cancer stem cells in breast cancer cell-lines (Hsu et al., 2015). I wanted to check whether knocking out ILK has an effect on normal stem and luminal progenitor frequency in the mammary gland.

To achieve ILK null cells, mice that have the ILK gene flanked by loxP sites and express CreESRTM protein were used. Similar to the β 1-integrin^{fx/fx}CreESRTM mice, primary MECs from ILK^{fx/fx}CreESRTM transgenic mice were isolated and treated them for 3-4 days with 4OHT. After that MECs were dissociated into single cells and plated for organoid formation assay. ILK knockout was confirmed using qRT-PCR (Figure 4.6, A). Although knocking out ILK caused a slight decrease in the solid organoids and a slight increase in the hollow organoid-forming cells, these changes were not significant (Figure 4.6 B and C). Therefore, deleting ILK did not have an effect on stem cell and luminal progenitor frequency. This suggests that β 1-integrin control of the IPP complex via ILK is not required for stem and luminal progenitor maintenance in-vitro.

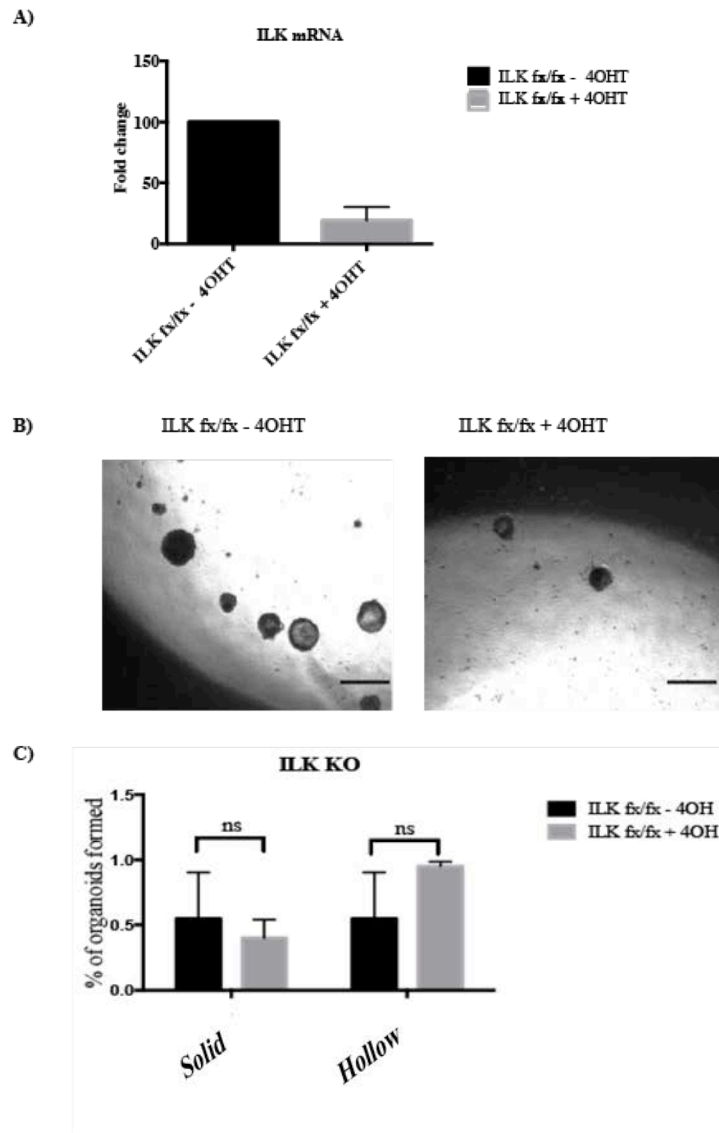


Figure 4.6 Knocking out ILK in primary cells had no effect on solid or hollow organoid-forming population. Primary MECs were isolated from $ILK^{f/fx};CreESR$ mice and cultured as single cells in organoid media at a density of 5×10^5 /well in an ultra-low attachment 6-well plate for 3 days $-/+4OHT$. MECs were then dissociated into single cells and cultured in organoid forming media in an ultra-low attachment 24-well plate at a density of $2 \times 10^3/cm^2$. a) Gene expression level measured by qRT PCR from cells obtained at day 3 post 4OHT treatment B) Phase contrast image at 4X magnification of organoids formed on day10, scale bar= 500 μm . C) Number of organoids formed was counted on day 10 and divided by the number of cells seeded at day 0 in order to calculate the percentage of organoids formed from $ILK^{f/fx}$ cells \pm 4OHT (n=2). Statistical significance was determined by Student's t test for paired samples. Error bars in the graph represent standard error of the mean.

4.2.3 Knocking out Rac1 as a downstream target of β 1-integrin reduced solid organoid frequency but had no effect on hollow organoid activity.

An alternative pathway that might link integrins to stem cells involves Rac1. This protein is important for the maintenance of many types of adult stem cells such as in the epidermis, small intestine, and lymphocytes. (Benitah et al., 2005; Guo et al., 2008; Stappenbeck and Gordon, 2000). Rac1 is also a downstream target of β 1-integrin in the mammary gland, as deletion of β 1-integrin caused a reduction in Rac1 activity (Jeanes et al., 2012).

β 1-integrin regulation of Rac1 is not through the regulation of adhesion complex (Moreno Layseca, 2015). Nothing is yet known about whether Rac1 is involved with normal mammary stem cells. To determine the role of Rac1 in mammary stem cells, I made use of our established Rac1^{fx/fx};CreESRTM mouse line. Similar to the β 1-integrin^{fx/fx};CreERTM and the ILK^{fx/fx};CreESRTM, the line expresses CReESRTM which is activated by addition of 4OHT to cause deletion of the floxed gene. In this case it is the Rac1 gene.

Total MECs were cultured with and without 100 nM 4OHT for 3 days and the Rac1 deletion was confirmed by qRT-PCR (Figure 4.7, A). MECs were then dissociated into single cells and plated for organoid formation assay for 10 days in organoid-formation media. Interestingly, the inhibition of Rac1 caused a significant reduction in stem cells but had no effect on the organoids formed from luminal progenitors (Figure 4.7 B,C).

These results suggest that, possibly downstream of integrins, Rac1, but not ILK, may have a significant role in maintaining mammary stem cell activity.

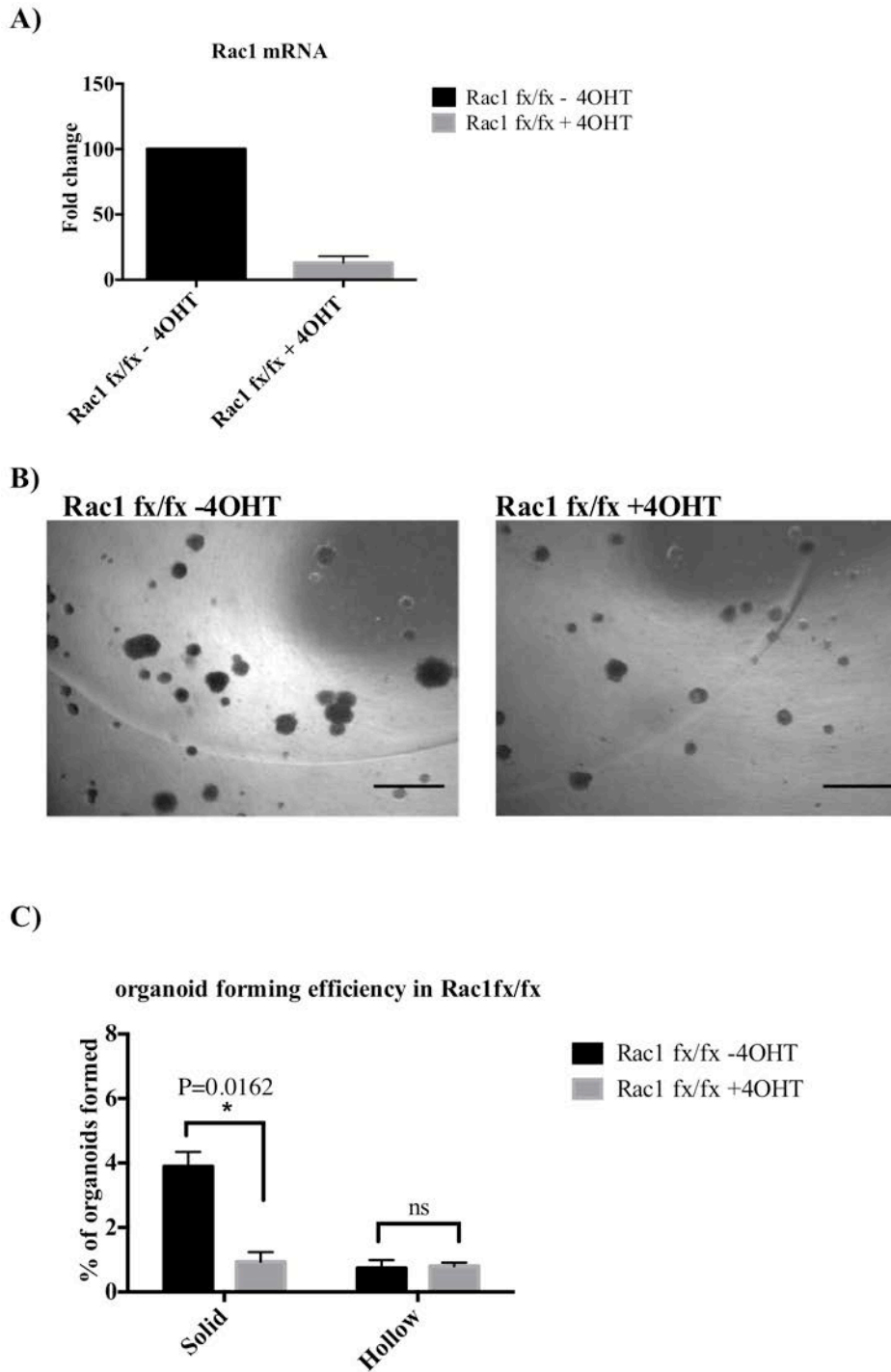


Figure 4.7 Knocking out Rac1 in primary MECs reduced solid but not hollow organoid-forming efficiency. Primary MECs were isolated from $Rac1^{fx/fx}; CreESR$ mice and cultured as single cells in organoid media at a density of 5×10^5 /well in an ultra-low attachment 6-well plate for 3 days with and without 4OHT. The MECs were then dissociated in single cells and cultured in organoid forming media in an ultra-low attachment 24-well plate at a density of $2 \times 10^3/cm^2$. A) Gene expression level measured by qRT PCR from cells obtained at day 3 post 4OHT treatment. B) Phase contrast image at 4X magnification of organoids formed on day10, scale bar= 500 μm . C) Number of organoids formed was counted on day 10 and divided by the number of cells seeded at day 0 in order to calculate the percentage of organoids formed from $Rac1^{fx/fx}$ cells +/- 4OHT (n=3). Statistical significance was determined by Student's t test for paired samples. Error bars in the graph represent standard error of the mean.

4.2.4 Inhibition of Rac1 activity using EHT reduces solid but not hollow organoid formation efficiency.

In order to confirm the stem cell phenotype obtained from the Rac1 null cells, studies with a Rac1 inhibitor (EHT 1864) were performed. This molecule is known to inhibit Rac1 as well as its related isoforms, Rac1b Rac2 and Rac3. The association of EHT 1864 with Rac promoted the loss of bound nucleotide, inhibiting both guanine nucleotide association and Tiam1 Rac1 guanine nucleotide exchange factor activity. EHT 1864 therefore places Rac1 in an inactive state, preventing its engagement with downstream effectors (Shutes et al., 2007).

Initially, an optimal dose was determined by which a significant amount of Rac1 inhibition in MECs was achieved without causing cell toxicity. Primary MECs were cultured with 0, 10 and 20 nM EHT 1864 for 3 days. Cells were then collected and a Rac1 activity assay was performed to determine which dose caused sufficient Rac1 inhibition. Treating MECs with a dose of 20 nM EHT 1864 caused more than 70% inhibition of Rac1 activity (Figure 4.8). This dose was used to test the effect of EHT 1864 on primary stem cells and luminal progenitor frequency. Primary MECs were incubated for 3 days with 20 nM EHT 1864, dissociated into single cells and plated in organoid formation media under low attachment conditions to form organoids. Similar to the Rac1 KO data, Rac1 inhibition with EHT resulted in the reduction of solid, but not in the reduction of hollow organoids.

Taking the results from the Rac1 inhibitor and Rac1 knockout cells together, it can be concluded that Rac1 is involved in maintaining mammary stem cells but not the luminal progenitor population.

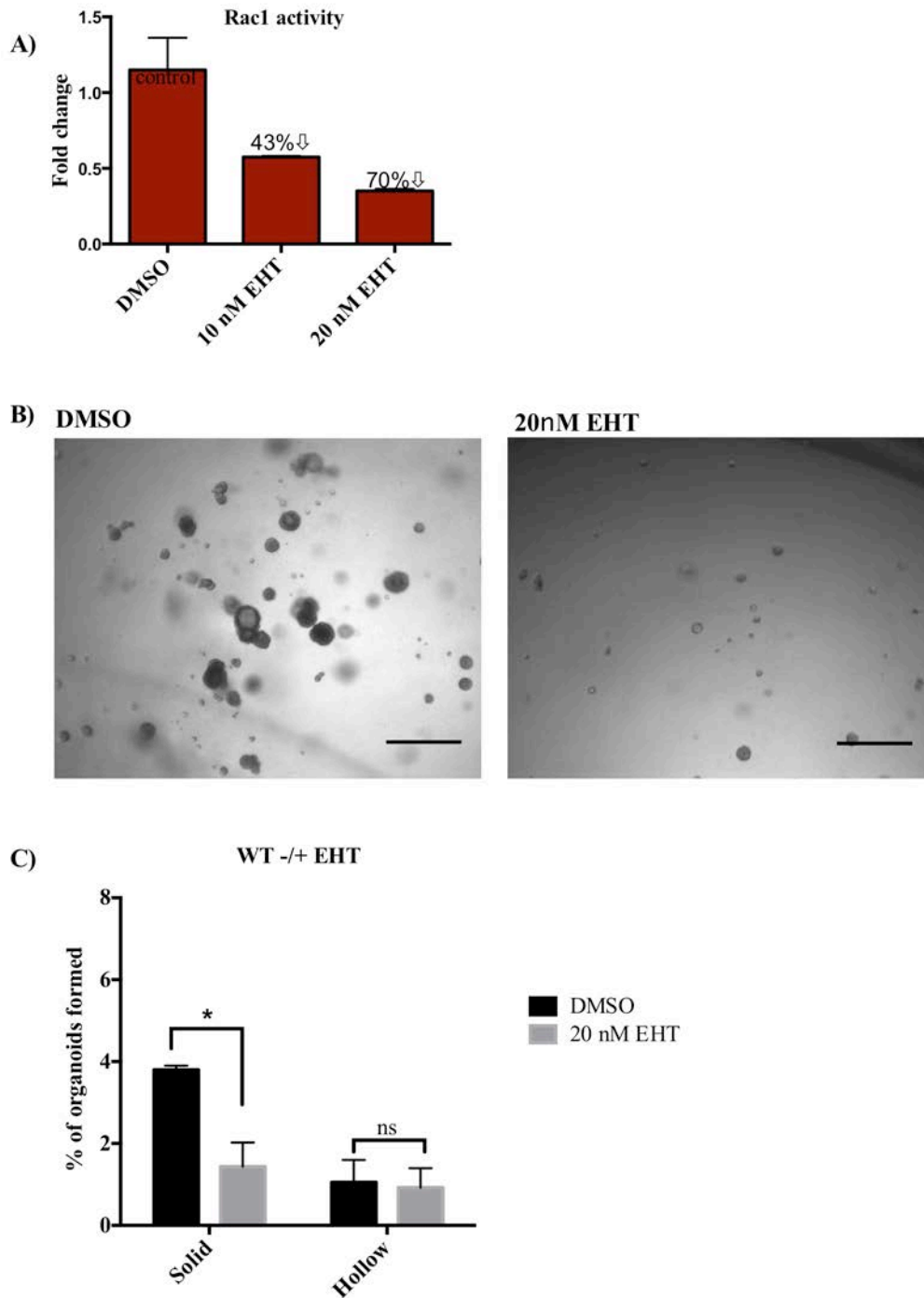


Figure 4.8 Inhibition of Rac1 activity using EHT reduces solid but not hollow organoid-formation efficiency.

