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# Function of the β4 Integrin in Cancer Stem Cells and Tumor Formation in Breast Cancer: A Masters Thesis

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# FUNCTION OF THE β4 INTEGRIN IN CANCER STEM CELLS AND TUMOR FORMATION IN BREAST CANCER

A Masters Thesis Presented

By

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## Abstract

The integrin α6β4 (referred to as β4) is expressed in epithelial cells where it functions as a laminin receptor. Integrin β4 is important for the organization and maintenance of epithelial architecture in normal cells. Particularly, β4 is shown to be essential for mammary gland development during embryogenesis. Integrin β4 also plays important roles in tumor formation, invasion and metastasis in breast cancer. However, the mechanism of how integrin β4 mediates breast tumor formation has not been settled. A few studies suggest that integrin β4 is involved in cancer stem cells (CSCs), but the mechanism is not clear. To address this problem, I examined the expression of β4 in breast tumors and its potential role involved in regulating CSCs. My data shows that β4 is expressed heterogeneously in breast cancer, and it is not directly expressed in CSCs but associated with a basal epithelial population. This work suggests that β4 can regulate CSCs in a non-cell-autonomous manner through the interactions between $\beta4^+$ non-CSC population and $\beta4^-$ CSC population. My data also shows that β4 expression is associated with $CD24^+CD44^+$ population in breast tumor. To further study the role of β4 in breast cancer progression, I generated a β4 reporter mouse by inserting a p2A-mCherry cassette before ITGB4 stop codon. This reporter mouse can be crossed with breast tumor models to track $\beta4^+$ population during tumor progression.

## Table of Contents

# LIST OF TABLES

**Chapter II**



**Chapter III**

# LIST OF FIGURES

**Chapter II**



**Chapter III**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AML | Acute myeloid leukemia |
| APC | Adenomatous polyposis coli |
| ATCC | American Type Culture Collection |
| BM | Basement membrane |
| BMP | Bone morphogenetic protein |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| CSC | Cancer stem cell |
| DCIS | Ductal carcinoma in situ |
| ECM | Extracellular matrix |
| EMT | Epithelial-to-Mesenchymal Transition |
| ER | Estrogen receptor |
| FACS | Fluorescence activated cell sorting |
| FN | Fibronectin |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GFP | Green Fluorescence Protein |
| HD | Hemidesmosome |
| HER2 | Human epidermal growth receptor |
| LM | Laminin |
| MCF | Michigan Cancer Foundation |
| MDA | MD Anderson |

| | |
|---|---|
| MET | Mesenchymal-to-Epithelial Transition |
| MMP | Matrix metalloproteinase |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NMuMG | Normal murine mammary gland |
| NSG | NOD scid gamma |
| PDX | Patient-derived xenografts |
| PI3K | PIP3 kinase |
| PR | Progesterone receptor |
| qPCR | Quantitative realtime PCR |
| RANKL | Nuclear factor kappa B ligand |
| RNA-seq | RNA deep sequencing |
| SUM | The University of Michigan Human Breast Cancer Cell Lines |
| TGF-β | Transforming growth factor β |
| UMMS | University of Massachusetts Medical School |
| VEGF | Vascular endothelial growth factor |

# Chapter I

# Introduction

## Overview

This thesis focuses on the role of integrin $\alpha 6\beta 4$ in cancer stem cells and tumor initiation in breast cancer. This issue is significant because integrin $\alpha 6\beta 4$ as an adhesion receptor is important for organization and maintenance of epithelial architecture. It also can mediate a variety of signaling pathways to promote tumor progression (Soung et al., 2011).

## Breast Cancer Overview

Breast cancer is a major health problem and the most commonly diagnosed cancer in women worldwide. The American Cancer Society estimates that about 231,840 new cases of female invasive breast cancer and 60,290 new cases of breast carcinoma in situ will be diagnosed in 2015 (Siegel et al., 2015). Breast cancer alone is expected to account for 29% of all new cancers among women in the United States (Siegel et al., 2015). It is also the second leading cause of cancer death in the United States, with about 40,290 deaths expected in 2015 for women representing 15% of all cancer-related deaths among women (Siegel et al., 2015).

It is believed that breast cancer develops stepwise from normal breast epithelial cells to atypical hyperplasia, ductal carcinoma in situ (DCIS), invasive ductal

carcinoma and metastatic breast cancer. Multiple genetic and epigenetic alterations may occur during this process. DCIS, though noninvasive, is greatly associated with invasive breast cancer and has a very high chance to develop into invasive breast cancer if at high grade (Allred, 2010; Ward et al., 2015). Studies have shown the mechanism of how DCIS turns into invasive breast cancer (Hu et al., 2008). However, early molecular alterations preceding DCIS have not been clearly demonstrated so far (Rivenbark et al., 2013).

Breast cancers are classified by immunohistochemistry to detect the expression of certain protein biomarkers, including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth receptor 2 (HER2) and various cytokeratins. Based on this clinical classification, breast cancer can be classified as $ER^+/PR^+HER2^-$, $ER^+/PR^+/HER2^+$, $ER^-/PR^-/HER2^+$, and $ER^-/PR^-/HER2^-$ breast cancers. Breast cancer can also be classified by gene expression (Perou et al., 2000; Sorlie et al., 2003; Sorlie, 2004) . Based on this molecular classification, breast cancer can be classified as six molecular subtypes, luminal A ($ER^+$), luminal B ($ER^+/HER2^+$), $HER2^+$, basal-like, claudin-low, and normal-like breast cancers (Rakha et al., 2008; Rivenbark et al., 2013).

$ER^+$ and $HER2^+$ breast cancers can be treated by effective targeted therapy of anti-estrogen treatment (tamoxifen) or anti-HER2 drug (trastuzumab), respectively, both in conjunction with chemotherapy (Mukai, 2010; Rimawi et al.,

2015). These targeted treatments have improved survival and the long-term outcomes for $ER^+$ and $HER2^+$ patients dramatically. However, basal-like and claudin-low molecular subtypes of breast tumors lack the expression of ER, PR as well as amplification of HER2 ($ER^-PR^-HER2^-$), and are therefore also referred to as triple-negative breast cancers (TNBCs). TNBCs tend to be more aggressive compared to $ER^+$ breast cancers, and there are no effective targeted therapies (Chacón and Costanzo, 2010; Dent et al., 2007; Li et al., 2013). Though they respond to some chemotherapies, the patients often have poor recurrence-free and overall survival outcomes (Kassam et al., 2009; Kennecke et al., 2010; Liedtke et al., 2008).

**Tumor Heterogeneity and Cancer Stem Cells**

A single tumor harbors a mixture of different subpopulations with distinct molecular properties (Meacham and Morrison, 2013; Skibinski and Kuperwasser, 2015). Two models are used to explain this intratumor heterogeneity, the clonal evolution model and the cancer stem cell model, which are not mutually exclusive but could also be integrated together (Campbell and Polyak, 2014; Kreso and Dick, 2014).

The clonal evolution model states that tumor cells acquire various mutations over time and cancer could initiate in any random single cell. Certain mutation could give cells growth advantage over other normal cells, and these cells will become

more aggressive and may leave a large number of offspring by chance. Thus, during tumor progression, any cell within the tumor could potentially be the one that drives tumor progression and results in therapy resistance and recurrence since mutations will occur in random cells over time.

The cancer stem cell model states that there is a small subpopulation of cells in the tumor that have stem cell-like properties, called cancer stem cells (CSCs). CSCs have the ability of self-renewal and can also differentiate into different cell types. They are thought to be responsible for cancer recurrence. Current therapies kill tumor cells but not CSCs, and CSCs can proliferate extensively. Thus, despite good therapeutic response by the bulk tumor, it shrinks but will grow back. Moreover, the recurred tumor will gain resistance to the previous therapies.

In this work, I focused on the CSC model. TNBCs harbor a relatively high frequency of CSCs compared with other breast cancer subtypes. This could be one of the reasons why TNBCs are more aggressive with poor recurrence-free and overall survival outcomes. Despite the evidence supporting the existence of CSCs, much less is known about the origin of these cells and the mechanisms that regulate their functions.

CSCs can be isolated by cell-surface markers, and a series of assays can be conducted to examine their stem-cell properties. The gold standard measure of CSCs is examining their ability to repopulate new tumors and maintain the long-term clonal growth by either transplantation into serial recipients or in situ tracking. Initial studies on CSCs were conducted in acute myeloid leukemia (AML), and the $CD34^{+}CD38^{-}$ subpopulation was identified as leukemia-initiating cells (Bonnet and Dick, 1997; Lapidot et al., 1994). After that many studies on CSCs in various solid tumors followed (Collins et al., 2005; Eramo et al., 2008; Hermann et al., 2007; Li et al., 2007; O'Brien et al., 2007; Patrawala et al., 2006; Prince et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004; Wu et al., 2007). The first identification of CSCs in a solid tumor was in human breast cancer (Al-Hajj et al., 2003). A $CD24^{-}CD44^{+}$ subpopulation was identified as CSCs in breast cancer by showing this subpopulation alone is sufficient to generate new tumors in immune-deficient mice while the other three subpopulations ($CD24^{+}CD44^{+}$, $CD24^{-}CD44^{-}$, and $CD24^{+}CD44^{-}$) cannot (Al-Hajj et al., 2003).