Cells from primary MECs of ICR mice were cultured at a density of 5×10^4 /well in a 6-well plate with 0,10,20 nM EHT .A) Cells were then collected for and a Rac1 activity assay was performed on fresh cell lysates using G-LISA Rac1 activation assay kit. B) Phase contrast image at 4X magnification of organoids formed on day10, scale bar = 500 μ m. C) Number of organoids formed was counted on day 10 and divided by the number of cells seeded at day 0 in order to calculate the percentage of organoids formed from Rac1^{fl/fl} cells +/- 4OHT (n=3). Statistical significance was determined by Student's t test for paired samples. Error bars in the graph represent standard error of the mean.

4.2.5 β 1-integrin knockdown phenotype can be rescued by overexpressing active Rac1

So far, the results with Rac1 knockout and Rac1-activity inhibition indicated that that Rac1 is important for stem cell maintenance. In order to test whether β 1-integrin maintains bi-lineage organoids by activating Rac1, an active form of Rac1 (F28L) (Lin et al., 1999) was over-expressed in the same plasmid that contains the shRNA sequence for β 1-integrin. This resulted in a plasmid encoding a GFP-tagged Rac1 under the control of EF1 α promoter and the shRNA to target β 1-integrin under the control of the H1 promoter (Figure 4.9. A). This is an advantageous system, since it contains the cDNA rescue construct and shRNA in the same vector. The plasmid was packaged into a lenti-virus system, and cells from the same mammary epithelial prep were infected with GFP-control, sh β 1, or sh β 1+Rac1. All these three plasmids contained a GFP marker.

The virus infection was done in organoid forming media on day 0 overnight, and a second infection was done on day 1. Media was then changed and cells were incubated for 3 days to achieve gene knockdown. They were then dissociated into single cells and sorted for GFP. Around 30-50% infection efficiency was achieved (Figure 4.9.B). The GFP positive cells were plated into an organoid forming assay.

Significant inhibition of β 1-integrin (more than 80%) was achieved in the sh β 1 and sh β 1+Rac1 infected cells (Figure 4.9 C). Also, GFP expression was stable throughout the 10 days of organoid formation (Figure 4.9 D). Similar to the results obtained from β 1-integrin knockout cells, knocking down β 1-integrin in primary MECs resulted in a significant reduction in stem cells and luminal progenitor frequency. Interestingly, overexpressing a fast-cycling Rac1 in sh β 1 cells restored stem cells but not luminal progenitor organoid formation ability (Figure 4.10)

Together these results suggest that β 1-integrin might control mammary stem cells through Rac1 signaling, but they affect luminal progenitors through a different mechanism.

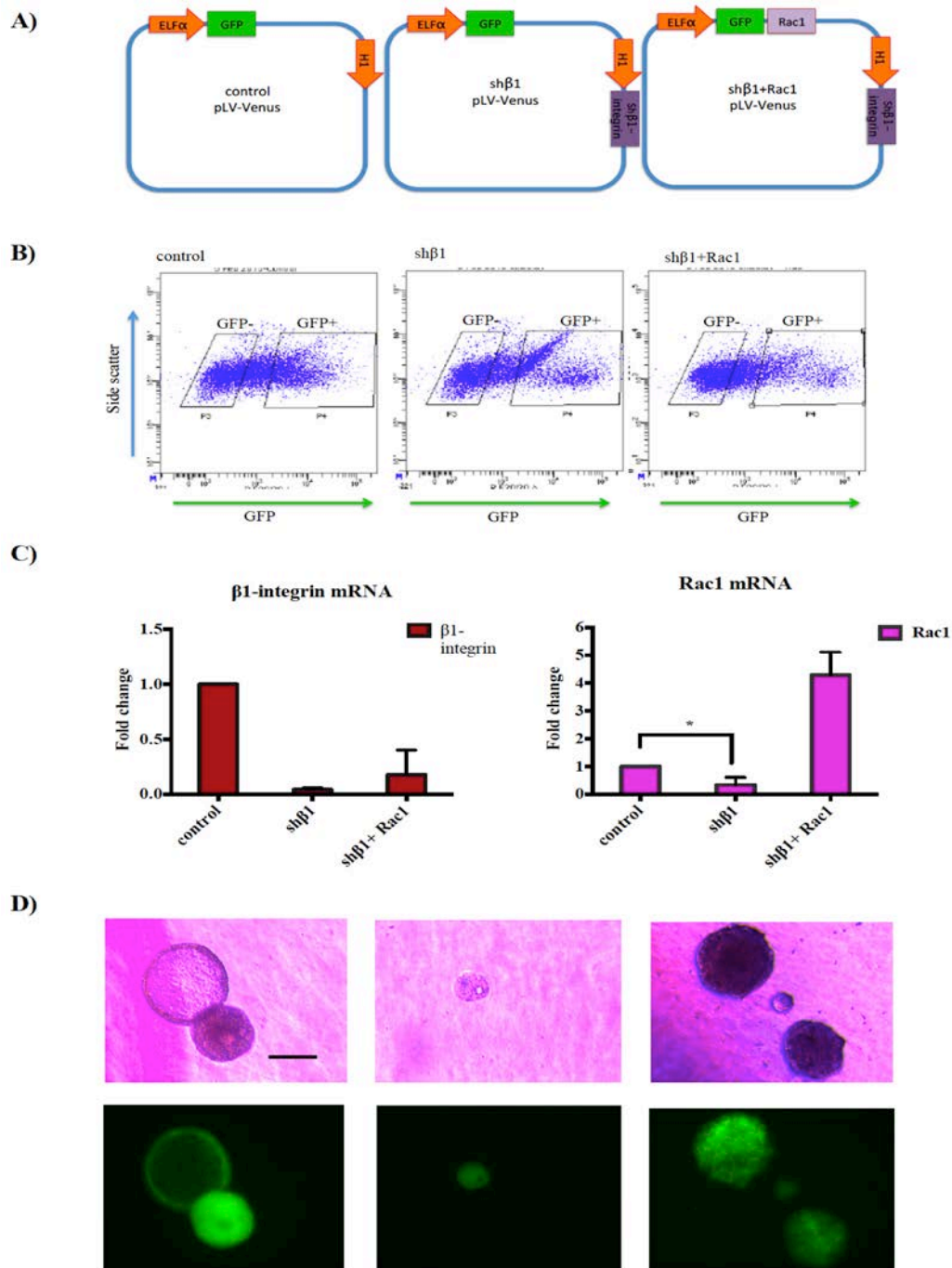


Figure 4.9 Infection of primary MECs with lenti-virus to knockdown β 1-integrin and overexpress active Rac1

Cells from primary MECs of ICR mice were cultured at a density of 5×10^4 /well in a 6-well plate and infected with either control, sh β 1 or sh β 1+ Rac1 viruses overnight then washed and re-infected on the next day with another dose of virus. 16 hours post the second infection; cells were washed with blank media and re-plated in organoid forming media for an extra 3 days. Infected MECs were then dissociated using trypsin/EDTA, passed through a 40 micron filter and sorted for GFP expressing cells A) plasmid map of pLV-Venus plasmid for control, sh β 1 and sh β 1+ Rac1. B) FACS blots showing gating and sorting of GFP positive cells. C) RNA was collected from cells directly after sorting and qRT PCR for β 1-integrin and Rac1 gene expression was performed on control, sh β 1 and sh β 1+ Rac1 infected cells. D) Fluorescent imaging at 10X magnification showing organoids formed on day 10 from control, sh β 1 or sh β + Rac1 GFP cells. Scale bar= 100 μ m.

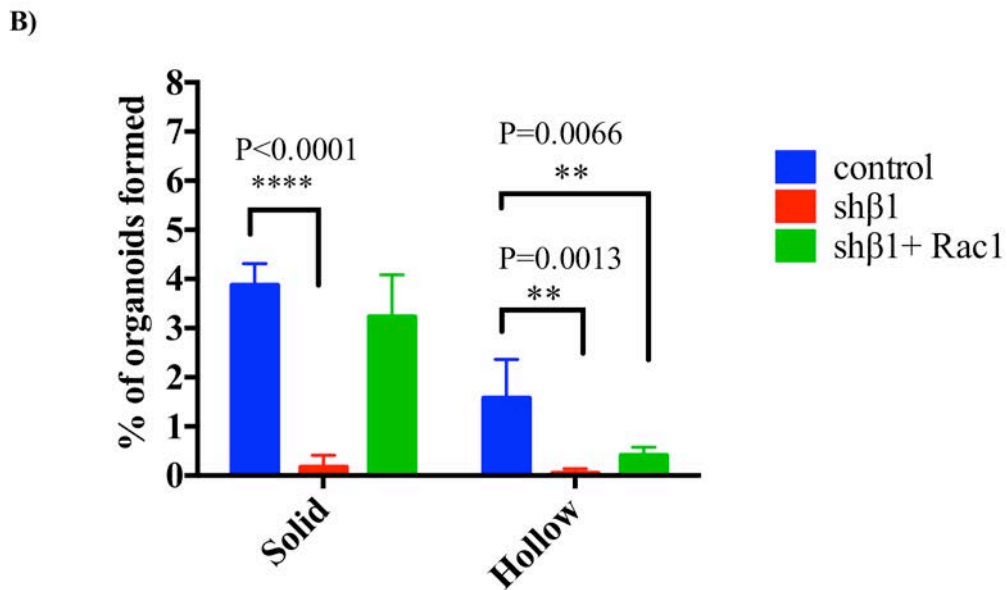
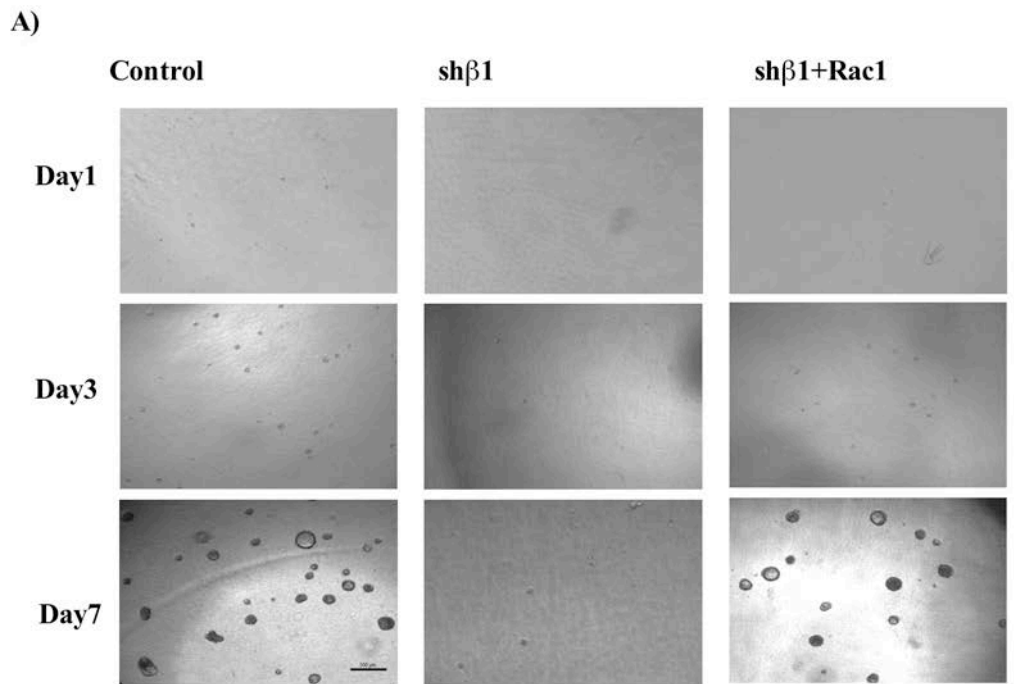


Figure 4.10 Active Rac1 rescues the formation of solid organoids in β 1-integrin knockdown cells.

Cells were collected post FACS sorting for GFP sorting from samples infected by control, sh β 1 or sh β 1+ Rac1 viruses (as explained in Figure 4.9) and then counted. $2 \times 10^3 / \text{cm}^2$ were plated in ultra-low attachment 24-well plates for 10 days. Solid and hollow organoids were then counted using 4X magnification on day 10 and the percentage of organoids formed was calculated. A) Phase contrast at 4X magnification on days 0, 3 and 7 of organoids formed from control, sh β 1 and sh β +Rac1 cells. Scale bar= 500 μm B) percentage of organoids formed from control, sh β 1 and sh β + Rac1 cells (n=4). Statistical significance was determined by one-way Anova test. Error bars in the graph represent standard error of the mean.

4.3 Discussion

Lineage tracing studies have provided evidence that within the mammary gland there are bi-potent stem cells that contribute to both myoepithelial and luminal lineages, as well as luminal-restricted progenitors that can only give rise to differentiated luminal cells (Rios et al., 2014a). The mechanisms that control stem cells and luminal progenitor maintenance might therefore differ.