**Integrins**

In this context, we are interested in integrins in breast cancer. Integrins are transmembrane receptors that form heterodimers composed of $\alpha$ and $\beta$ subunits. In mammals, the integrin family comprises 18 $\alpha$ and 8 $\beta$ subunits that bind noncovalently, generating 24 unique integrins, and they bind to specific ligands including collagen, fibronectin, vitronectin and laminin (Hynes, 2002). Integrin

subunits span the cell membrane with a large extracellular domain, a single transmembrane domain and a short cytoplasmic tail of 40-70 amino acids. The exception is the β4 subunit, which has a cytoplasmic domain of 1088 amino acids, one of the largest known cytoplasmic domains of any membrane protein (Campbell and Humphries, 2011; Srichai and Zent, 2010; Takada et al., 2007).

As adhesion receptors, integrins mediate cell-cell adhesions as well as cell adhesion to extracellular matrix proteins. All integrins except integrin α6β4 are connected to actin, while the integrin β4 subunit connects to keratin intermediate filaments. They can form adhesion structures such as focal adhesion and hemidesmosome which connect ligands in the ECM with the cytoskeleton. Other than functioning as adhesion receptors, integrins also mediate signaling transduction and play important roles in cell proliferation, division, apoptosis, survival, motility, and differentiation (Guo and Giancotti, 2004; Levental et al., 2009; Glukhova and Streuli, 2013). Each integrin plays distinct, nonredundant roles shown by the phenotypes of genetic knockout mice, such as in development, vasculogenesis and immune responses (Takada et al., 2007).

Unlike other transmembrane receptors, integrins mediate signaling in a bidirectional way (Hynes, 2002). They connect the extracellular and intracellular environments by binding ligands in the ECM and adaptor molecules in the cytoplasm through outside-in signaling or inside-out signaling. Outside-in

signaling involves the extracellular domain of integrins binding to their ligands to promote conformational changes first, which is followed by integrin clustering and the assembly of large intracellular adhesion complexes. Inside-out signaling involves an internal signaling molecule binding to the cytoplasmic tail of integrins first, which then induces conformational changes and influences the binding of integrins to their ligands. Moreover, integrins also participate in signaling crosstalk, where in addition to the activation of their own downstream signaling cascades, they interact with and regulate the activation of growth hormones and G-protein coupled receptor-induced signaling pathways (Mariotti et al., 2001; Trusolino et al., 2001; Guo et al., 2006).

## Integrin α6β4 in Epithelial Biology

Integrin α6β4 is referred to as β4 integrin since its only partner is the α6 subunit. Integrin β4 is special among all integrin subunits in that it has an extremely large cytoplasmic domain and it connects to keratin intermediate filaments instead of the actin cytoskeleton (Borradori and Sonnenberg, 1999; Dowling et al., 1996; Green and Jones, 1996). It is expressed primarily on the basal surface of most epithelia and in a few other cell types, preferentially binding to most of the known laminins (Lee and Streuli, 2014; Mercurio, 1995). In normal cells, β4 integrin is essential for the organization and maintenance of epithelial architecture by serving as an important component of the stable and rigid adhesive structures termed hemidesmosomes (HDs) that link the intermediate filament cytoskeleton

with laminins in the basement membrane (BM) (Borradori and Sonnenberg, 1999; Dowling et al., 1996; Green and Jones, 1996).

Homozygous knockout reveals the function of β4 in maintaining the integrity of epithelia, especially the epidermis. In the absence of β4 expression, the epidermis detaches from the dermis in response to mechanical stress, which leads to death of β4 knockout mice shortly after birth (Dowling et al., 1996; van der Neut et al., 1996; 1999a). This critical role for β4 derives from its ability to mediate the formation of HD on the basal cell surface (Borradori and Sonnenberg, 1999; Green and Jones, 1996). HDs are most pronounced in stratified epithelia, but rudimentary forms exist in most epithelia (Green and Jones, 1996).

Although many studies have been conducted on the contribution of β4 to epithelial biology in the past two decades, its role in mammary gland development is unclear and confusing (Klinowska et al., 2001). During embryogenesis, integrin β4 is expressed at least from embryonic day 12.5 (E12.5), restricted in the mammary epithelial bud which begins to elongate to form the rudimentary mammary tree after E14.5. The adult mammary glands are composed of two layers of cells: luminal cells and myoepithelial cells. Integrin β4 is expressed primarily in myoepithelial cells and anchors them to the basement memberane (Gjorevski and Nelson, 2011). Seminal studies by Bissell and others

using three-dimensional cultures revealed the importance of laminins in mammary morphogenesis and the contribution of the α6 integrins (α6β4 and α6β1) to this process (Nisticò et al., 2014). Our previous work shows that integrin β4 regulation of parathyroid hormone related protein (PTHrP) contributes to the ability of this integrin to regulate mammary bud development during embryogenesis (Li et al., 2015).

**Rational for the Thesis Work**

In breast cancer, β4 has been shown to be important for tumor formation, invasion and metastasis (Soung et al., 2011). The role of β4 in tumor migration and invasion has been extensively studied. In cancer cells, β4 integrin switches from associating with HD to interacting with actin cytoskeleton and redistributes to cell protrusions, which can stimulate carcinoma migration and invasion (Lipscomb and Mercurio, 2005; Rabinovitz and Mercurio, 1997; Rabinovitz et al., 1999). Integrin β4 also promotes tumor invasion by regulating several downstream effector molecules and activating various signaling pathways. Integrin β4, together with growth factor receptors, initiates signaling cascades involving Shp2 and Src, which leads to activation of PI3K and Ras pathways (Shaw et al., 1997; Mercurio, 2003; Bertotti et al., 2006). As a result, integrin β4 enhances expression of cancer related genes such as VEGF, ERBB3, NFAT, NF-kB, STAT3 and c-Jun at the translational or transcriptional levels (Chung et

al., 2002; Folgiero et al., 2007; Gerson et al., 2012a; Jauliac et al., 2002; Yoon et al., 2006; Zahir et al., 2003).

Integrin β4 has also been found to be essential for breast tumor formation. Our lab showed that β4 is important for breast tumor growth by demonstrating that SUM-159 breast cancer cells with β4 knocked down generate fewer and smaller tumors in vivo compared to control cells (Lipscomb et al., 2005). Moreover, this tumor growth phenotype could be rescued by overexpressing β4 back into the knockdown cells. Another study also shows that β4 promotes tumor formation by amplifying ERBB2 signaling (Guo et al., 2006). However, the mechanism of how integrin β4 regulates tumor formation still needs to be further investigated.

The role of CSCs in tumor formation has arisen much interest in the field studying tumor initiation. Several integrins such as α6 and β1 have been shown to be stem cell markers (Pontier and Muller, 2009). However, less is known about the role of β4 in CSCs. Data in our lab suggests that β4 may be involved in stem cell properties by showing that β4 knockout mammary buds cannot develop into mature mammary glands when transplanted into immune-deficient mice (Li et al., 2015). Moreover, several studies suggest that β4 is involved in CSCs of breast cancer and lung cancer in the past two years (Zheng et al., 2013; Vieira et al., 2014). In non-small-cell lung cancer, integrin β4 has been identified as a CSC

marker by showing that a rare population of $CD24^{+}ITGB4^{+}Notch^{hi}$ cells are able to form new tumors in serial transplantation assay (Zheng et al., 2013). Another study shows that P-cadherin signaling induces stem cell properties in basal-like breast cancer cells through integrin β4 (Vieira et al., 2014). This study demonstrates that P-cadherin directly interacts with β4 and regulates the expression of both integrin α6 and β4 subunits, which are essential for stem cell properties shown by mammosphere assay (Vieira et al., 2014). However, the mechanism of β4 regulation on CSCs is still unclear. Given these data and the importance of CSCs in tumor initiation, I was interested in investigating the role of integrin β4 in CSCs and tumor initiation in breast cancer.

# Chapter II

## Integrin β4 Regulates Breast Cancer Stem Cells in a Non-cell-autonomous Manner


### Abstract

Integrin β4 is not only very important for the organization and maintenance of epithelial architecture in normal epithelia, but it also plays essential roles in cancer. It has been shown to be important for tumor formation, invasion and metastasis. However, the mechanism of how integrin β4 is involved in tumor formation is still unclear. My study shows that integrin β4 is heterogeneously expressed in breast cancer and the $\beta4^+$ population is critical for tumor formation. It further shows that β4 affects CSCs in a non-cell-autonomous manner and I discuss the possibility of its regulation in CSCs through PTHrP paracrine signaling.


### Introduction

Integrin β4 has been shown to be important for tumor formation and survival in breast cancer (Guo et al., 2006; Lipscomb et al., 2005). However, the mechanism involved is still not very clear. Breast CSCs were identified as $CD24^-CD44^+$ by showing that it is necessary and sufficient to form new tumors in vivo while other three subpopulations ($CD24^-CD44^-$, $CD24^+CD44^-$, $CD24^+CD44^+$) cannot (Al-Hajj et al., 2003). Our lab showed that β4 is important for mammary

gland development during embryogenesis through regulating parathyroid hormone-related protein (PTHrP) expression (Li et al., 2015). In this study, we knocked out integrin β4 using MMTV-Cre and found that the mammary bud elongation was blocked, which is very similar to the phenotype of PTHrP knockout mice. Upon further investigation, we found that PTHrP expression was decreased in β4 knockout mammary bud, and the mammary bud elongation phenotype could be rescued by adding additional PTHrP when β4 knockout mammary bud was cultured in vitro. Moreover, in the study, the fact that β4 knockout mammary buds cannot develop into mature mammary glands when transplanted into immune-deficient mice suggests that β4 could be potentially involved in stem cell properties. Compared to integrin α6 and β1, not much is known about β4 in mammary stem cells (Pontier and Muller, 2009). Integrin β4 has been indicated in progenitor cells (Chapman et al., 2011; Yoshioka et al., 2013). Moreover, β4 is identified as a marker for CSCs in non-small-cell lung cancer (Zheng et al., 2013). In breast cancer, a study has shown that P-cadherin regulates CSC functions through integrin β4 (Vieira et al., 2012; 2014). Given all these studies and that CSCs are important for tumor formation, I wanted to investigate whether integrin β4 is involved in CSC functions and the mechanisms involved.

Previously, tumor formation is believed to be associated with epithelial-to-mesenchymal transition (EMT) (Thiery et al., 2009). It has been shown that EMT

generates cells with properties of stem cells, and CSCs express mesenchymal markers with decreased epithelial markers (Mani et al., 2008). However, integrin β4 is a gene associated with epithelial cells, and our lab showed that β4 is downregulated dramatically during EMT induced by TGF-β or Snail (Chang et al., 2013; Yang et al., 2009). In another system that our lab has been using, we sorted out two populations from Src-transformed MCF10A cells based on α6 staining (Goel et al., 2014). $\alpha6^{hi}$ population has epithelial traits while $\alpha6^{lo}$ population is mesenchymal based on both cell morphology and molecular markers (Goel et al., 2014). Moreover, $\alpha6^{lo}$ population has higher CSC properties but lower β4 expression compared to $\alpha6^{hi}$ population. Based on these observations, it seems that β4 expression is not directly expressed by CSCs. Since β4 is important for tumor formation while it is not expressed in CSCs, I propose that β4 is expressed heterogeneously in breast tumors and this $\beta4^{+}$ non-CSC subpopulation is important for tumor formation.

## Results and Discussion

*2.1 Integrin β4 is expressed heterogeneously in breast tumors.*

We have a patient-derived xenograft (PDX) model in our lab. I used PDX tumors derived from two TNBC patients labeled as #1126 and #1258, respectively (Fig 1A). β4 expression was first examined in these two tumor samples by immunofluorescence (IF) staining (Fig 1B). The result shows that β4 is expressed

heterogeneously by a subpopulation of cells in the tumor. To examine the molecular difference between $\beta4^+$ and $\beta4^-$ populations, I sorted out $\beta4^+$ and $\beta4^-$ cells by FACS in PDX tumor #1126 (Fig 1C).

qPCR data shows that the $\beta4^+$ subpopulation expresses a lower level of CSC marker OCT4 compared to $\beta4^-$ subpopulation (Fig 1D), which confirms that β4 is not directly expressed by CSCs. Since our lab showed that β4 is associated with basal-like breast cancer (Lu et al., 2008) and it is downregulated during EMT together with epithelial markers (Chang et al., 2013; Yang et al., 2009), I checked the expression of several basal and epithelial markers.The $\beta4^+$ subpopulation expresses higher levels of basal and epithelial genes compared to the $\beta4^-$ subpopulation (Fig 1D).

The Ewald group showed that cell dissemination during invasion needs an epithelial program (Shamir et al., 2014). Moreover, a basal epithelial population is shown as leader cells at the invading front in breast cancer (Cheung et al., 2013). These studies indicate that not all cells undergo EMT during tumor progression, and there is a basal epithelial population driving tumor invasion. Interestingly, two back-to-back papers recently showed that EMT is not required for metastasis and epithelial cells can intravasate into the blood system and form metastasis (Cheung et al., 2013; Fischer et al., 2015; Shamir et al., 2014; Zheng et al., 2015). These results contradict with the previous model that cells need to

undergo EMT and become mesenchymal-like to break away from the primary site (Thiery et al., 2009). Overall, these studies address the important role of a basal-epithelial population within the tumor during tumor progression other than the cells that undergo EMT which are previously believed to drive tumor progression.

In cancer, integrin β4 switches from associating with HD to connecting with actin and redistributing to cell protrusions (Lipscomb and Mercurio, 2005; Rabinovitz and Mercurio, 1997; Rabinovitz et al., 1999). Moreover, the invading front of several carcinomas are enriched in the expression of β4 and its ligand laminin-332 (Kim et al., 2011a; Pyke et al., 1995; Xue et al., 2011). β4 has been shown to drive tumor invasion by regulating several downstream effector molecules and signaling pathways (Gerson et al., 2012; Shaw et al., 1997). My data also shows that the $\beta4^+$ population is associated with a basal epithelial program (Fig 1D). Based on all these findings, I propose that $\beta4^+$ population may be the leader cells during invasion that Ewald group reported in their studies (Cheung et al., 2013; Shamir et al., 2014). To examine whether the $\beta4^+$ population is present at the invasive front to drive tumor invasion and to track β4 expression during tumor progression and metastasis, I made a β4 reporter mouse using CRISPR (see Chapter III). In this work, I'm interested in investigating whether the $\beta4^+$ subpopulation is important for tumor formation and the mechanism involved other than their potential role in tumor invasion.

*2.2 Integrin β4 regulates CSCs in a non-cell-autonomous manner through PTHrP paracrine signaling*

I checked some genes that are important for CSC functions, and I found an interesting gene expression pattern that $\beta4^-$ and $\beta4^+$ cells have in certain important paracrine signaling pathways for example, the notch signaling pathway. qPCR data show that $\beta4^+$ cells express higher level of JAG1 while $\beta4^-$ cells express higher NOTCH3 as well as HEY1, which is a downstream target in Notch signaling (Fig 1D). Notch signaling is established to be important for CSC self-renewal (Bouras et al., 2008; Dontu et al., 2004; Harrison et al., 2010). One study showed that NF-kB regulates CSCs in a non-cell-autonomous manner through Notch paracrine signaling. CSCs express notch3 receptors, and NF-kB regulates JAG1 expression in non-CSCs (Yamamoto et al., 2013). This non-cell-autonomous regulation model is very similar with what my results indicate. Thus, I proposed that $\beta4^+$ non-CSCs can interact with $\beta4^-$ CSCs through paracrine signaling.

To show that $\beta4^+$ and $\beta4^-$ populations can interact with each other, I explored the Src-transformed MCF10A system in our lab where $\beta4^{hi}$ and $\beta4^{lo}$ populations were isolated (Goel et al., 2014). It has been shown that the $\beta4^{lo}$ population has CSC properties compared to the $\beta4^{hi}$ population (Goel et al., 2014). Part of the results from RNA-seq on $\beta4^{hi}$ and $\beta4^{lo}$ populations is shown in Table 1. I labeled $\beta4^{hi}$

population with GFP and $\beta4^{lo}$ population with mCherry. I mixed them together and plated them in single cells on Matrigel. The result shows that they grow into cell clusters together in 3D culture (Fig 2A). This indicates that $\beta4^{hi}$ and $\beta4^{lo}$ populations can interact with each other and grow together in 3D culture.

Our lab showed that integrin $\beta4$ regulates PTHrP expression during embryonic mammary gland development and there are many signaling pathways during embryogenesis that have parallel signaling pathways in cancer, I checked the expression of PTHrP and its receptor PTH1R in $\beta4^{+}$ and $\beta4^{-}$ populations sorted from PDX tumors (Li et al., 2015). $\beta4^{+}$ cells express higher PTHrP while $\beta4^{-}$ cells express higher PTHrP as well as BMP4 which is a downstream target of PTH1R signaling (Fig 1D). It is known that PTHrP is expressed primarily by epithelial-derived cells, and its receptor PTH1R expression is restricted to mesechymal cells. Thus PTH1R signaling is activated in the paracrine manner. PTHrP promotes increased tumor cell growth in breast cancer lines as well as in vivo (Dittmer et al., 2006). PTHrP also drives breast tumor progression and metastasis examined by mouse model (Li et al., 2011). However, no one has shown that PTHrP is involved in CSCs functions.