In this project, I aimed to study the role of integrins in stem cells and luminal progenitors, as they are known to be major microenvironmental sensors that control cell phenotype and fate decisions. I focused on β 1-integrin, which are important adhesion proteins in the mammary gland. They have been used to sort for stem cells, and to distinguish them from luminal progenitors as well as differentiated luminal cells (Shackleton et al., 2006; Stingl et al., 2006). β 1-integrins were previously suggested to have a key role in stem cells, because deleting them in K5-expressing basal cells abolished the gland's regenerative potential (Taddei et al., 2008b). However one can argue that knocking out the β 1-integrin under K5 promoter might cause a secondary regenerative defect, due to the basal cells losing contact with the ECM.

It was therefore important to conduct in-vitro assay from single cells to test for the regenerative capacity of β 1-integrin KO cells. The organoid assay also provided an advantage, which was to test whether β 1-integrin has a role in both luminal progenitors and the stem cell population. In this chapter, I showed that β 1-integrin pathway is required for maintaining stem/progenitor cells, as shown by FACS and organoid-forming assay. There are several explanations for why loss of β 1-integrin results in the loss of stem cell properties. For example, it could be due to defects in proliferation. However, my FACS data suggested that β 1-integrin loss induces differentiation into the differentiated lineages, as shown by the increase in differentiated luminal population.

Knocking out β 1-integrin had a dramatic effect on both stem cells and luminal progenitors, which suggests that it might regulate multiple components of the stem and progenitor signaling pathways. To dissect the mechanism by which β 1-integrin controls stem cells, I therefore knocked out two important downstream targets. I first looked at ILK protein as a major component of the adhesion complex of β 1-integrin. ILK is an essential downstream component of integrin signaling involved in mammary cell differentiation and milk

production (Akhtar et al., 2009). ILK is involved breast cancer stem cell activation by cooperating with Wnt1 signaling (Oloumi et al., 2010). However my results suggested that ILK knockout had no effect on stem cells or luminal progenitor populations. In fact, a small increase in the luminal progenitor population was observed in the ILK null cells. These results suggested that β 1-integrin affects stem cells through a different mechanism other than through ILK signaling.

I therefore examined another important signaling protein downstream β 1-integrin, which is the small Rho GTPase (Rac1) (Jeanes et al., 2012b). Nothing is yet known about whether Rac1 is important for stem cells and luminal progenitors in the mammary gland. Here I showed for the first time that Rac1 is involved in maintaining stem cells, but not luminal progenitors. Therefore, it seems that the β 1-integrin controls luminal progenitor signaling through a different mechanism unrelated to Rac1 activation. Overexpressing an active form of Rac1 also rescued stem cell activity but not luminal progenitors, which confirmed the knockout and Rac1 inhibition results.

Taken together, the data provide new insights about how β 1-integrin controls stem cells and progenitor fate. It would therefore be interesting to investigate how the β 1-integrin-Rac1 signaling might be necessary to maintain the currently unknown profile of transcription factors that control stem cells and luminal progenitors. β 1-integrin is required for Stat5 transcription factor activity in differentiated luminal cells, but nothing is known about its role in regulating other transcription factors (Naylor et al., 2005).

Therefore the next aim will be to try to understand the mechanisms linking integrin-mediated control of the stem cell niche, with the profile of transcription factors that determine the identity of both stem cells and luminal progenitors.

Chapter 5-Results: β 1-integrin signaling and transcriptional regulation of stem cells

5.1 Introduction

In the mammary gland, β 1-integrin involvement in controlling key transcription factors were mainly studied in alveoli differentiation, milk production and cell cycle (Li et al., 2005; Naylor et al., 2005). Nothing is yet known on whether β 1-integrin regulates transcription factors that are important in mammary stem and progenitor cells. In the previous chapter, I showed how deleting β 1-integrin and its downstream effector Rac1 had a key role in maintaining solid organoid forming cells, and therefore might play an important role in maintaining stem cells. From these results, it can be hypothesised that β 1-integrin-Rac1 signaling might be important in transducing signals to TFs in the nucleus and then trigger self-renewal or differentiation of mammary stem cells.

Studies that used gene expression microarray analysis of different subpopulations within the mammary gland had identified signature transcription factors such as β -catenin, cMyc, Slug, MEF2, P63 and Twist (Lim et al., 2010). These are key regulators with multiple roles in mammary cell stem cell activity, fate determination, and tumour initiation and progression (Chakrabarti et al., 2014; Guo et al., 2012; Moumen et al., 2012; Vesuna et al., 2009). The transcription factors Sox9, Elf5, Sox10, and Hey1 (as a target gene for Notch signaling) are regulators of luminal restricted progenitor (Bouras et al., 2008; Chakrabarti et al., 2012; Guo et al., 2012).

In this chapter, I aimed to investigate the mechanisms linking β 1 integrin-mediated stem cell phenotype with transcription factors that determine the identity of both stem cells and luminal progenitors. I therefore looked at the mRNA expression for a panel of transcription factors (or their direct transcriptional target genes) that were known to be involved in stem and progenitor cells.

In this project, I tested for the expression of these transcription factors, and then focused on Wnt/ β -catenin signaling and its downstream transcription targets (Axin2 and Lef1). The reason why I focused on canonical Wnt signaling, is because it has a fundamental role in stem cell activity in the mammary gland (Shackleton et al., 2006). In breast cancer, Wnt/

β -signaling signaling was also shown to be particularly hyperactive in the basal-like subtype and cancer stem cells that express high levels of β 1-integrin (Li et al., 2003). In the embryo tissue, the pathway promotes placode development and is required for initiation of mammary gland morphogenesis (Boras-Granic et al., 2006; Chu et al., 2004; Veltmaat et al., 2004) . It is also important in the postnatal mammary gland, Wnt/ β -catenin signaling controls branching morphogenesis, bud and alveolar formation during pregnancy (Badders et al., 2009; Brisken et al., 2000; Lindvall et al., 2009; Teulière et al., 2005) Lineage tracing experiments showed that Wnt/ β -catenin controls both luminal and basal lineages depending on the developmental stage of the mammary gland (van Amerongen et al., 2012). However, how stem cells sense the microenvironment via adhesion receptors and then activate Wnt/ β -catenin signaling in order to maintain their stem cell property, remains poorly understood.

Axin2 is a direct target gene of the canonical Wnt/ β -catenin pathway and therefore, its mRNA level will be used as a direct readout for Wnt activity (Gehrke et al., 2009; Jho et al., 2002; Leung et al., 2002). Moreover, Axin2-expressing cells have stem cell activity in the mammary gland (Zeng and Nusse, 2010). Based on this, I will be using Axin2 as a functional stem cell marker, which is under the control of Wnt/ β -catenin signaling.

Activating the canonical pathway requires extracellular Wnt ligand binding to receptor complexes containing Frizzled and Lrp5/6 proteins. This results in the phosphorylation of GSK3 β , a direct inhibitor of β -catenin nuclear translocation. The degradation of GSK3 β results in the stabilization of cytoplasmic β -catenin and its translocation into the nucleus. This is followed by transcriptional activation mediated by β -catenin/TCF complexes and transcription of target genes like Lef1 and Axin2 (Jho et al., 2002)

Wnt3a protein is a prototypical example of a ligand for frizzled receptor that consistently activate this pathway (Jho et al., 2002; Many and Brown, 2014). Recombinant human and mouse wnt3a proteins are commercially available and are used in experiments that activate Wnt/ β -catenin signaling in-vitro (Willert and Nusse, 2012; Zeng and Nusse, 2010). Wnt signaling can also be activated chemically by adding lithium chloride. There are also a number of GSK3 β inhibitors available, these inhibitors work on preventing GSK3 β binding to β -catenin and inducing its degradation (Meijer et al., 2003).

Although β 1-integrin signaling and Wnt signaling are crucial in stem cells maintenance, not much work has been done in order to understand whether or not these two pathways interact. In chick embryos, β 1-integrin activity regulates epithelialisation by controlling downstream Wnt and Notch signaling during somite border formation (Rallis et al., 2010). Rac1 was also found to be a crucial component of the Wnt signaling in lymphoid and fibroblasts by its key role in controlling beta-catenin translocation into the nucleus. In response to Wnt activation by Wnt3a, Rac1 activates Jun N-terminal kinase-2 (JNK2), which phosphorylates β -catenin and promotes its nuclear translocation (Jamieson et al., 2015; Wu et al., 2008b). Rac1 activity was also shown to be directly activated by Wnt3a (Valls et al., 2012; Wu et al., 2008b).

In this chapter, I investigated whether there is a link between β 1-integrin-Rac signaling and Wnt signaling by knocking out β 1-integrin, knocking out Rac1 and overexpressing active Rac1 in β 1-integrin knockdown cells. I then checked for Wnt/beta-catenin activity by looking at Wnt target gene expression. I also investigated whether activating Wnt signaling with Wnt3a or with GSK3 inhibitor can rescue the β 1-integrin knockdown stem cell phenotype.

5.2 Results

5.2.1. β 1-integrin regulates multiple transcription factors involved in mammary stem cells and luminal progenitors maintenance

I showed previously that knocking out β 1-integrin affects both solid and hollow organoid formation. In order for the stem and luminal progenitor to commit to a certain fate, it must regulate specific transcription factors that are important in its self-renewal and differentiation.

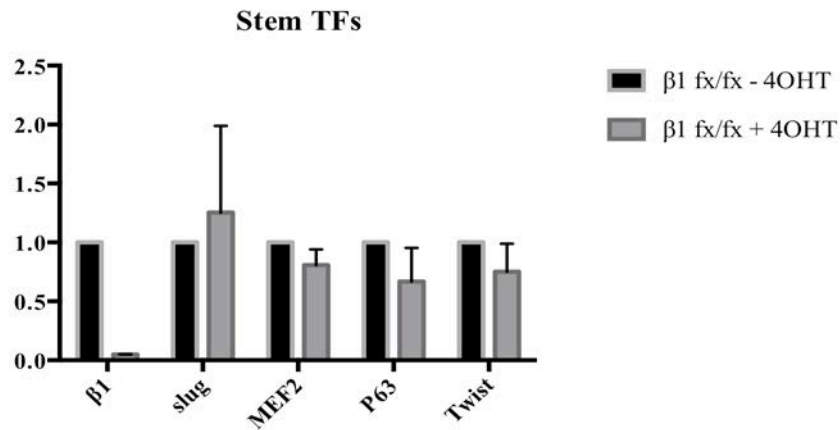
To find candidate transcription factors that might be regulated by β 1-integrin signaling, a screen of β 1-integrin null cells for a number of transcription factors that were known to be important in stem and progenitor maintenance was performed. To achieve that aim, first a

knockout β 1-integrin was done in cells extracted from β 1-integrin^{fx/fx}-CreESRTm female virgin mice. Cells were seeded at a density of 5×10^4 in organoid media in low-attachment 6-well plate with and without 4OHT for 3 days to insure complete β 1-integrin deletion.

The cells were then trypsinised into single cells and cultured under organoid forming conditions (i.e. in organoid media at a density of 2×10^3 in low attachment 24-well plate). 2 days later, cells were collected and RNA was extracted using TriFast RNA extraction reagent, 100 ng of RNA were used to make cDNA using Revers Transciptase cDNA synthesis method. I then performed qPCR reactions using SYBR Green master mix on transcription factors that are expressed in mammary stem cells. These were Slug, MEF2, P63 and Twist (Lim et al., 2010). My results showed that none of these transcription factors were down regulated in β 1-integrin knock out cells, suggesting that β 1-integrin might be regulating stem cells through a different pathway (Figure 5.1 A).

I then looked at transcription factors involved in luminal-restricted progenitors: these included Sox9, Elf5 and Sox10 (Bouras et al., 2008; Chakrabarti et al., 2012; Guo et al., 2012). Knocking out β 1-integrin significantly reduced the expression of Sox9 and Elf5 transcription factors, suggesting a role of β 1-integrin controlling these transcription factors in luminal progenitors (Figure 5.1 B). These results also confirmed my organoid-formation experiments that showed β 1-integrin to be important in luminal-restricted progenitors and not only in basal stem cells.

A)



B)

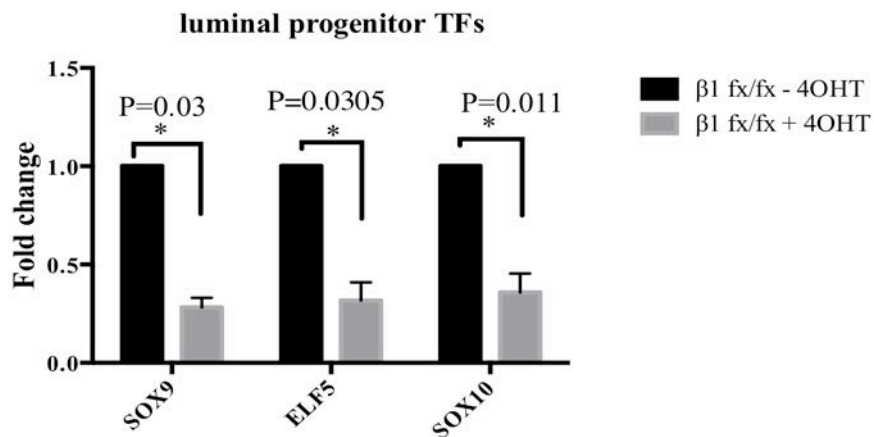


Figure 5.1 Effect of knocking out β1- integrin on stem and progenitor transcription factors.

Primary MECs were enzymatically extracted from β1-integrin^{fx/fx}/CRreESRTM mice and cultured at a density of 5X10⁴ in a 6-well plate +/- 4OHT for 3 days to achieve β1- integrin knockout. Single cells were then cultured in organoid media in a 24-well low attachment plate at a density of 4X10³ cells/well. On day 2, cells were collected and RNA was extracted using TriFast RNA extraction reagent. 100 ng of RNA was used to synthesize cDNA using Reverse Transcriptase reaction. Specific primers were designed for each gene of interest in order to perform qPCR reactions and check for mRNA expression. The ΔCT method was used to determine the fold change of gene expression in β1- integrin knock out cell compared to the control (β1- integrin^{fx/fx} -4OHT). A) Expression of stem cell transcription factors Slug, MEF2 ,P63 and Twist in control and β1- integrin knockout cells. B) Expression of luminal progenitor transcription factors SOX9, Elf5 and SOX10 in control and β1-integrin knockout cells. n=3 error bars are representative of +/- standard error of the mean. Statistical test determined using one-way Anova statistical test.

5.2.2 β 1-integrin and Rac1 knockout down regulate Wnt/ β -catenin signaling

In the previous chapter, β 1-integrin signaling through Rac1 was found to be important in maintaining stem cells but not luminal progenitors. This means that it might be involved in pathways that are active in the basal/stem cell population. One of the important pathways that are active in the basal/stem population is the Wnt/ β -catenin signaling. This pathway is the main pathway, which is involved in maintaining bi-potent basal stem cells in adult and embryonic mammary gland (van Amerongen et al., 2012).