To better characterize $\beta4^{+}$ and $\beta4^{-}$ population within a breast tumor, RNA-seq was performed on $\beta4^{+}$ and $\beta4^{-}$ populations sorted from PDX tumor #1258 (Fig 1E). Part of the results was shown in Table 2. I first used mouse lineage markers

CD31, CD45 and Ter-119 to exclude the potential mouse stromal cells recruited to the PDX tumor, and then sorted out the $\beta4^+$ and $\beta4^-$ cells in $Lin^-$ cells. I confirmed β4 expression in $\beta4^+$ and $\beta4^-$ subpopulations before sending them for RNA-seq (Fig 1E). RNA-seq data is similar with the previous qPCR data in that $\beta4^+$ population expresses lower level of CSC markers while is associated with a basal epithelial program (Table 2).

PTHrP expression is always associated with β4. I checked this association in another two systems. In the Src-transformed MCF10A system, PTHrP is associated with $\beta4^{hi}$ population (Table 1). I overexpressed VEGF promoter with GFP tag in MDA-MB-231 cells and sorted out $GFP^+$ cells which have higher VEGF activity (VEGF-high). Our lab has shown that VEGF-high cells have CSC properties (Goel et al., 2014). I checked β4 and PTHrP expression in VEGF-low and VEGF-high cells by qPCR, and found that PTHrP expression is associated with β4 in VEGF-low population (Fig 2B). I knocked down β4 with siRNA in MCF10A cells, and found that PTHrP expression decreased at both mRNA and protein level (Fig 2C). When I ligated integrin α6β4 by plating the cells on laminins, PTHrP expression increase (Fig 2D). This indicates that PTHrP expression is directly induced by ligation of integrin α6β4. Based on these results, I propose that $\beta4^+$ non-CSCs and $\beta4^-$ CSCs interact with each other through

paracrine signaling, which potentially involves β4 regulation on PTHrP expression.

*2.3 Integrin β4 is essential for CSC functions.*

Our lab has shown that β4 integrin is important for mammary tumor formation and growth by knocking down β4 using shRNA in SUM-159 cells (Lipscomb et al., 2005). The shβ4 cells form fewer and smaller tumors when injected into the mammary fat pad of immunocompromised mice compared to shGFP cells. Overexpressing β4 back in to the shβ4 cells can rescue the tumor formation phenotype. To investigate the mechanism involved, I studied the same system by knocking down β4 in SUM-159 cells using shRNAs (Fig 3A). Since CSCs are known to be very important for tumor formation, I first examined if the tumor formation phenotype we got after β4 knocking down was due to decrease in the amount of CSC population. Interestingly, I found that knocking down β4 doesn't decrease the $CD24^{-}CD44^{+}$ population which is identified as CSCs in breast cancer (Al-Hajj et al., 2003), but it dramatically reduces $CD24^{+}CD44^{+}$ population (Fig 3B).

To further investigate the mechanism, I sorted out $CD24^{-}CD44^{+}$ and $CD24^{+}CD44^{+}$ populations in SUM-159 cells, and checked gene expression in these two populations by qPCR. The data show that $CD24^{-}CD44^{+}$ CSCs express lower levels of integrin β4 but higher PTH1R. The $CD24^{+}CD44^{+}$ population

expresses higher level of basal and epithelial markers as well as PTHrP (Fig 3C). I cultured these two populations separately for 1 week and checked CD24 and CD44 expression by FACS again. The $CD24^{-}CD44^{+}$ population can give rise to the cell profiles in the parental SUM-159 cells, both $CD24^{-}CD44^{+}$ and $CD24^{+}CD44^{+}$ populations (Fig 3D). However, $CD24^{+}CD44^{+}$ population can only give rise to itself (Fig 3D). As one major property of stem cells, they should be able to differentiate into different types of cells. This result confirms that $CD24^{-}CD44^{+}$ population is CSCs while $CD24^{+}CD44^{+}$ population doesn't have this stem cell property. Moreover, this result also indicates that integrin β4 is essential for CSC functions. As it is discussed above, I expected that intact CSC population ($CD24^{-}CD44^{+}$) in SUM-159 cells should be able to differentiate into all cell subtypes in SUM-159 cells, just as shown in Fig 3D. However, after knocking down β4, there was a dramatic reduce in $CD24^{+}CD44^{+}$ population (Fig 3B), which means that $CD24^{-}CD44^{+}$ cells in shβ4 cells failed to give rise to $CD24^{+}CD44^{+}$ population in the parental SUM-159 cells. This indicates that CSC function of $CD24^{-}CD44^{+}$ population is defective without β4. Thus, I proposed that integrin β4, though it is not directly expressed by CSCs, is essential for maintaining CSC functions.

Since shRNAs don't fully knock down β4, I used CRISPR to target β4 in MDA-MB-231 cells. I sorted out single cells and grew them into colonies before selecting the clones with β4 completely knocked out. I selected one single cell

clone from each of the two guide RNAs sg3 and sg4, and performed RNA-seq (Fig 3E). β4 expression was examined in control and two knockout clones before RNA-seq (Fig 3F). Part of the RNA-seq results is shown in Table 3.

*2.4 Integrin β4 is associated with $CD24^+CD44^+$ population in breast tumor.*

I checked β4 expression by FACS in a Luminal A type human breast tumor. There are β4-low and β4-high populations, and β4-low cells are mostly $CD24^-CD44^+$ while β4-high cells are mostly $CD24^+CD44^+$ (Fig 4A). If the tumor cells are analyzed first by CD24 and CD44 expression, there are two subpopulations $CD24^-CD44^+$ and $CD24^+CD44^+$ (Fig 4A). Interestingly, $CD24^+CD44^+$ subpopulation has higher level of β4 expression compared to $CD24^-CD44^+$ subpopulation.

The model I propose from my data is described in fig 4B. β4 is expressed heterogeneously in breast tumor with $\beta4^+$ and $\beta4^-$ populations. The $\beta4^+$ population is $CD24^+CD44^+$ expressing basal and epithelial markers, and the $\beta4^-$ population is $CD24^-CD44^+$ with CSC properties. $\beta4^+$ non-CSCs are important for and interact with $\beta4^-$ CSCs by providing the ligands for paracrine signaling. Together, β4 regulates CSC functions in a non-cell-autonomous manner, and both $\beta4^+$ and $\beta4^-$ populations are needed for tumor formation.

## Materials and Methods

**Antibodies.** β4 (505, for western blot) (Rabinovitz et al., 1999); β4 (4399B, for FACS, IF and IHC); actin (Sigma-Aldrich); phycoerythrin-conjugated anti-CD44 (BD Bioscience); Alexa Fluor 647 anti-CD24 (BD Bioscience); PTHrP (a gift from Dr. Jiarong Li).

**Cell lines.** SUM-159 cells were provided by Dr. Steve Ethier (Medical College of South Carolina). MCF10A cells were obtained from the Barbara Ann Karmanos Cancer Institute. MDA-MB-231 cells were purchased from American Type Culture Collection. ER-Src-transformed MCF10A cells were provided by Dr. Kevin Struhl (Harvard Medical School). Isolation of the $CD24^{-}CD44^{+}$ population from these Src-transformed MCF10A cells and characterization of the $\alpha6^{hi}$ and $\alpha6^{lo}$ populations have been described (Goel et al., 2014). The SUM-159 shGFP and β4-shRNAs (sh68 and sh70) human breast carcinoma cell lines were generated and maintained as described previously (Chung et al., 2004). MDA-MB-231 cells stably expressing GFP under control of the VEGF promoter were generated and maintained as described previously (Goel et al., 2014). MCF10A siCtril and siβ4 cells were generated as described previously (Gerson et al., 2012b). A6hi and a6low cells stably expressing GFP and mCherry, respectively were generated by PCDH-GFP and PLV-mCherry (Addgene) lentiviral plasmids and selected on puromycin. MDA-MB-231 cells stably expressing sgRNAs were generated by cloning indicated sgRNA sequences into v2 lentiviral backbone

plasmid (Addgene). Single-cell sorting was performed to select clones with β4 knockout.

**RNA-seq**. RNA was extracted from the indicated cells and sent to Applied Biological Materials (ABM) for quantification and sequencing. Sequencing was performed on the paired ends of the mRNA fragment with a reading length of 75bps. Each sample had a sequencing depth of 8 million.

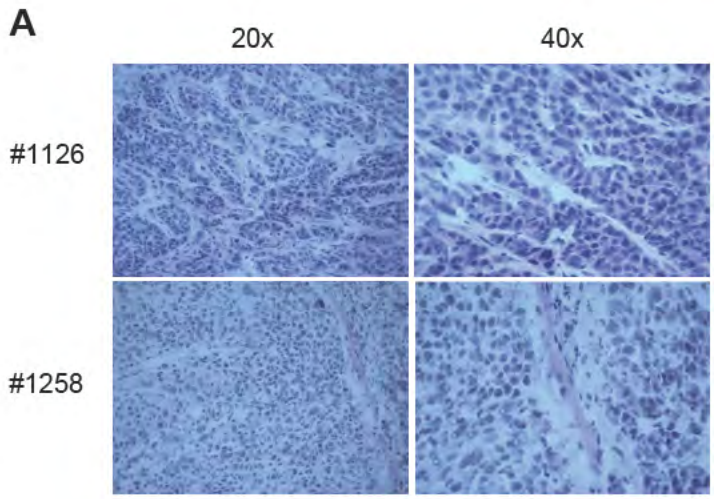
**Cell-Based Assays.** Flow cytometry was used to analyze surface expression of the β4 integrin, CD44, CD24 and lineage markers (CD31, CD45, Ter-119) in breast cancer cell lines and breast tumors. Cells in culture were detached, stained with antibodies and analyzed by FACS. PDX tumors and primary human breast tumors were dissociated into single cell suspensions as described before staining and FACS analysis (Goel et al., 2013). For 3D co-culture, $\alpha6^{hi}$-GFP and $\alpha6^{lo}$-mCherry cells were suspended in single cells and plated on Matrigel-coated plates for 1 week. For integrin α6β4 ligation assay, MCF10A cells were cultured on FN, LM111 and LM511-coated plates for 4 hours before collecting for RNA isolation.

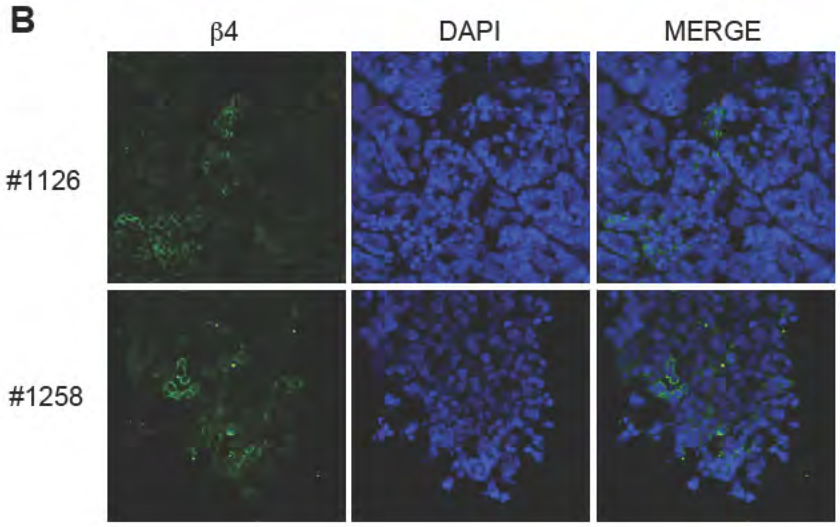
**Tumor samples.** For PDX tumors, triple-negative breast tumor tissue samples #1126 and #1258 were obtained from breast cancer surgery patients, through Brigham and Women's Hospital and Massachusetts General Hospital. These

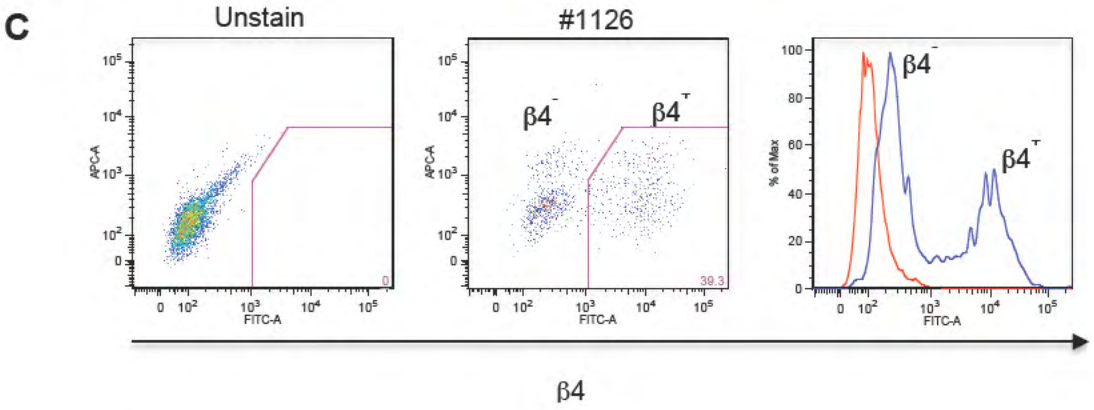
breast tumor samples were cut into small pieces, which were then surgically implanted into the fourth mammary fat pad of NSG mice. Tumor xenografts were expanded into multiple recipient NSG mice for several passages. For primary human tumors, two Luminal A type breast tumors were obtained from Tissue Bank at UMass Medical School.

**Immunoblotting and qPCR.** Cells were extracted in RIPA (50mM Tris-HCL [pH 7.4], 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1mM PMSF, and protease inhibitors). The proteins were separated by SDS-PAGE and immunoblotted using Abs as specified in the figure legends. For qPCR, RNA isolation was done with the NucleopSpin RNA kit (Macherey-Nagel) and 1ug of total RNA was used to produce cDNAs using Roche First strand synthesis kit (Roche). qPCR was performed using a SYBR green (Applied Biosystems) master mix as described by the manufacturer. Sequences for qPCR primers used are provided in Table 4.