To test whether β 1-integrin and Rac1 regulate Wnt/ β -catenin signaling, the expression of Wnt target genes (Axin2 and Lef1) were measured in β 1-integrin and Rac1 null cells. To do so, cells were first isolated from β 1-integrin^{fx/fx}-CreESRTM and Rac1^{fx/fx}-CreESRTM. They were then cultured with and without 4OHT in organoid media for 3 days to knockout β 1-integrin and Rac1 genes. The cells were then trypsinised and seeded in organoid formation assay. At day 2 of the assay, cells were collected, RNA was extracted, and cDNA was synthesised from these cells. The level of Axin2 and Lef1 was measured using qPCR in control, β 1-integrin and Rac1 null cells. Interestingly, knocking out β 1-integrin and Rac1 significantly reduced Axin2 and Lef1 expression (Figure 5.2).

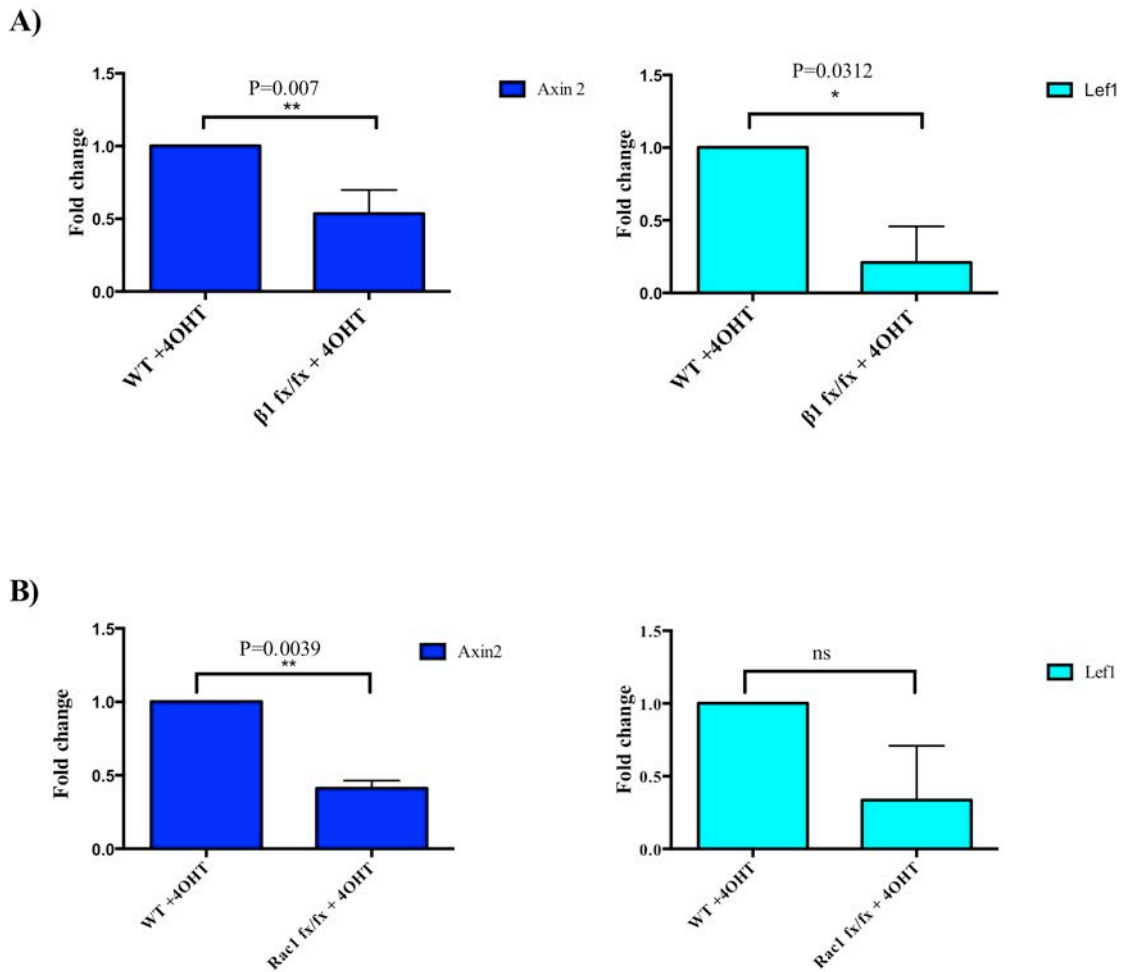


Figure 5.2 Knocking out $\beta 1$ - integrin and Rac1 down regulates Wnt/ β -catenin signaling in MECs.

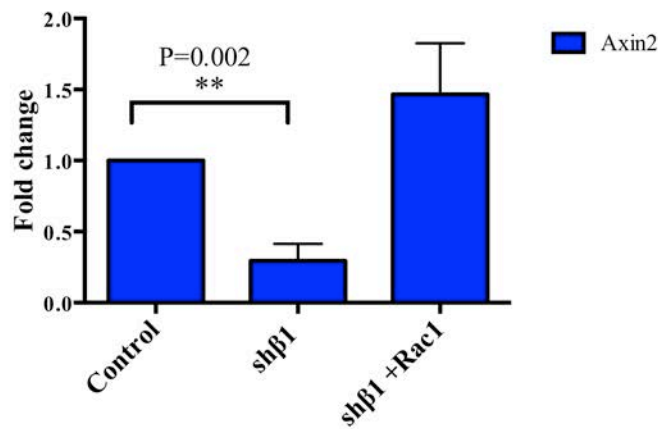
Primary MECs were enzymatically extracted from $\beta 1$ - integrin^{fx/fx/CR^eTM} and Rac1- integrin^{fx/fx/CR^eTM} mice and cultured at a density of 5×10^4 in a 6-well plate +/- 4OHT for 3 days to achieve $\beta 1$ - integrin and Rac1 knockout. Single cells were then cultured in organoid media in 24-well low attachment plate at a density of 4×10^3 cells/well. On day 2, cells were collected and RNA was extracted using TRiFast RNA extraction reagent. 100ng of RNA was used to synthesis cDNA using Reverse transcriptase reaction. Specific primers were designed for Axin2 and Lef1 to perform qPCR reactions and check for mRNA expression. The Δ CT method was used to determine the fold change of gene expression in $\beta 1$ - integrin and Rac1 knock out cell compared to the control ($\beta 1$ - integrin^{fx/fx}-4OHT and Rac1- integrin^{fx/fx}-4OHT).

A) Expression of Axin2 and Lef1 in control and $\beta 1$ -integrin knockout cells. B) Expression of Axin2 and Lef1 in control and Rac1 knockout cells. n=3 error bars are representative of +/- standard error of the mean. Student t-test was done to determine the statistical significance of data.

5.2.3 Active Rac1 can rescue Wnt signaling in β 1-integrin knockdown cells.

I then investigated whether Rac1 can rescue Wnt signaling in β 1-integrin knockdown cells. This was done by infecting cells isolated from ICR mice with control, sh β 1 and sh β 1+ Rac1 viruses. The infection was done in organoid media using the lentivirus stem cell infection protocol. 3 days following infection, cells were sorted for GFP expression and seeded in organoid-forming assay. On Day 2, of the assay, cells were collected and RNA was extracted. A qPCR was then performed on cDNA from those cells and the expression of Axin2 and Lef1 genes in control, sh β 1 and sh β 1+ Rac1 cells was measured. Activating Rac1 in β 1-integrin knockdown cells rescued the expression of Wnt target genes, indicating rescuing of the Wnt/beta-catenin signaling in β 1-integrin knockdown cells (Figure 5.3).

A)



B)

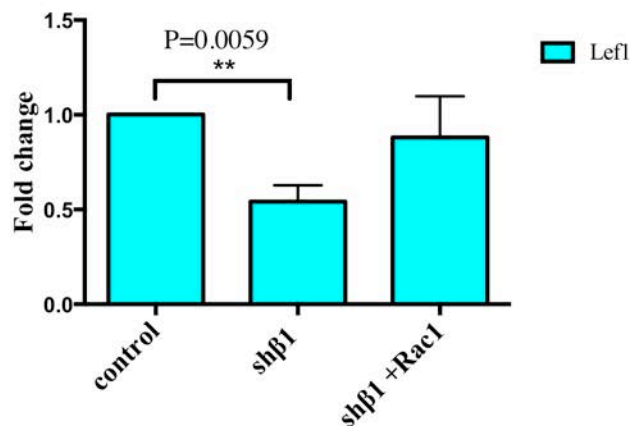


Figure 5.3 Active Rac1 can rescue Wnt/ β -catenin signaling in sh β 1 cells.

Primary MECs were enzymatically extracted from ICR adult female mice and cultured at a density of 5×10^4 in a 6-well plate. Cells were infected with viruses expressing control, sh β 1, and sh β 1+ Rac constructs. 3 days post infection, the cells were sorted for GFP expression and cultured in organoid media in 24-well low attachment plate at a density of 4×10^3 cells/well. On day 2, cells were collected and RNA was extracted using TriFast RNA extraction reagent. 100ng of RNA was used to synthesis cDNA using Reverse transcriptase reaction. Specific primers were designed for Axin2 and Lef1 to perform qPCR reactions and check for mRNA expression. The Δ CT method was used to determine the fold change of gene expression in control, sh β 1, and sh β 1+ Rac1 cells. A) Expression of Axin2 in control, sh β 1, and sh β 1+ Rac1 cells. B) Expression of Lef1 in control, sh β 1, and sh β 1+ Rac cells. n=3 error bars are representative of \pm standard error of the mean. One-way Anova test was done to determine the statistical significance of data.

5.2.4 Effect of β 1-integrin and Rac1 knockdown on Notch signaling:

Notch signaling is one of the most established pathways in luminal progenitors and their differentiation. Notch signaling restricts stem and progenitor cells self-renewal and promotes their commitment/differentiation to a luminal fate (Aasen et al., 2008; Bouras et al., 2008; Buono et al., 2006). Notch signaling is regulated by Wnt/ β -catenin pathway and Wnt signaling suppresses Notch in order to prevent stem cells from differentiating into the luminal lineage (Rodilla et al., 2015). I showed in the previous chapter that when β 1-integrin was deleted, there was an increase in the luminal population, particularly in the differentiated luminal cells. This pathway could therefore, have a direct link with the β 1-integrin signaling pathway.

In order to investigate the link between β 1-integrin and Notch signaling, qPCR was performed for Notch targets Hes1, Hes5, Hey1 and Hey2 (Borggreffe and Oswald, 2009) on cells obtained from β 1-integrin and Rac1 knockouts. All of these targets were up regulated in β 1-integrin knockout cells (Figure 5.4A). In the Rac1 knockout cells, those target genes remained unchanged (Figure 5.4 B). These results show that β 1-integrin might have a direct effect on Notch signaling and provide a possible explanation for the increase in luminal cells in the β 1-integrin knockout cells as shown the previous chapter.

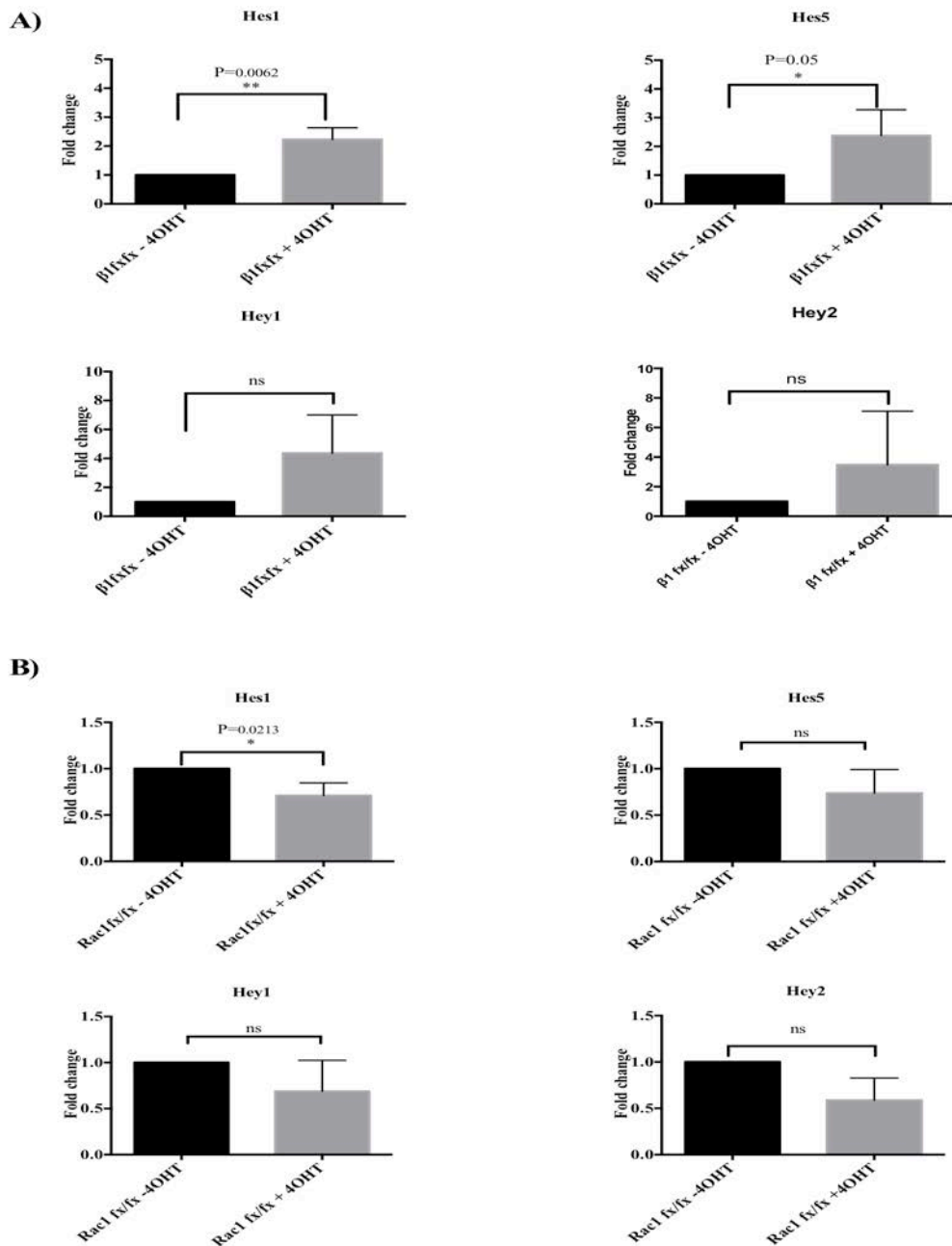


Figure 5.4 Effect of knocking out β1- integrin and Rac1 on Notch signaling.