## Figures


A
20x
40x
#1126
#1258


B
β4
DAPI
MERGE
#1126
#1258


C
Unstain
#1126
APC-A
FITC-A
β4⁻
β4⁺
% of Max
β4

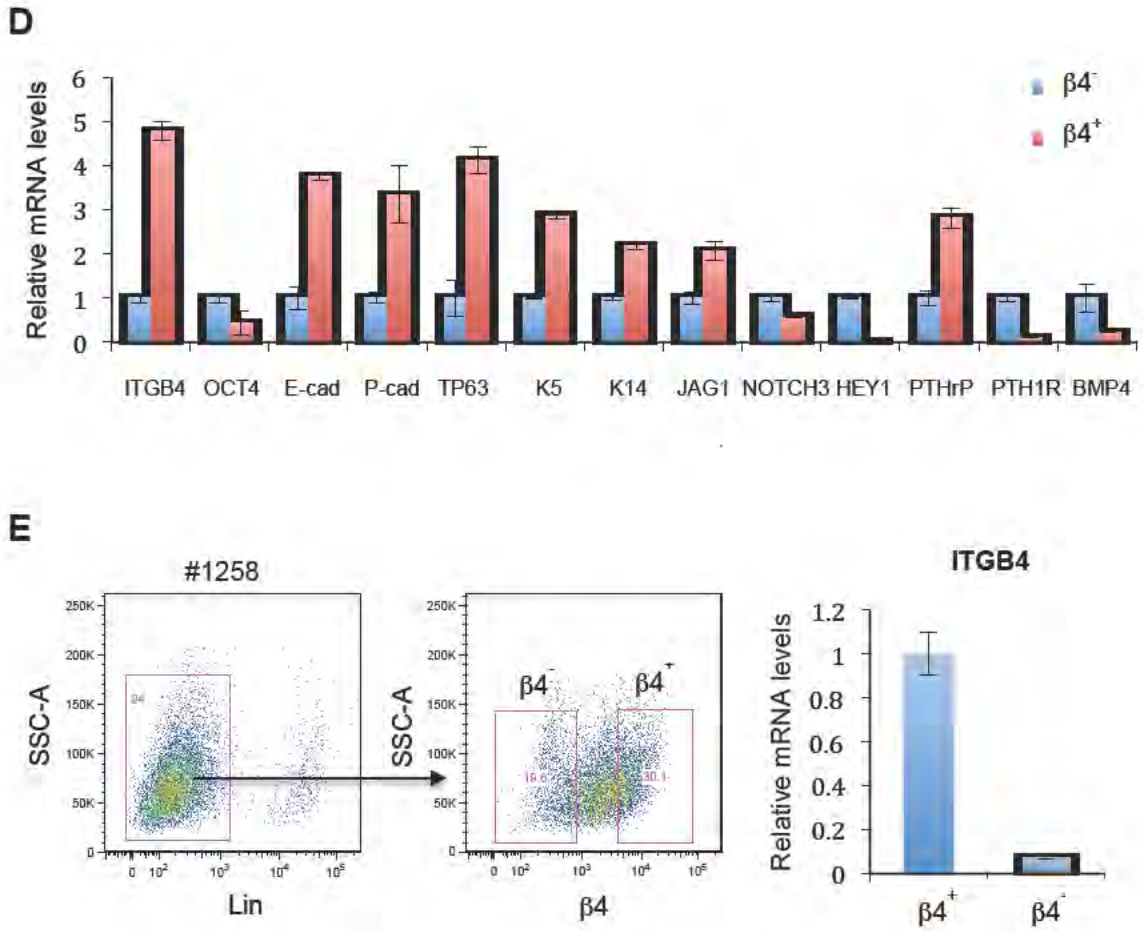
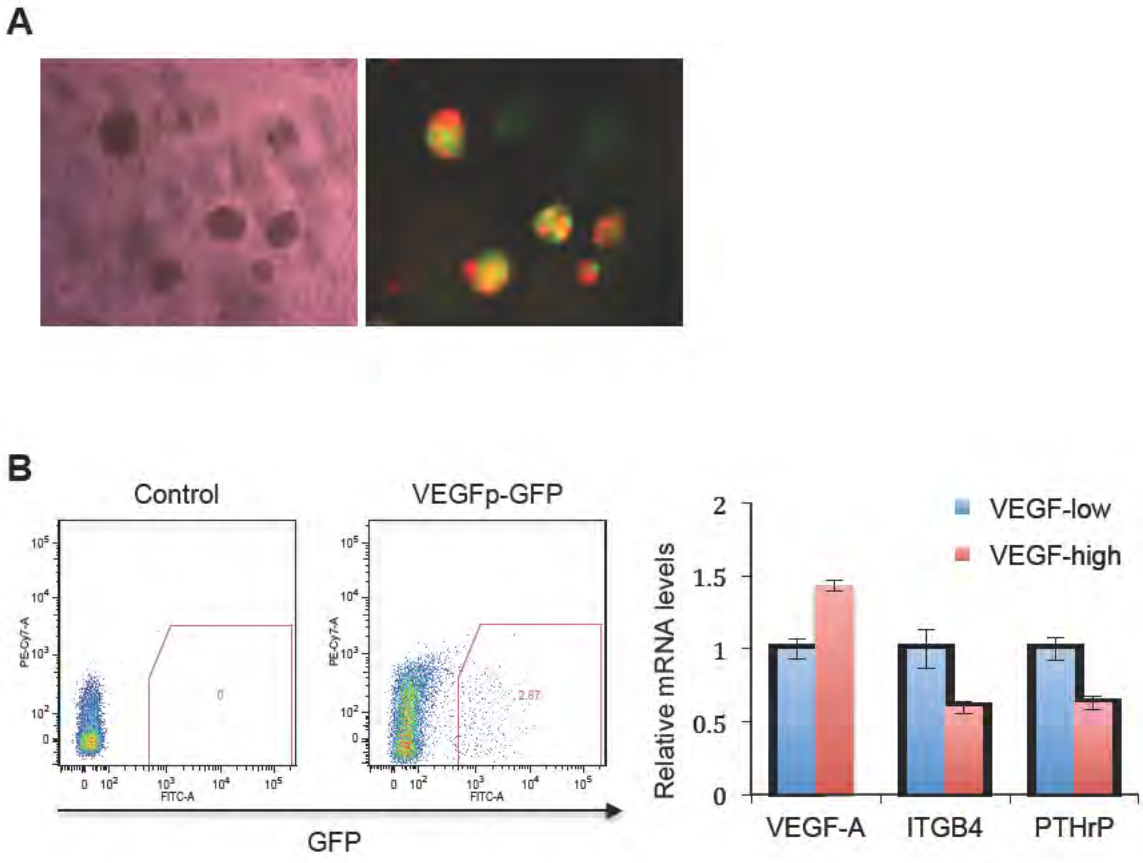



**Figure 1. Integrin β4 is expressed hetergeneously in breast tumor.**

(A) H&E staining of PDX tumor #1126 and #1258. (B) Expression of integrin β4 in PDX tumor #1126 and #1258 was examined by IF staining. (C) Integrin β4 expression in PDX tumor #1126 is analyzed by FACS, and $\beta4^+$ and $\beta4^-$ populations were sorted. (D) Relative mRNA levels of indicated genes were quantified in $\beta4^+$ and $\beta4^-$ populations by qPCR. (E) $\beta4^+$ and $\beta4^-$ populations were sorted in PDX tumor #1258 by FACS, and β4 expression was examined by qPCR.


A
B
Control
VEGFp-GFP
PE-Cy7-A
FITC-A
2.87
GFP
Relative mRNA levels
VEGF-low
VEGF-high
VEGF-A
ITGB4
PTHrP
C
Untreat
siCtrl
siβ4
β4
PTHrP
Actin
Relative mRNA levels
ITGB4
PTHrP

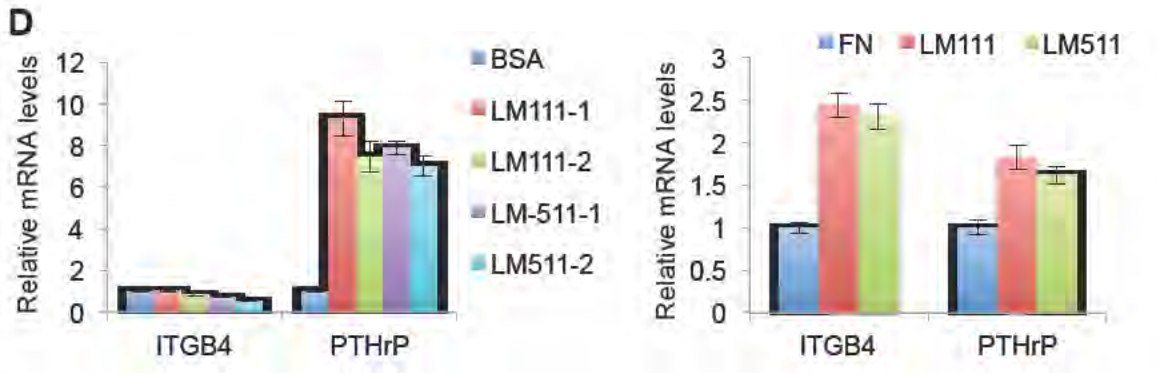
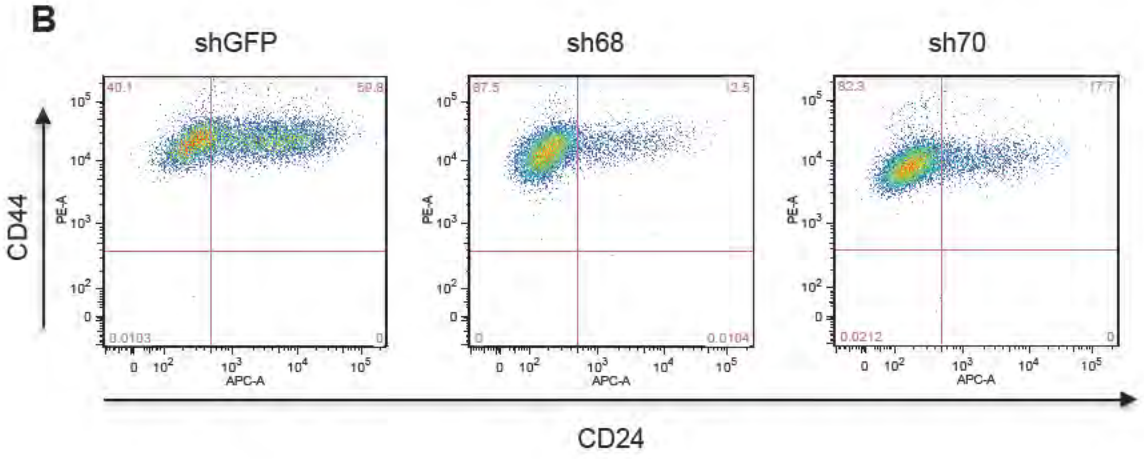
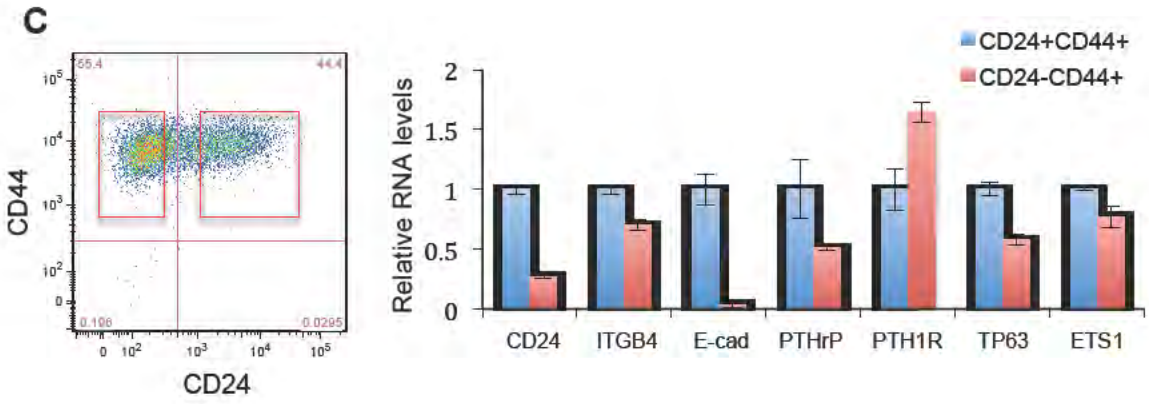