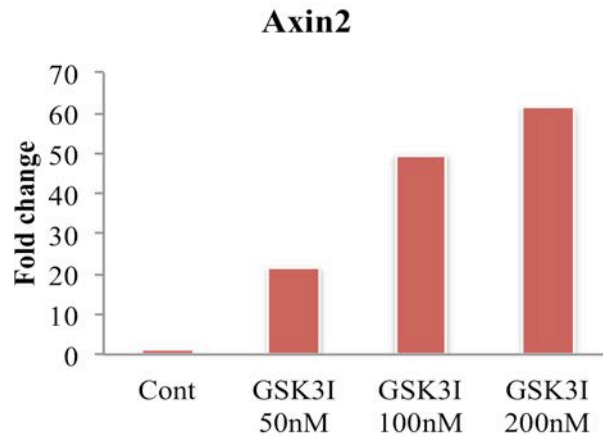
Primary MECs were enzymatically extracted from β1- integrin^{fx/fx}/CRETM and Rac1^{fx/fx}/CRETM mice and cultured at a density of 5X10⁴ in a 6-well plate +/- 4OHT for 3 days to achieve β1-integrin and Rac1 knockout. Single cells were then cultured in organoid media in 24-well low attachment plate at a density of 4X10³ cells/well. On day 2, cells were collected and RNA was extracted using TriFast RNA extraction reagent. 100ng of RNA was used to synthesis cDNA using Reverse transcriptase reaction. Specific primers were designed for Hes1, Hes5, Hey1 and Hey2 Notch targets to perform qPCR reactions and check for their mRNA expression. The ΔCT method was used to determine the fold change of gene expression in β1- integrin and Rac1 knock out cell compared to the control (β1- integrin^{fx/fx} -4OHT and Rac1^{fx/fx} -4OHT). A) Expression of Notch targets in control and β1- integrin knockout cells. B) Expression of Notch targets in control and Rac1 knockout cells. n=3 error bars are representative of +/- standard error of the mean. Student T-test was done to determine the statistical significance of data

5.2.5 Activation of Wnt/ β -catenin signaling using wnt3a and GSK3 inhibitor induces solid and reduces hollow organoid-forming cells

For this project, I chose to focus on the Wnt/ β -catenin signalling pathway, as I found that β 1-integrin – Rac1 signaling is linked to the Wnt/ β -catenin pathway. The next experiment will be to try and rescue the β 1-integrin and Rac1 knockout stem cell phenotype by activating canonical Wnt pathway and its downstream targets. To activate Wnt signaling, I used recombinant mouse wnt3a ligand protein (Sato et al., 2004; Willert and Nusse, 2012) and GSK3 β inhibitor (GSK3I) (known commercially as CHIR 99021) (Ring et al., 2003).

Before performing rescue experiments, I first needed to find the optimal dose of wnt3a and GSK3I that activates Wnt signaling in primary MECs. To do so, a dose optimisation experiment was done where different doses (50, 100, and 200 ng/ml) of wnt3a and (50, 100 and 200 nM) of GSK3I were added to primary MECs in an organoid formation assay. Each dose was done in duplicate in a 24-well low attachment plate in organoid media. 4×10^3 were added to each well. One well from each dose was collected at 48 hours post treatment for RNA extraction and the second well was kept for organoid formation. qPCR was performed for Axin2 gene as a readout of the Wnt/ β -catenin activity. GSK3I enhanced the expression of Axin2 in a dose dependent manner (Figure 5.5 A). The 20 ng/ml of Wnt3a dose did not increase Axin2 expression. However, doses 100 and 200 ng/ml activated Axin2 expression by 5 and 8 fold respectively (Figure 5.5 B).

A)



B)

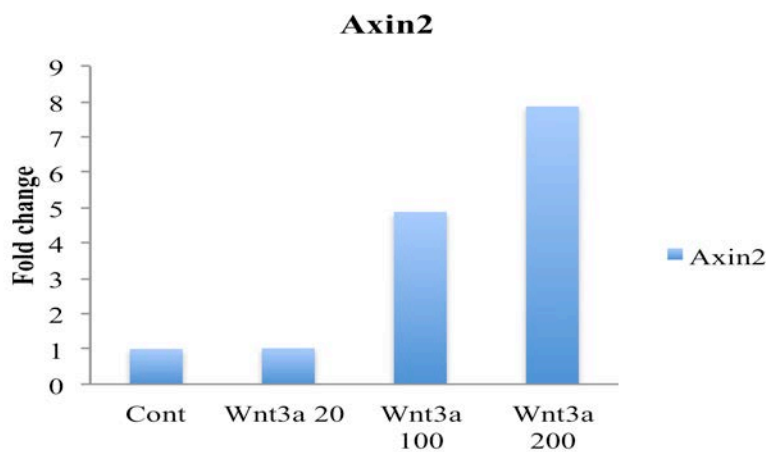


Figure 5.5 Activating Wnt signaling using different doses of Wnt3a and GSK3I

Primary MECs were enzymatically extracted from ICR mice and single cells were then cultured in organoid media in 24-well low attachment plate at a density of 4×10^3 cells/well. Cells were then treated with concentrations of 0, 50, 100 or 200 nM GSK3I or with 0, 20, 100, or 200 ng/ml of Wnt3a. On day 2, cells were collected and RNA was extracted using TriFast RNA extraction reagent. 100 ng of RNA was used to synthesis cDNA using Reverse transcriptase reaction. Specific primers were designed for Axin2 Wnt target to perform qPCR reactions and check for its mRNA expression. The ΔCT method was used to determine the fold change of gene expression in different samples compared to the untreated control. A) Expression of Wnt target gene Axin2 in GSK3I treated cells B) Expression of wnt target gene Axin2 in wnt3a treated cells. n=1.

An organoid assay was also performed on cells treated with different doses of GSK3I and Wnt3a. GSK3I at dose of 50nM induced the highest stem cell activity (5 fold increase). The doses 20,100 and 200 ng/ml of Wnt3a all enhanced solid organoid activity at a similar level. Interestingly, both GSK3I and Wnt3a reduced hollow organoid formation. They also suppressed the expression of Notch target genes Hes1, Hes5, Hey1 and Hey2 (Figure 5.6). These results confirmed previous studies that showed that Wnt signalling supresses Notch signalling in order to maintain bi-potent stem cells and prevent them from differentiating into luminal cells (Gu et al., 2013). Based on this experiment, The dose of 50 nM GSK3I and the 100ng/ml concentration of Wnt3a were chosen to activate the Wnt/ β -catenin pathway in the rescue experiments.

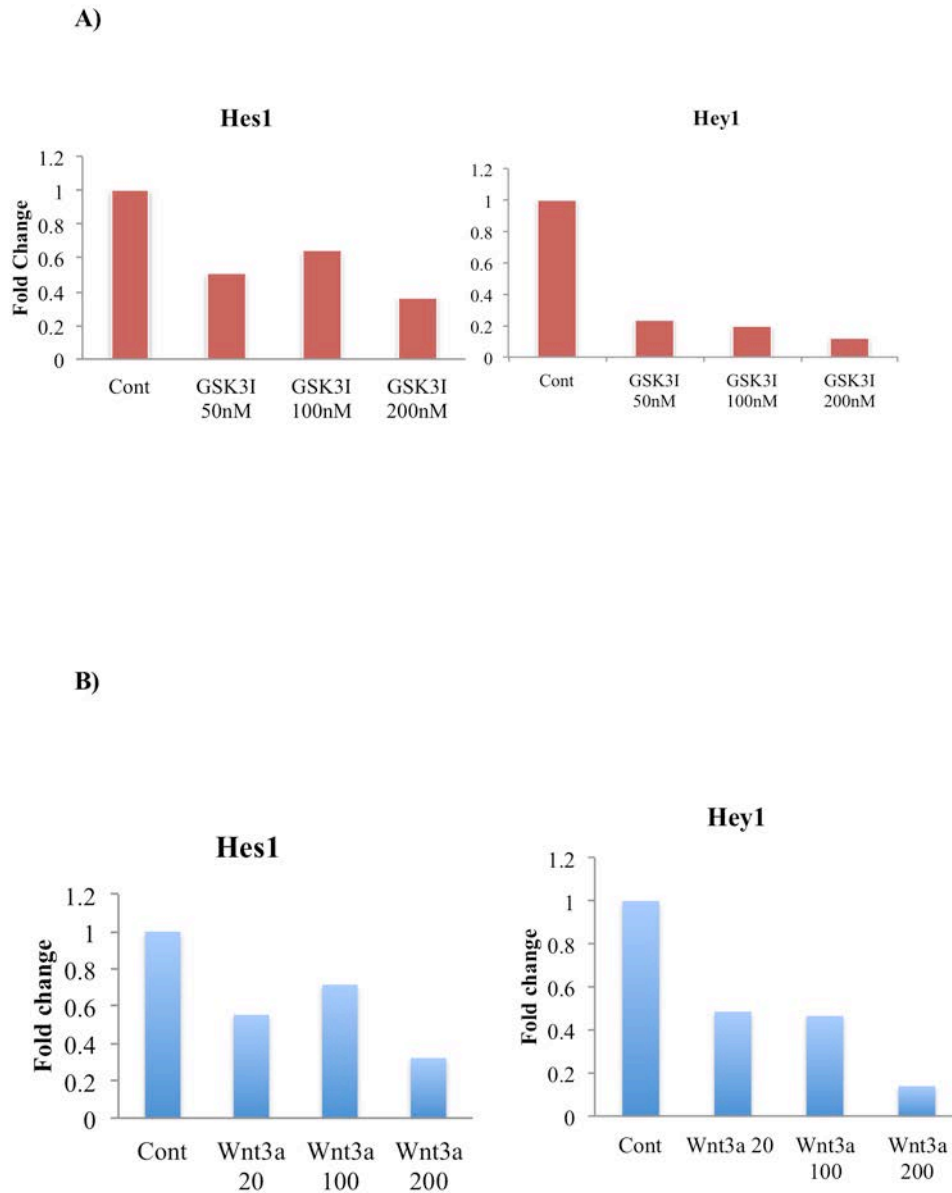


Figure 5.6 Activating Wnt signaling using different doses of Wnt3a and GSK3I suppresses Notch target gene expression

Primary MECs were enzymatically extracted from ICR cells and single cells were then cultured in organoid media in 24-well low attachment plate at a density of 4×10^3 cells/well. Cells were treated with 0, 50, 100 or 200nM GSK3I or with doses 0, 20, 100, or 200 ng/ml of Wnt3a. On day 2, cells were collected and RNA was extracted using TriFast RNA extraction reagent. 100 ng of RNA was used to synthesise cDNA using Reverse transcriptase reaction. Specific primers were designed for Hes1 and Hey1 Notch targets to perform qPCR reactions and measure mRNA expression. The ΔCT method was used to determine the fold change of gene expression in different samples compared to the untreated control.

A) Expression of Notch target gene Hes1 and Hey1 in GSK3I treated cells B) Expression of Notch target gene Hes1 and Hey1 in wnt3a treated cells. n=1.

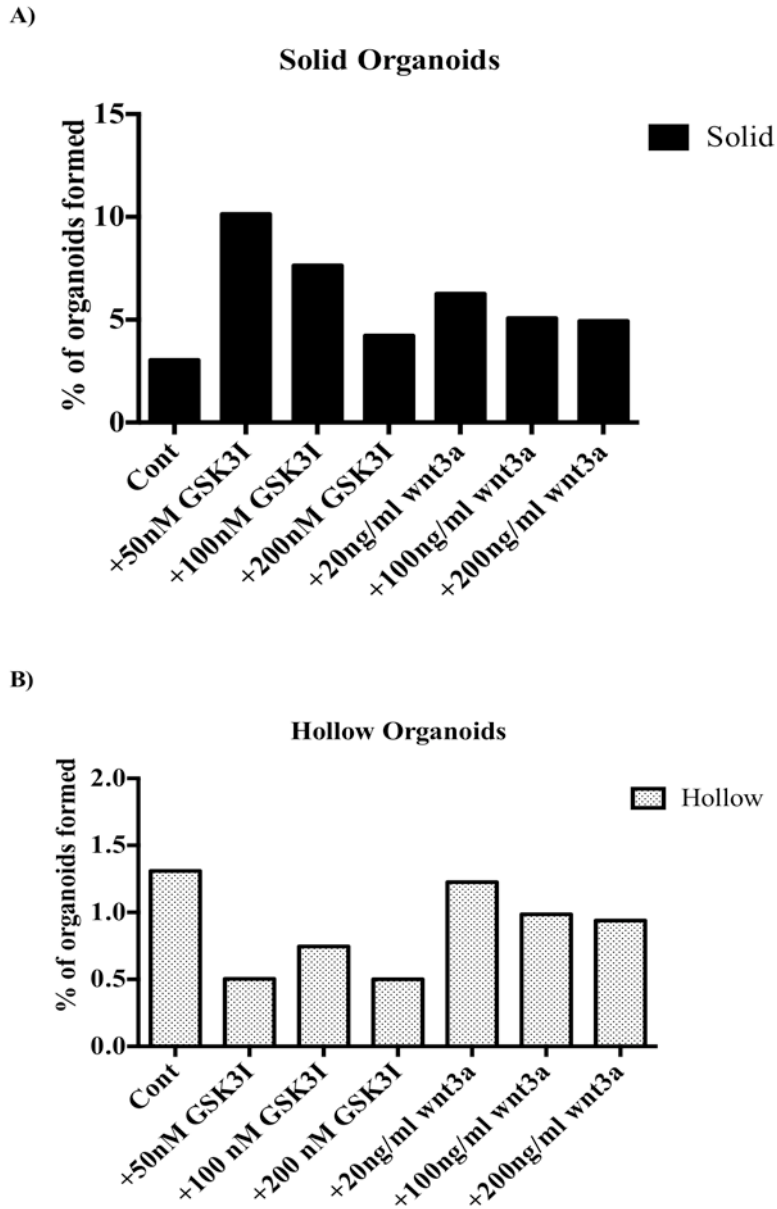


Figure 5.7 Activating Wnt signaling by different concentrations of Wnt3a and GSK3I increases solid and suppresses hollow organoid-forming activity
 Cells were collected from primary preps of ICR mice. MECs were dissociated into single cells and cultured in organoid forming assay at a density of $2 \times 10^4 / \text{cm}^2$. Cells were treated in different wells with doses of 0, 50, 100 or 200nM GSK3I or with doses 0, 20, 100, or 200 ng/ml of wnt3a and left for 10 days to form stem and luminal progenitor organoids. Organoids were counted using 4X magnification and divided by the number of cells seeded to obtain a % of organoid formed. A) Quantification of organoids formed from stem cells. B) Quantification of Organoids formed from luminal progenitors.

5.2.6 Activation of Wnt signaling by GSK3I and not Wnt3a rescue stem cells in β 1-integrin null MECs

In order to investigate the link between β 1-integrin signaling and Wnt signaling, I tried to rescue the β 1-integrin knockout solid organoids by activating Wnt signaling in those cells either by adding 100 ng/ml Wnt3a or 50 nM GSK3I to the organoid culture media. This was done by first obtaining β 1-integrin knockout cells after 3 days of 4OHT treatment. Single cells from control and β 1-integrin knockout were cultured in an organoid assays with and without Wnt3a or with and without GSK3I. The addition of Wnt3a to β 1-integrin null cells did not rescue the stem cell phenotype. Interestingly, it activated the formation of luminal organoids. This was not expected, especially that wnt3a suppressed luminal progenitor formation in the presence of β 1-integrin. This is suggesting a new role of β 1 integrin in Wnt3a mediated stem cell activity and suppression of luminal progenitors.

The addition of GSK3I to β 1-integrin cells caused a rescue of solid organoid-formation activity. Hollow organoid formation was still suppressed in the control+GSK3I and in the β 1-integrin KO+ GSK3I cells. This suggested that β 1-integrin is controlling Wnt/ β -catenin signaling upstream of GSK3 β (Figure 5.8).

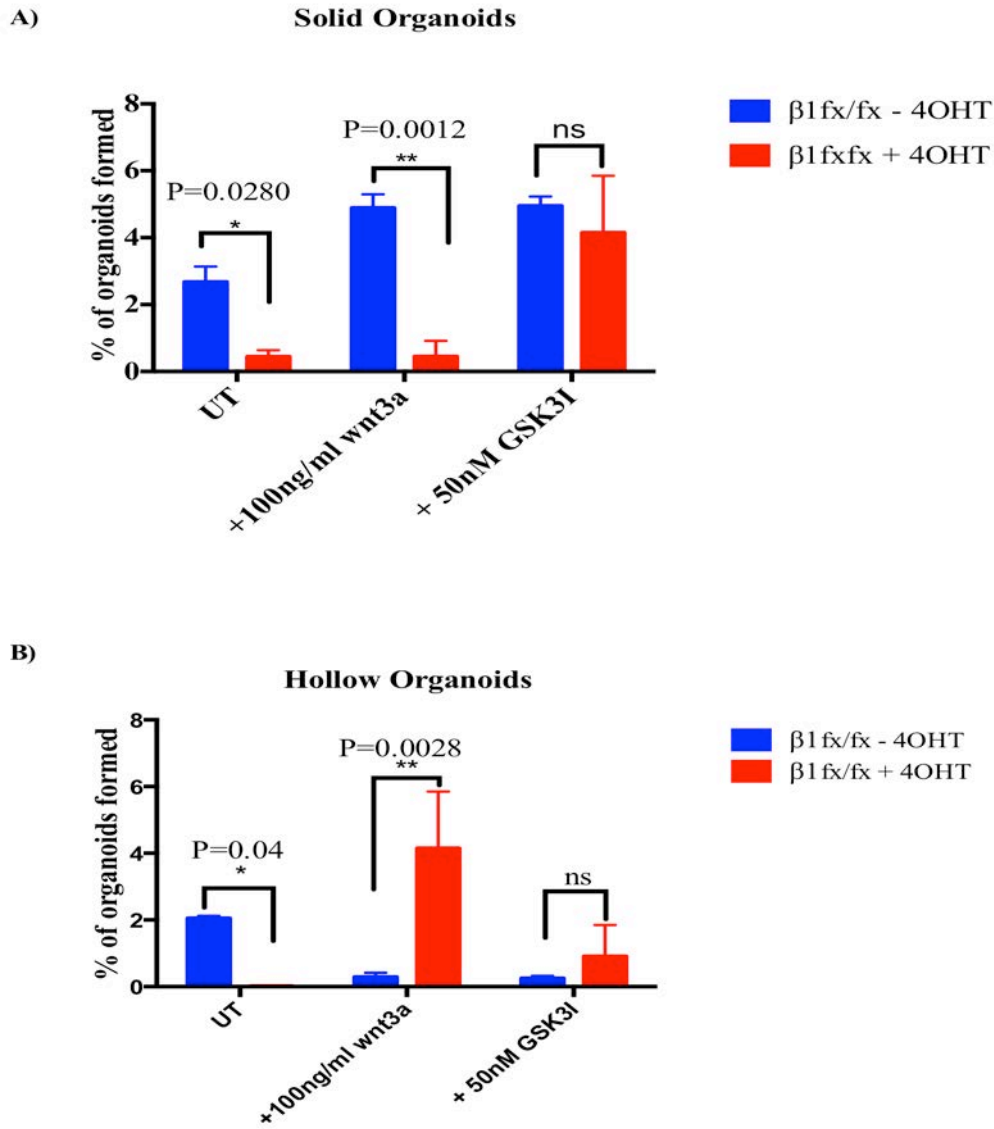


Figure 5.8 Effect of Wnt3a and GSK3I treatment on the formation of solid and hollow organoid in $\beta 1$ -integrin knockout cells.

Cells were collected from primary preps of $\beta 1$ -integrin^{fx/fx}/CR^eTM mice. MECs were dissociated into single cells and cultured in organoid forming assay at a density of $2 \times 10^4/cm^2$. Cells of control and $\beta 1$ -integrin knockout were treated in different wells with 100 ng/ml wnt3a and 50 nMGSK3I. Cells were left for 10 days in organoid media to form solid and hollow organoids. Organoids were counted using 4X magnification and divided by the number of cells seeded at day 0 and multiplied by 100 to obtain % of organoid formed. A) Quantification of solid organoids formed from control and $\beta 1$ -integrin knockout cells. B) Quantification of hollow organoids formed from control and $\beta 1$ -integrin knockout. Error bars represent standard error of the mean. N=2 . ns= non significant.

This experiment was then repeated but protein from cells was obtained. A nuclear and cytoplasmic fractionation was then performed on cells obtained from day 2 of the organoid assay. A western blot on the nuclear fraction of these cells was done to check for β -catenin translocation into the nucleus after Wnt3a and GSK3I treatment. Due to the low cell number, this assay was challenging and hard to repeat. It was possible however to obtain one western blot that showed that β -catenin was translocated into the nucleus in control cells activated with Wnt3a and GSK3I. Interestingly, when activating β 1-integrin knockout cells using Wnt3a, there was much less of β -catenin translocation compared to the control Wnt3a activated cells. GSK3I however induced as similar level of nuclear β -catenin translocation in control and β 1-knockout cells (Figure 5.9). This suggests an explanation on why Wnt3a did not rescue the β 1-integrin knockout stem cells .

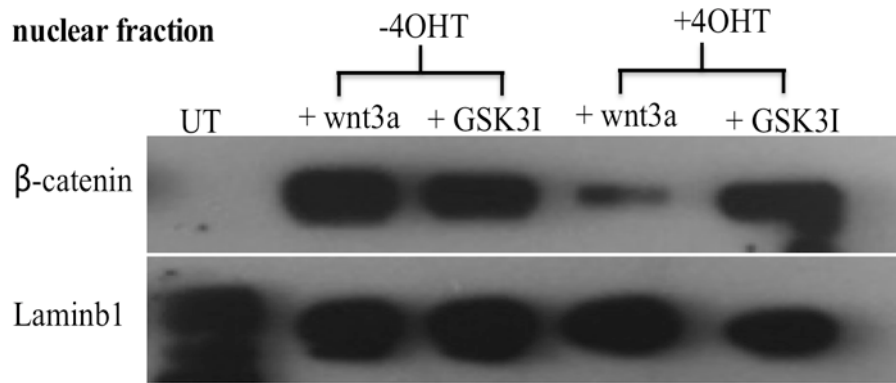


Figure 5.9 β 1-integrin knock out and nuclear translocation of β -catenin in response to wnt3a and GSK3I treatment. Cells were collected from primary preps of β 1- integrin^{fx/fx}/CreTM mice. MECs were dissociated into single cells and cultured in organoid forming assay at a density of $2 \times 10^4/\text{cm}^2$. This time the cells were cultured in a 6-well plate to obtain higher number of cells. Cells of control and β 1-integrin were treated in different wells with 100 ng/ml Wnt3a and 50 nM GSK3I. Cells were left for 2 days in organoid media and then collected for nuclear and cytoplasmic protein fractionation. 20 μ g of nuclear protein was loaded in each well and nuclear β -catenin was detected using β -catenin antibody (cell signaling - 9582). Laminb1 was used as an internal loading control and was detected using Rabbit anti-Laminb1 antibody (AbCAM-ab16048).

5.2.7 Expression of active Rac1 in β 1-integrin knockdown and stem cell activation using wnt3a and GSK3I

I showed previously that active Rac1 could rescue Axin2 RNA expression in β 1-integrin knockdown cells. I also showed that adding wnt3a to β 1-integrin knockout cells couldn't rescue the stem cell phenotype. Next, it was important to test whether adding wnt3a can activate stem cells in β 1-integrin knockdown cells that express active Rac1. This will help in understanding whether Rac1 is required in wnt3a stem cell rescuing of β 1-integrin knockdown cells.

To achieve this aim, total MECs were isolated from ICR mice and infected with a virus containing control, sh β 1 and sh β 1+Rac1 plasmid in organoid-forming conditions. The cells were sorted for GFP expression and seeded in organoid forming media with and without wnt3a and GSK3I for 7-10 days. Basal and luminal organoids were then counted on at 4X magnification.

Similar to the β 1-integrin knockout experiments, Wnt3a did not rescue the solid organoids but activated hollow organoid formation in sh β 1 cells. GSK3I rescued solid organoid formation in sh β 1 and maintained its suppression of hollow organoids. In the sh β 1 cells that express active Rac1, wnt3a activated solid organoid formation and did not cause an increase in hollow organoid frequency. This suggests that active Rac1 can activate Wnt3a induction of stem cell and suppression of luminal progenitors activity.

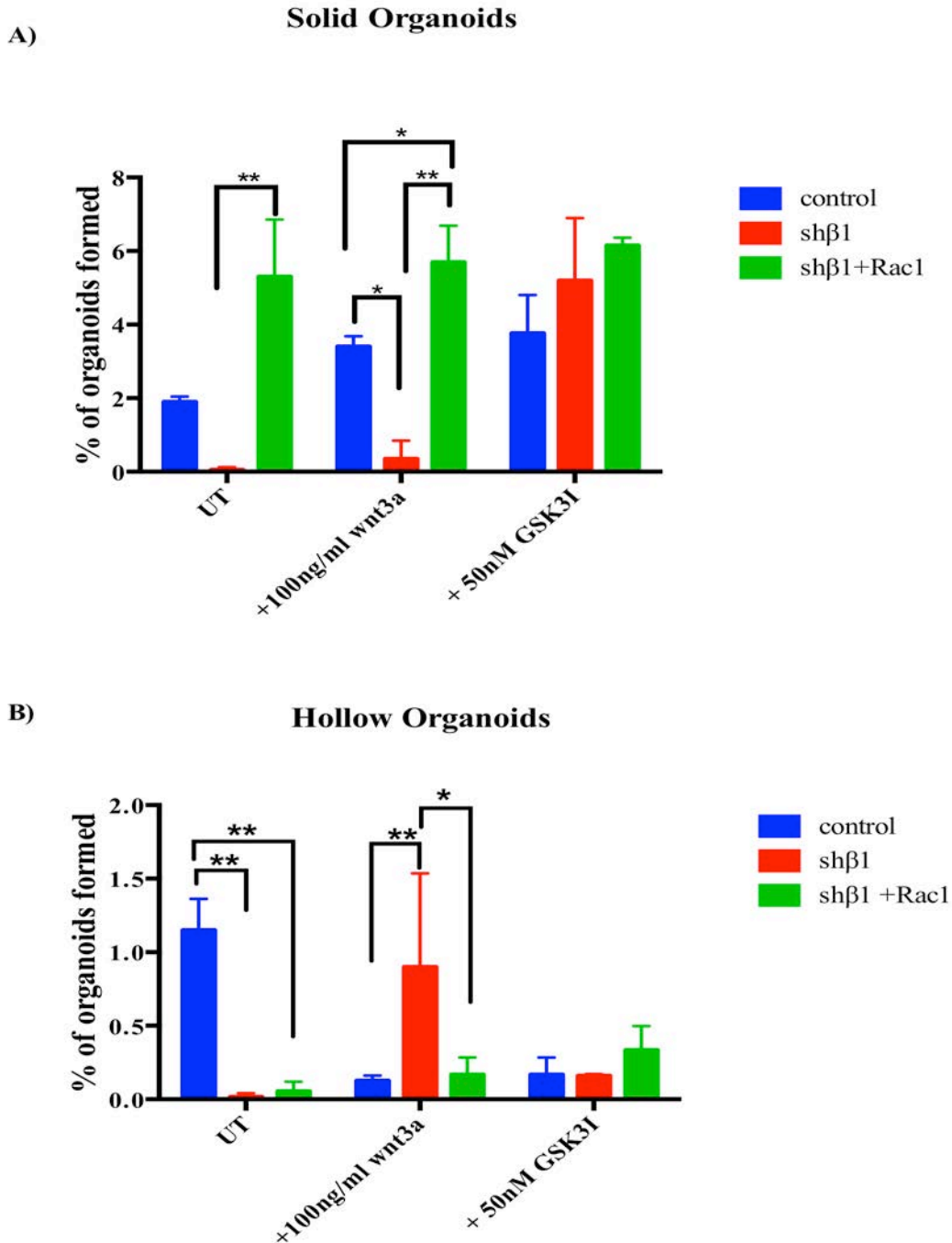


Figure 5.10 effect of Wnt3a and GSK3I on solid and hollow organoid formation in shβ1, and shβ1+ Rac cells. Primary MECs were enzymatically extracted from ICR adult nulliparous female mice and cultured at a density of 5×10^4 in a 6-well plate. Cells were infected with viruses expressing control, shβ1, and shβ1+ Rac1 constructs. 3 days post infection; the cells were sorted for GFP expression and cultured in organoid media in 24-well low attachment plate at a density of $2 \times 10^3/\text{cm}^2$. Cells from each virus-infected group were cultured either -/+ wnt3a or -/+ GSK3I. Cells were then left for 10 days to form organoids. The number of organoids obtained was divided by the number of cells seeded at day 0 and multiplied by 100 to obtain % of organoids formed. A) Quantification of solid cell organoids for each group. B) Quantification of Hollow organoids for each group. n=3 error bars are representative of +/- standard error of the mean. One-way Anova statistical test was done in order to determine the statistical significance of data.