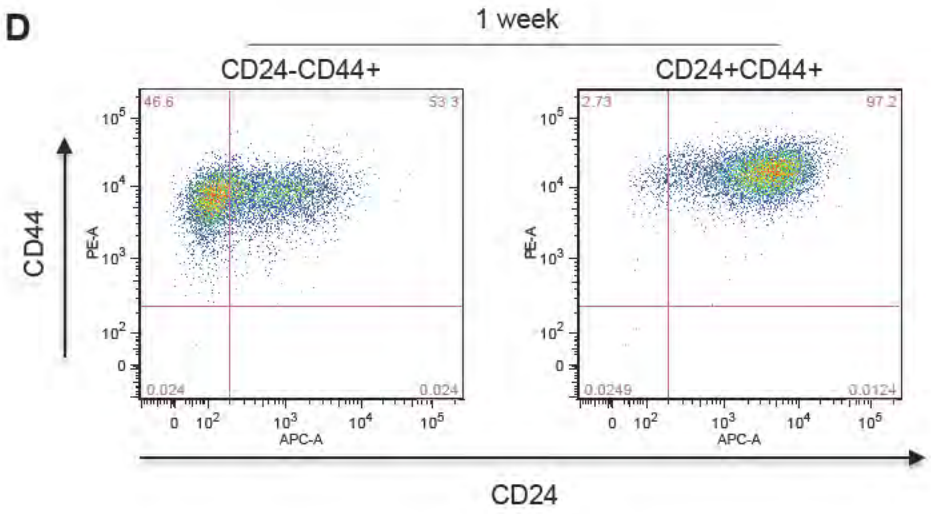

**Figure 2. Integrin β4 regulates PTHrP expression.**

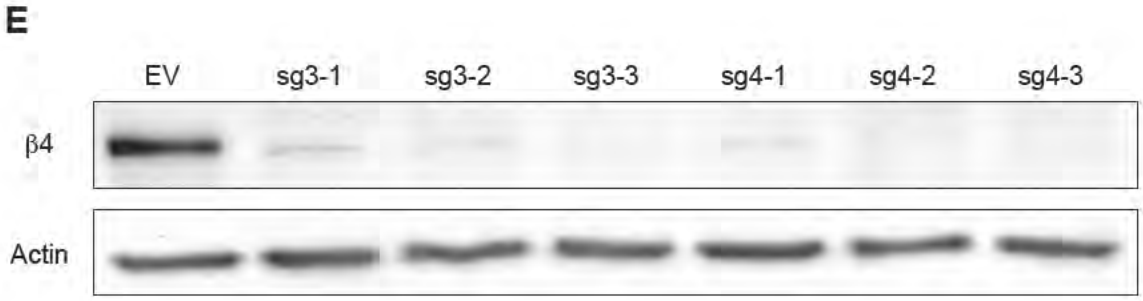
(A) $\alpha6^{hi}$-GFP and $\alpha6^{lo}$-mCherry cells were mixed in single-cell suspension and cultured on Matrigel for 1 week. (B) $GFP^+$ cells were sorted from MDA-MB-231 cells stably expressing GFP under control of the VEGF promoter. Relative mRNA levels of VEGF-A, ITGB4 and PTHrP are examined by qPCR in VEGF-low ($GFP^-$) cells and VEGF-high ($GFP^+$) cells. (C) Expression of β4 and PTHrP were examined by western blot and qPCR in MCF10A cells transfected with siCtrl or siβ4. (D) MCF10A cells were plated on BSA, fibronectin (FN) or indicated laminins (LM) for 4hr before collected for qPCR.


A
shGFP
sh68
sh70
β4
Actin


B
shGFP
sh68
sh70
CD44
CD24
PE-A
APC-A
40.1
59.8
0.0103
0
87.5
12.5
0
0.0104
82.3
17.7
0.0212
0


C
55.4
44.4
0.106
0.0295
CD44
CD24
CD24+CD44+
CD24-CD44+
Relative RNA levels
2
1.5
1
0.5
0
CD24
ITGB4
E-cad
PTHrP
PTH1R
TP63
ETS1


D
1 week
CD24-CD44+
CD24+CD44+
46.6
53.3
2.73
97.2
0.024
0.024
0.0249
0.0124
PE-A
APC-A
CD44
CD24


E
EV
sg3-1
sg3-2
sg3-3
sg4-1
sg4-2
sg4-3
β4
Actin

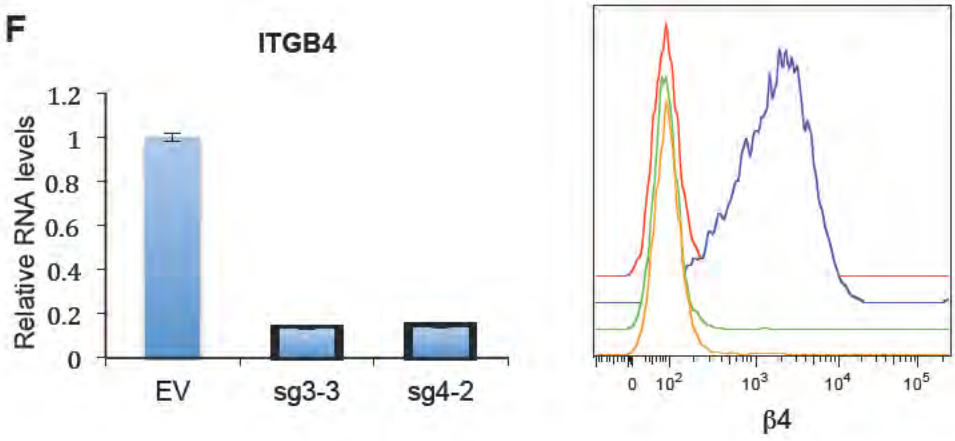

F
ITGB4
Relative RNA levels
1.2
1
0.8
0.6
0.4
0.2
0
EV
sg3-3
sg4-2
β4

**Figure 3. Knocking down integrin β4 affects CSC functions.**

(A) Integrin β4 was knocked down in SUM-159 cells by two shRNAs, sh68 and sh70. (B) $CD24^{+}CD44^{+}$ population was reduced dramatically after β4 was knocked down. (C) $CD24^{-}CD44^{+}$ and $CD24^{+}CD44^{+}$ populations in SUM-159 cells were sorted, and the expression of indicated genes were examined by qPCR. (D) $CD24^{-}CD44^{+}$ and $CD24^{+}CD44^{+}$ populations were cultured separately for 1 week before CD24 and CD44 expression were analyzed by FACS again. (E) MDA-MB-231 cells stably expressing sgRNAs (sg3 and sg4) targeting integrin β4 were generated. Three clones derived from single cells for each sgRNA were selected, and β4 expression was examined by western blot. (F) Expression of integrin β4 is examined by qPCR and FACS in two selected clones sg3-3 and sg4-2.