5.2.8 Inhibition of Rac1 inhibits Wnt -induced stem cell activation.

I then wanted to investigate the role of Rac1 in activating Wnt/ β -catenin signaling. To do so, MECs from ICR mice were obtained and cultured for 3 days with and without Rac1 inhibitor (EHT1864). This was done in low attachment 6-well plates in organoid media. The cells from control and EHT- treated cells were then trypsinised into single cells and cultured in organoid formation assay. Both control and EHT-treated cells were cultured either alone, with 100ng/ml Wnt3a, or with 50nM GSK3I in organoid formation conditions. The cells were then cultured for 7-10 and organoids of luminal and stem cells were then counted using 4X magnification.

The addition of the Rac1 inhibitor alone resulted in the activity inhibition of solid but not hollow organoids. Adding Wnt3a or GSK3I to Rac1-inhibited (EHT-treated) cells did not rescue the stem cell phenotype. There was a slight increase in the hollow organoid frequency when adding Wnt3a to EHT-treated cells. However, this increase was not significant. Similar to the control cells, GSK3I inhibited the formation of hollow organoids in EHT-treated cells (Figure 5.11). These results suggest that without Rac1 activity, Wnt3a and GSK3I cannot activate solid organoid forming cells. Also, active Rac1 seems to have no role in Wnt mediated suppression of hollow organoid formation.

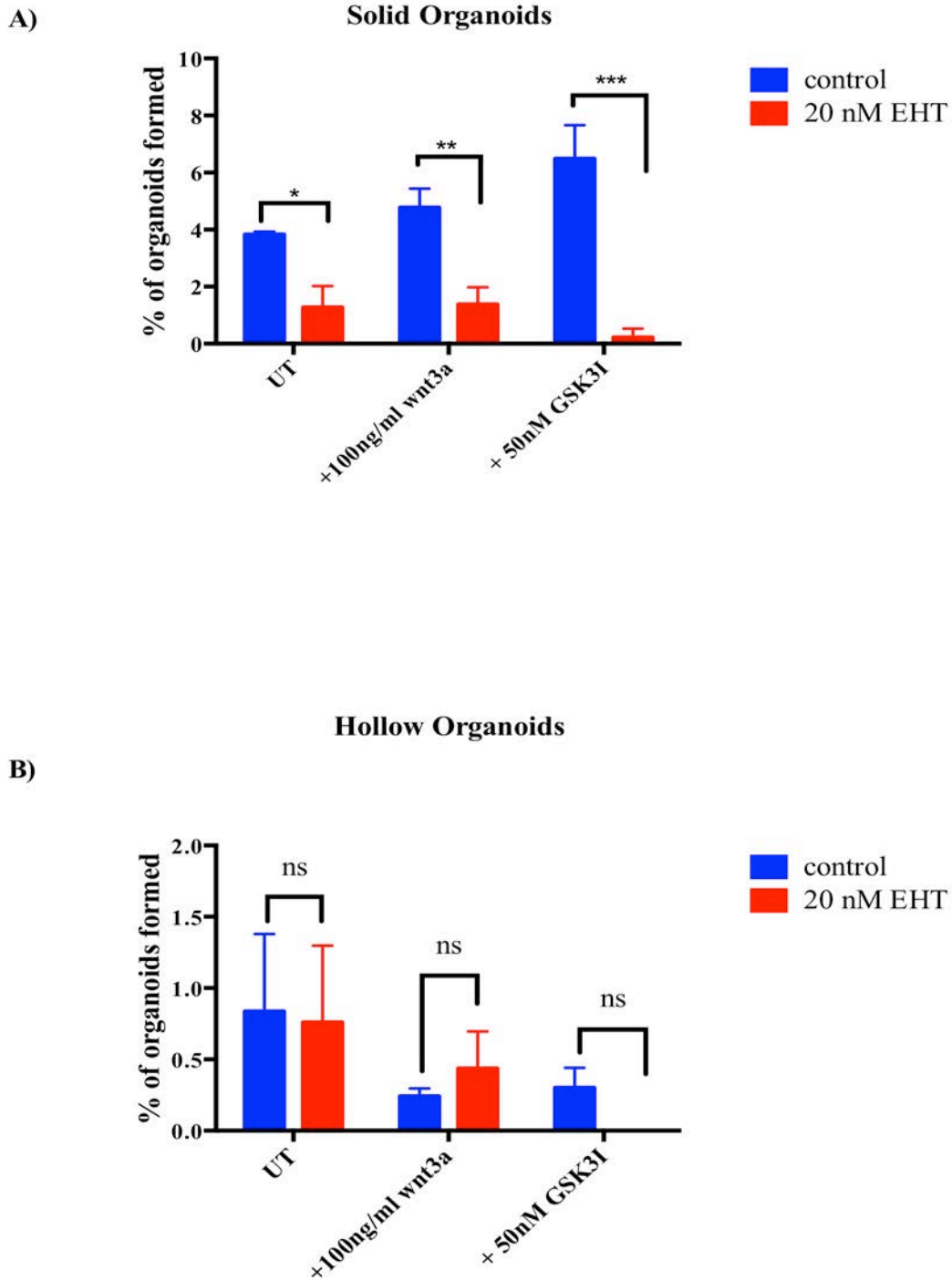


Figure 5.11 Inhibiting Rac1 by EHT1864 compound prevents induction of solid organoid activation by wnt3a and GSK3I.

Primary MECs were enzymatically extracted from ICR adult nulliparous female mice and cultured at a density of 5×10^4 in a 6-well plate. Cells were treated for 3 days with and without 20 nM Rac1 inhibitor (EHT 1864). Control and EHT-treated MECs were dissociated into single cells and cultured in organoid forming assay. Each of these two groups was also cultured with and without 100ng/ml wnt3a or 50 nM GSK3I. Cells were then left for 10 days to form organoids. The number of organoids obtained was divided by the number of cells seeded at day 0 and multiplied by 100 to obtain % of organoids formed. A) Quantification of solid organoids formed from each group. B) Quantification of hollow organoids formed from each group. n=2 error bars are representative of \pm standard error of the mean. One -way Anova statistical test was done in order to determine the statistical

5.3 Discussion:

5.3.1:Beta1-integrin control the expression of luminal transcription factors

Sox9:

(Sex-determining region Y [SRY]-box 9 protein) Sox9 is a high mobility group box transcription factor that is critical in regulating embryo development and in the development, differentiation, and lineage commitment of adult stem cells (Kiefer, 2007). Sox9 is important in the maintenance of stem or progenitor cells in liver, hair follicle, intestine, and pancreas (Cheung and Briscoe, 2003; Vidal et al., 2005). In the mammary gland ectopic expression of Sox9 together with Slug was sufficient in reprogramming differentiated cells into mammary stem cells. When Sox9 was expressed alone the differentiated luminal cells turned into luminal progenitors (Guo et al., 2012) when deleting Sox9 in the mammary gland, Sox9 affected the luminal cells and not basal cells, lineage commitment and proliferation (Malhotra et al., 2014). My results showed that Slug expression was not affected when knocking out β 1-integrin. Sox9 was however down regulated significantly. This suggests that β 1-integrin might be controlling luminal progenitor activity through Sox9. These results are novel and are worthy of further investigations as nothing is known so far about β 1- integrin control of Sox9 in stem cells.

Elf5:

The transcription factor E74-like factor 5 (Elf5) functions as a downstream of the prolactin receptor signaling pathway and plays an important role in mammary gland development (Cordero et al., 2015). The conditional mouse knockout Elf5-null mammary glands exhibit a complete failure of alveologenesis during pregnancy (Oakes et al., 2008b). Elf5 is also expressed in long-lived ductal luminal progenitors and contributes to the maintenance of the adult nulliparous mammary gland (Rios et al., 2014b). It also regulates luminal progenitors through suppressing Notch signaling in these cells (Chakrabarti et al., 2012). The down regulation of Elf5 in β 1-integrin KO cells might therefore provide an explanation of why there has been an elevated expression of Notch targets in β 1-integrin KO cells. Thus these results will contribute to the underlying molecular mechanism for the altered cell lineage decisions in β 1-integrin null mammary epithelial cells.

5.3.2: β 1-integrin controls Wnt signaling

Activation of canonical Wnt signaling requires the presence of a Wnt proteins that binds to the Wnt receptor Frizzled and LRP5/6. Without Wnt signaling, the β -catenin transcription factor cannot translocate into the nucleus and is degraded by a degradation complex made of the following proteins: Protein phosphatase 2A (PP2A), Axin, Adenomatous polyposis coli (APC), glycogen synthase kinase3 (GSK3) casein kinase (CKI). That complex binds to β -catenin, degrades it, and subsequently sends it to the proteasome to be digested.

However, when Wnt proteins like (Wnt3a) binds to Frizzled and LRP5/6 receptors, the negative Wnt regulator translocate from the complex into the cytoplasm. The destruction complex function becomes disrupted. Activating Wnt/ β -catenin pathway causes an accumulation of β -catenin in the cytoplasm, followed by its translocation into the nucleus. Active β -catenin then acts as a transcriptional co-activator for the TCF/LEF transcription factors. The direct downstream targets of Wnt/ β -catenin signaling are Axin2 and Lef1. The mRNA expression of these two genes are therefore direct indicators of the Wnt signaling activity (Jho et al., 2002). Thus, the mRNA expression of these two genes was measured in my experiments as an indicator of Wnt/ β -catenin signaling activation.

Wnt/ β -catenin signaling in the mammary stem cells is important for maintaining their self-renewal and bi-potency (Gu et al., 2013). My results showed that when adding Wnt3a and GSK3 inhibitor to the organoid culture, it causes an increase in solid organoid activity and a suppression hollow organoid formation. I also found that it directly down regulates Notch signaling as shown by a decrease in Notch targets expression. This could explain why there has been a reduction in the hollow organoid frequency in wnt activated cells, as Notch signaling enhances progenitor commitment to the luminal lineage (Bouras et al., 2008).

Integrins act at signaling crossroads, and their interactions with other signal transduction pathways are key to the regulation of many types of normal and cancer stem cells (Campos et al., 2006b; Guan, 2010). Like the Wnt/ β -catenin signaling, β 1-integrin is highly expressed in stem cells (Shackleton et al., 2006). Nothing is yet known whether these two pathways interact in the mammary gland and control stem cells. I found from my deletion studies that β 1-integrin is important in mammary stem cells, and therefore I wanted to

investigate the effect of deleting $\beta 1$ -integrin on Wnt / β -catenin signaling. My results showed that deleting $\beta 1$ -integgrin by adding 4-OHT to $\beta 1$ intgerin^{fx/fx} cells resulted in a significant down regulation of Wnt/signaling. This was shown by a decrease in Wnt target gene Axin2 and Lef1 expression. I confirmed these results by knocking down $\beta 1$ -integrin using $\beta 1$ -shRNA expressing lentivirus and found that knocking down $\beta 1$ -intgerin with shRNA resulted in the down regulation of Axin2 and Lef1 expression.

Next, I wanted to investigate whether over activating this pathway can rescue the stem cells. For this, I used Wnt3a as an extracellular ligand and GSK3 β inhibitor. The GSK3 β inhibitor causes the degradation of negative regulator of β -catenin GSK3 β causing β -catenin translocation into the nucleus followed by transcription of Wnt target gene (Meijer et al., 2003). Interestingly, wnt3a did not rescue solid organoid activity in $\beta 1$ -integrin knockdown cells. Instead it increased hollow organoid formation.

In the absence of $\beta 1$ -integrin, wnt3a was not able to translocate β -catenin into the nucleus to a similar extend like the control. However, there was a slight increase in nuclear β -catenin levels compared to the untreated control. This might explain why wnt3a did not rescue solid organoids in $\beta 1$ -integrin null cells. However, the reason behind Wnt3a activating hollow organoids in $\beta 1$ -deleted cells is difficult to explain. One explanation could be because of the slight increase in the level of nuclear β -catenin, that was caused by adding Wnt3a to the culture media. It might be that different levels of β -catenin in the nucleus activates different stem cell pathways. The low amount of β -catenin in the nucleus could activate luminal progenitors while the high amounts activate stem cell activity. It has been shown before, that the cellular decisions of self-renewal or differentiation are dependent on the integration and reciprocal titration of stem cell regulatory networks. It its known from different types of adult stem cells that Notch and Wnt/ β -catenin signaling often intersect in stem and progenitor cells and regulate each others titer in the cell. The biological outcome of signaling through each pathway often depends on the context and timing as cells progress through stages of differentiation (Huelsenken et al., 2001; Kwon et al., 2011). This might be the case in the mammary gland. It could be that different levels of Wnt/ β -catenin signaling activated different stem cell and luminal progenitor pathways. However, one need to test this hypothesis by performing titration experiments that involves

overexpressing different levels of active β -catenin in β 1-integrin null cells. This will help in understanding whether different levels of β -catenin in the nucleus activate different stem cell pathways.

When treating β 1-integrin knockout cells with GSK3 inhibitor, the solid organoid activity was rescued. With GSK3I treatment, β -catenin was able to translocate into the nucleus. This indicates that β 1-integrin control of Wnt signaling might be upstream of GSK3 β in the Wnt/ β -catenin pathway. Taken these results together, it can be concluded that β 1-integrin controls canonical Wnt signaling activity and its activation of mammary stem cells by controlling β -catenin translocation into the nucleus. My results are the first studies that link Wnt pathway and β 1-integrin in normal primary MECs. These results are therefore important in understanding the role of β 1-integrin in controlling mammary stem cells through Wnt signaling.

5.3.3 Rac1 and Wnt signaling in stem cells

I found that β 1-integrin controls Wnt/ β -catenin signaling, therefore I wanted to find signaling proteins that might be linking these two pathways together. Rac1 is a good candidate, as I previously showed that β 1-integrin controls stem cell activity via Rac1. Rac1 is also important in many stem cells like skin and intestine (Benitah et al., 2005; Myant et al., 2013). Also, I showed that Rac1 is important in maintaining stem cells but not luminal progenitors which makes it more likely to be involved in a stem cell specific pathway.

Rac1 was also shown in other studies to play a critical role in β -catenin translocation into the nucleus in fibroblast cell (Jamieson et al., 2015; Wu et al., 2008b). Therefore, I investigated whether deleting Rac1 affected Wnt pathway in MECs. Indeed I found that Wnt signaling was significantly reduced when knocking out Rac1. This was similar to the effect of deleting β 1-integrin on Wnt signaling. Over-expressing an active Rac1 in β 1 knockdown cells enabled wnt3a to activate stem cells in culture. Finally when inhibiting Rac1, using EHT1864 molecule, GSK3I and wnt3a were not able to rescue the stem cell activity. This indicates that Rac1 might be controlling Wnt/ β -catenin signaling

downstream of the GSK3I inhibitor. It might therefore affect β -catenin translocation into the nucleus or β -catenin transcriptional activation of Wnt targets.

Figure 5.12 suggests a molecular model by which β 1-integrin signaling through Rac1 regulates Wnt/ β -catenin signaling in mammary stem cells.

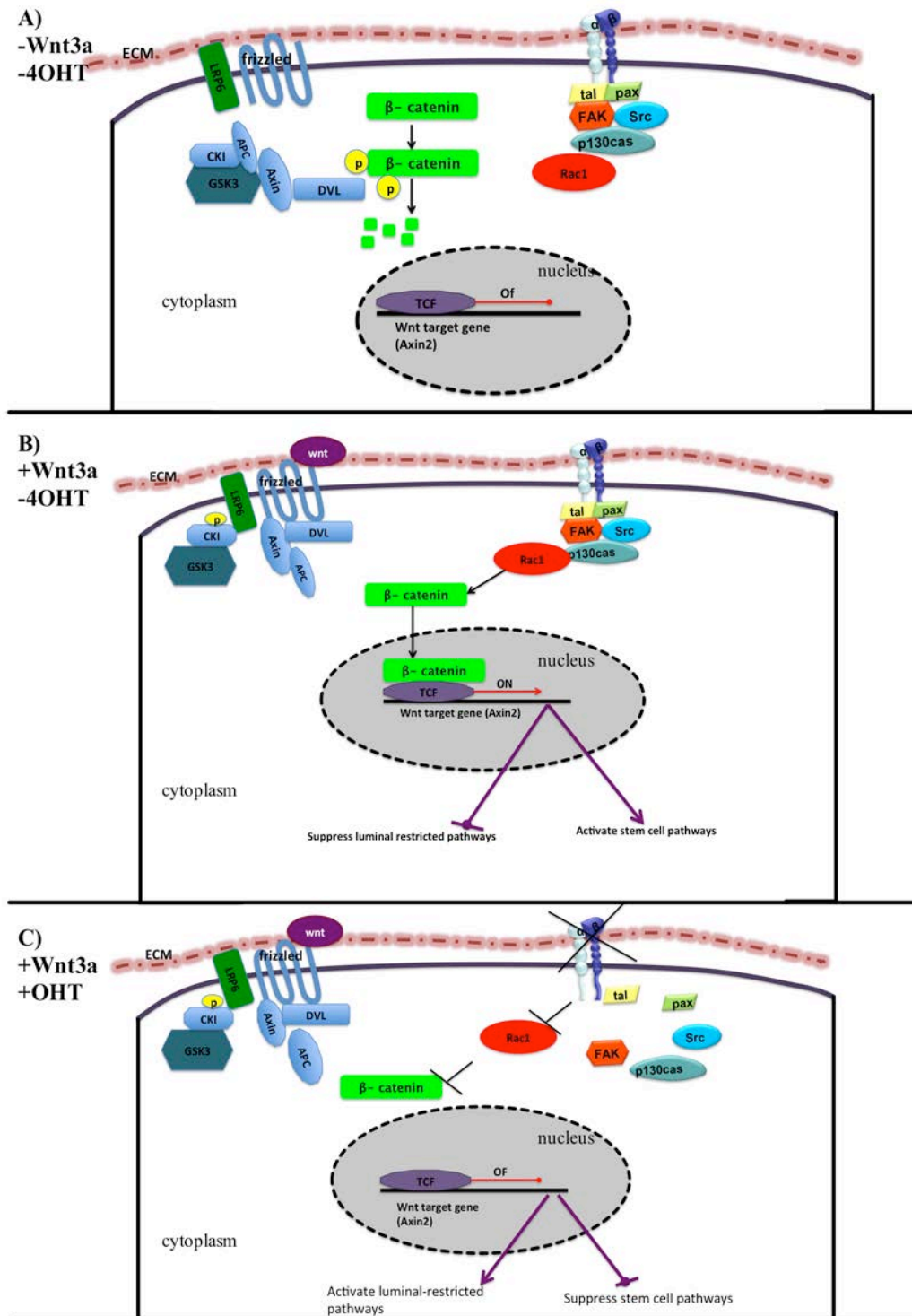


Figure 5.12 suggested model of β 1-integrin wnt/ β -catenin signaling cross talk in stem cells. Cells from β 1-integrin^{fx/fx} CreESRTM A) when the cells are not activated by wnt3a, β -catenin is phosphorylated and degraded by the degradation complex GSK3, APC and Axin. Without wnt3a activation β -catenin cannot translocate to the nucleus. B) Once Wnt3a is bound to the frizzled/ LPR5/6 receptor, negative regulators of β -catenin are dissociated into the cytoplasm. This causes β -catenin translocation to the nucleus and activation of the transcription of Wnt target genes. The translocation of β -catenin into the nucleus requires active Rac1. C) When deleting β 1-integrin, the activity of Rac1 is decreased, preventing β -catenin translocation into the nucleus despite of its release from the degradation complex

Chapter 6: General Discussion

6.1. Overview

This thesis focused on delineating the signals provided by β 1-integrin adhesion receptor to drive stem cells and luminal progenitor activity in MECs and on studying how the dependence on these signals is important in the activation of other stem cell pathways. Identifying the signals involved in normal stem cells are important in breast cancer research, as normal stem cells share functional properties and signaling pathways with cancer stem cells (Dontu et al., 2004; Guan, 2010; Lahlou and Muller, 2011). This could provide useful information in understanding the origin of the disease as well as in finding potential therapies for the disease.

To study the role of β 1-integrin in stem cells, I made use of β 1-integrin^{fx/fx};CreERTM mice. I deleted β 1-integrin in MECs isolated from these mice and showed that this deletion had a dramatic effect on stem cells and progenitor frequency. I confirmed these results using shRNA for knocking down β 1-integrin expression. I also investigated the involvement of two target proteins downstream β 1-integrin, these were ILK and Rac1. I showed that ILK knockout had no effect on stem cell or progenitor frequency, while deleting Rac1 significantly reduced stem cell activity in-vitro but not luminal progenitor activity. These results were novel and were done for the first time in primary MECs.

I then showed how Rac1 is downstream β 1-integrin and can rescue stem cell activity in β 1-integrin deleted cells. These rescue experiments were performed using my optimised lentivirus approach to knockdown β 1-integrin and rescue using active Rac1, which its cDNA was cloned in the same vector as the sh β 1 sequence to insure the overexpression of Rac1 and the knockdown of β 1-integrin at the same time. This approach is not used frequently in studying proteins involved in mammary stem cells due to the technical difficulties associated with infecting primary MECs. These difficulties were overcome by using my developed lentivirus infection approach.

Finally, I tried to link β 1-integrin-Rac1 signaling axis with Wnt/ β -catenin signaling, which

is a major signaling pathway in stem cells. No studies were done previously to link these two pathways in the mammary gland or other adult stem cells. I showed that deleting $\beta 1$ -integrin and Rac1 significantly down regulated the Wnt/ β -catenin signaling pathway. The down regulation of Wnt signaling was rescued in $\beta 1$ -integrin null cells using active Rac1, suggesting an explanation on why active Rac1 rescued the stem cell activity in cells with depleted $\beta 1$ -integrin.

I also found that activating Wnt signaling using GSK3 β inhibitor could rescue the stem cell activity in the $\beta 1$ -integrin null cells. However, activating Wnt signaling with an external ligand (Wnt3a) it did not rescue the stem cell phenotype but significantly activated the luminal progenitor activity, which was unexpected.

Finally, I showed that Wnt3a and GSK3I were not able to rescue stem cell activity when Rac1 activity was blocked using Rac1 inhibitor (EHT1862), suggesting that activating stem cells in-vitro by Wnt/ β -catenin signaling activators requires the presence of an active Rac1 protein.

In this section, I will discuss the significance of these findings in normal MEC stem cells and luminal progenitor. A critical appraisal of the methods used in this project and a comprehensive outline for future work is included.

6.2 Critical appraisal of key methodology

The methodologies described in this thesis allowed me to identify key components in the stem cell pathway controlled by $\beta 1$ -integrin. The genetic deletion in primary MECs that were extracted from $\beta 1$ -integrin^{fx/fx};CreERTM was very useful as it caused complete depletion of the $\beta 1$ -integrin protein. Thus experiments that were done using this approach gave me very significant clear results. The problem however with using transgenic mice, is that mice with the correct genotype are not always available. All the mice born in this colony were coming from parents that were homozygous $\beta 1$ -integrin^{fx/fx} gene but heterozygous for the CreERTM. Therefore, not all female litter from those colonies were suitable for experiments as they might not have the CreERTM gene. Also, a general problem that accord with transgenic in-bred mice lines is that they sometimes had

problems breeding during my project. This required me to wait a long time until I managed to obtain mice with the correct genotype for my experiments.

The use of lentivirus to genetically manipulate gene expression was optimised in this project and showed to be useful for rescue experiments. However, although I achieved an infection level of 40-50%, which is considered high for primary MECs, I still needed to sort GFP cells post infection, to obtain sufficient amount of gene knockdown. Cell sorting had a toxicity effect on the cells as more than half of the cells died after sorting. The cell number obtained from every sort was the limiting factor for my experiments. The organoid formation method does not require a high number of cells and therefore, I was able to perform this assay on sorted GFP cells. I was not able however, to perform biochemical assays to check for protein expression and interaction in these cells. It would have been much better if higher than 80% infection efficiency was achieved, as this will not require cell sorting and therefore, increase the chances of obtaining higher cell number.

Another problem with the low cell number obtained from the lentivirus-infected cells was that I was not able to perform in-vivo transplant experiments to verify the results obtained from in-vitro assay. For example, I wanted to perform an experiment where I overexpress active Rac1 in β 1-integrin knockdown cells, and then see if it can rescue the stem cell phenotype in-vivo, like it did in the organoid formation assay. This assay requires a high number of transduced cells for each experiment in order to have sufficient number of repeats and obtain statistical data. Unfortunately, due to the low number of cells obtained from each of my sorts I was not able to perform in-vivo experiments.

6.3. β 1-integrin in normal mammary stem cells –transducing stem cells from micro-environment into the cells

Integrins are micro-environmental sensors on stem cells that bind to extra-cellular matrix and respond to different environmental signals, causing the stem cells to either maintain dormant, self-renew, or differentiate. Deregulated integrins- ECM interaction was shown be pathogenic and cause many disease including breast cancer (Lahlou and Muller, 2011; Rosenblatt et al., 2011). I aimed to study the role of integrins in normal stem cells as understanding the role of integrins in stem cells might help understanding how these

adhesion receptors control cancer stem cells. My findings that β 1-integrin control stem cells through Rac1 but not ILK were novel in the normal mammary gland. I also showed the specific involvement of Rac1 downstream β 1-integrin in the stem cells but in luminal progenitor cells of primary MECs. The fact that Rac1 was specifically involved in stem cells, confirms previous studies from lineage tracing that within the mammary gland, that there are distinct populations of stem cells in the mammary gland. These include the bi-potent stem cells that contribute to both myoepithelial and luminal lineages, and the luminal progenitors that can only give rise to differentiated luminal cells. Different pathways control these two populations (Rios et al., 2014a). My in-vitro organoid – formation assay and my FACS sorting method enabled me to distinguish and quantify these two populations. However, in the future more experiments can be done in order to confirm my findings and obtain more mechanistic data. These experiments will be discussed below.

6.3.2 Future work for investigating the role of β 1-integrin-Rac1 in mammary stem cells

Animal studies:

An important experiment that could be done with the Rac1 rescue of β 1-integrin knockdown cells is to perform an in-vivo experiment which involves, infecting primary cells isolated from FVB mice with viruses expressing control, β 1-integrin and Rac1. The cells that are positive for GFP will then be sorted and transplanted at different densities in different mammary glands of 3-weeks old mice. Before transplanting the cells into the mammary glands the endogenous epithelial cells should be surgically cleared. Cell concentration will include (1000, 5000, 10000, and 50,000/ cells per gland) for every cell group. At least each concentration should be repeated 5 times in order to determine the stem cell frequency in each group. The glands will then be left for 8-12 weeks until the mice become adults. The glands will then be examined under the fluorescent microscope and those cells with stem cell property will produce glands that are GFP positive. Although I attempted many times to perform that experiment, I was not able to obtain sufficient cell numbers from the FACS sorting even though I tried to increase the number of mice used per prep from 4-mice to 12 mice per prep. I also tried double the amount of virus used for each infection in order to obtain better infection efficiency, however this was toxic for the cells and caused great amount of cells clumping. It is therefore, important for future in-

vivo experiments to make sure that the lentivirus infection is better optimised.

Understanding the role of β 1-integrin-Rac1 signaling in different populations:

My loss and gain of function studies were done in total MECs. It would be interesting however, to sort luminal and basal populations separately and then knockout β 1-integrin-Rac1 signaling in these cells to find out if that pathway has different effects in different populations. From my flow cytometry analysis of β 1-integrin knockouts, I found that there has been an increase in differentiated luminal cells and a decrease in both stem and luminal progenitors. It would be interesting to find out whether these differentiated luminal cells originated from basal or from luminal progenitor cells. This would give a better understanding on the role of β 1-integrin on stem cell fate decision.

6.4 β 1-integrin cross talk with Wnt signaling

In this project I obtained a model, by which β 1-integrin-Rac1 signaling control Wnt/ β -catenin signaling, which is a major signaling pathway in stem cells. I deleted β 1-integrins and Rac1 in primary cells and checked for Wnt-signaling targets. The main finding was that Wnt signaling requires β 1-integrin and active Rac1 activating stem cells and β -catenin translocation into the nucleus. No studies were done previously to link these two pathways in the mammary gland or other adult stem cells.

My results also showed that wnt signaling requires β 1-integrin for preventing stem cells from differentiating into luminal lineage. These findings will help in understanding how β 1-integrin interactions with the microenvironment influence stem cells fate decision.

6.4.1. Future work on β 1-integrin and Wnt/ β -catenin cross talk

Rescuing stem cells in β 1-integrin using active β -catenin:

An experiment that could be done and could help in confirming my results with the GSK3I and the wnt3a signaling is over expressing an active form of β -catenin in β 1-integrin null cells. There is a form of β -catenin mutant that cannot be phosphorylated by GSK3, and thereby constitutively translocate into the nucleus without the need of activation using wnt3a or GSK3 Inhibitor. This active form of β -catenin can be cloned into a vector that contains an shRNA sequence for β 1-integrin and then produced into a lentivirus for

rescuing experiments. The rescuing experiments can be tested both in-vitro using the stem cell assay and in-vitro using the mammary transplant assay.

7.0 Conclusion

The results of this thesis showed that β 1-integrin signaling controls stem cells via Rac1 and signals and cross talks with other important stem cell pathways such as Notch and wnt signaling. These results will provide better understanding on how β 1-integrin controls mammary stem and progenitor fate in both normal and cancer cells. It will also open new exciting areas of research that involve studying β 1-integrin signaling as a target pathway for cancer therapy.

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