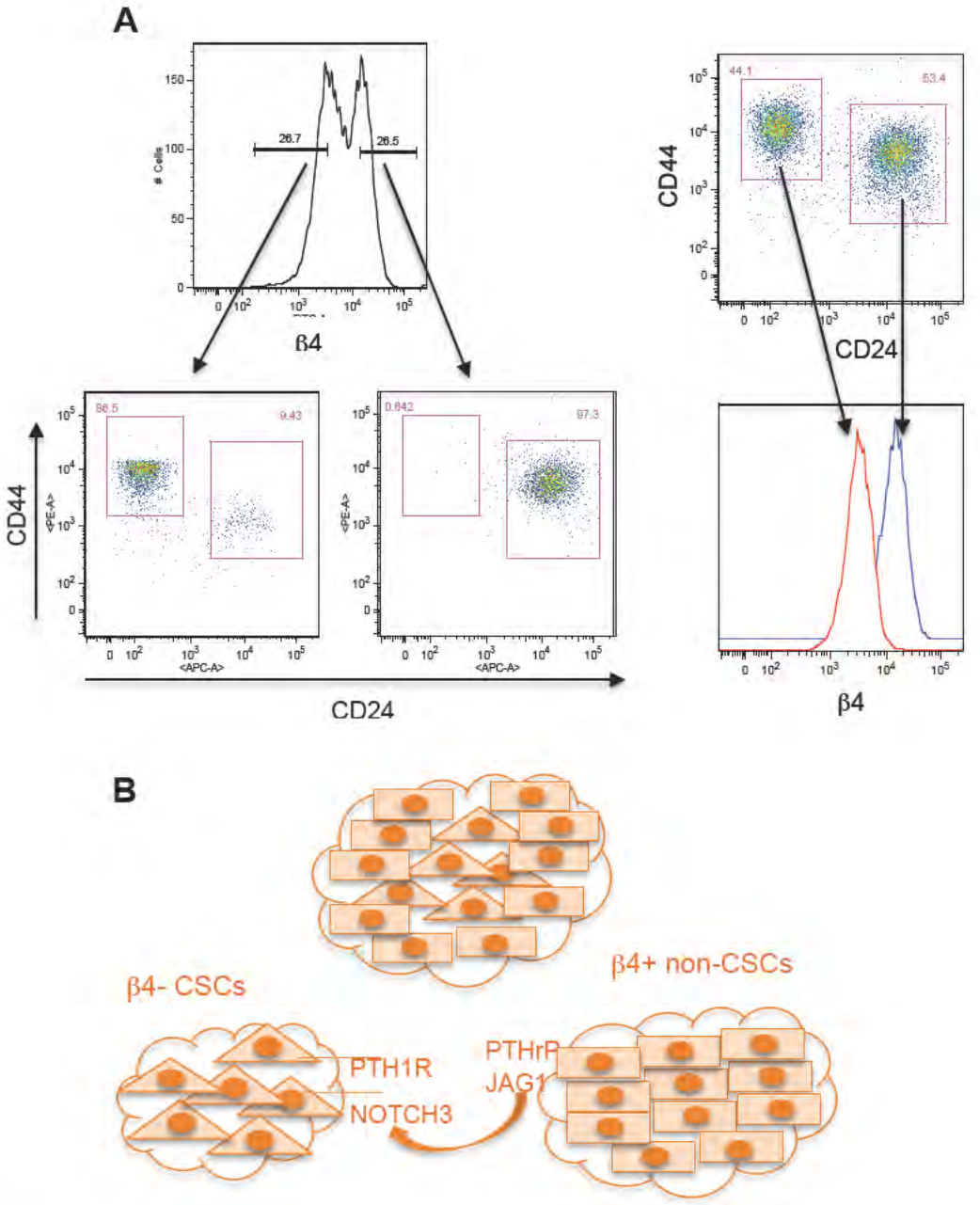



**Figure 4. Integrin β4 is associated with CD24+CD44+ population in breast tumor.**

(A) Expression of integrin β4, CD24 and CD44 were analyzed by FACS in Luminal A type human breast tumor. (B) Model of the study.

Table 1. Summary of RNA-seq of $\beta4^{hi}$ and $\beta4^{lo}$ populations in Src-transformed MCF10A system

| Top genes in $\beta4^{hi}$ population | $\log_2$ ratio $\beta4^{hi}$ / $\beta4^{lo}$ | Top genes in $\beta4^{lo}$ population | $\log_2$ ratio $\beta4^{lo}$ / $\beta4^{hi}$ |
|---|---|---|---|
| CDH1 | 4.3402873 | POSTN | 6.093040102 |
| TP63 | 3.743782825 | BMP4 | 3.157352242 |
| PTHrP | 3.211881568 | NOTCH3 | 3.13051063 |
| LAMA3 | 2.986767935 | WNT5A | 2.973761667 |
| ALDH1A3 | 2.304530856 | WNT5B | 2.543005934 |
| CD24 | 2.267123678 | TGFB3 | 1.832512552 |
| KRT14 | 2.201647226 | PVRL3 | 1.45199799 |
| CDH3 | 1.879796858 | CXCR4 | 1.451083445 |
| PVRL1 | 1.762919545 | TGFB2 | 1.224635659 |
| ITGA6 | 1.351495691 | WNT3 | 1.157352242 |
| LAMC2 | 1.326901226 | TGFB1 | 0.919192505 |
| LAMB3 | 1.123110904 | TWIST1 | 0.902375611 |
| JAG1 | 1.119903245 | ERBB2 | 0.838043658 |
| PVRL4 | 0.889953473 | COL4A2 | 0.815580486 |
| KRT18 | 0.809841409 | COL4A1 | 0.779803164 |
| KRT5 | 0.746395911 | SNAI1 | 0.731534904 |
| NOTCH1 | 0.594431214 | PVRL2 | 0.584585038 |
| IGF1R | 0.412021182 | ITGB1 | 0.476975791 |
| CD44 | 0.362532557 | NOTCH2 | 0.416051599 |

Table 2. Summary of RNA-seq of $\beta4^+$ and $\beta4^-$ populations in PDX tumor

Top 100 genes in $\beta4^+$ population

| Rank | gene_id | fold.change. $\beta4^-$ / $\beta4^+$ | genename |
|---|---|---|---|
| 1 | ERP27 | 0.055810447 | endoplasmic reticulum protein 27 |
| 2 | EDN2 | 0.068085924 | endothelin 2 |
| 3 | GABRP | 0.07806187 | gamma-aminobutyric acid (GABA) A receptor, pi |
| 4 | ITGB4 | 0.078433946 | integrin, beta 4 |
| 5 | SCGB2A2 | 0.08094378 | secretoglobin, family 2A, member 2 |
| 6 | PROM2 | 0.081264473 | prominin 2 |
| 7 | SOSTDC1 | 0.086105138 | sclerostin domain containing 1 |
| 8 | KRTAP19-5 | 0.088997743 | keratin associated protein 19-5 |
| 9 | LINC00617 | 0.089003394 | |
| 10 | CAPS | 0.090619562 | calcyphosine |
| 11 | EEF1E1-MUTED | 0.091320952 | |
| 12 | ITGB6 | 0.100665881 | integrin, beta 6 |
| 13 | DEFB109P1 | 0.105029219 | defensin, beta 109, pseudogene 1 |
| 14 | KRTAP5-2 | 0.105787866 | keratin associated protein 5-2 |
| 15 | SLC34A2 | 0.108548112 | solute carrier family 34 (type II sodium/phosphate cotransporter), member 2 |
| 16 | SNORA58 | 0.10884567 | small nucleolar RNA, H/ACA box 58 |
| 17 | CLDN8 | 0.111889128 | claudin 8 |
| 18 | MIR205HG | 0.112553915 | MIR205 host gene |
| 19 | ZNF20 | 0.114010716 | zinc finger protein 20 |
| 20 | S100A14 | 0.114327068 | S100 calcium binding protein A14 |
| 21 | EPHA1 | 0.115049589 | EPH receptor A1 |
| 22 | AZGP1 | 0.115545491 | alpha-2-glycoprotein 1, zinc-binding |
| 23 | EPN3 | 0.116073033 | epsin 3 |
| 24 | PROM1 | 0.116701246 | prominin 1 |
| 25 | SCARNA10 | 0.119531437 | small Cajal body-specific RNA 10 |
| 26 | PRODH | 0.121185626 | proline dehydrogenase (oxidase) 1 |
| 27 | FXYD3 | 0.121581886 | FXYD domain containing ion transport regulator 3 |
| 28 | KRT17 | 0.125540397 | keratin 17, type I |
| 29 | KRTAP21-2 | 0.127566641 | keratin associated protein 21-2 |
| 30 | HIST1H2AB | 0.128848047 | histone cluster 1, H2ab |
| 31 | S100P | 0.132093822 | S100 calcium binding protein P |
| 32 | VIPR1 | 0.132249714 | vasoactive intestinal peptide receptor 1 |
| 33 | LOC93432 | 0.138217499 | maltase-glucoamylase (alpha-glucosidase) |
| 34 | EFNA3 | 0.139139826 | ephrin-A3 |
| 35 | DEFB109P1B | 0.141477134 | defensin, beta 109, pseudogene 1B |
| 36 | LOC642423 | 0.142028508 | golgin A2 pseudogene |
| 37 | TRIM29 | 0.142518236 | tripartite motif containing 29 |
| 38 | LCN2 | 0.142842554 | lipocalin 2 |
| 39 | TMPRSS13 | 0.142981928 | transmembrane protease, serine 13 |
| 40 | KRTCAP3 | 0.143176989 | keratinocyte associated protein 3 |

| | | | |
|---|---|---|---|
| 41 | MB | 0.145079255 | myoglobin |
| 42 | NAALADL2 | 0.147429106 | N-acetylated alpha-linked acidic dipeptidase-like 2 |
| 43 | INHBB | 0.147610419 | inhibin, beta B |
| 44 | BSPRY | 0.1478731 | B-box and SPRY domain containing |
| 45 | LIPG | 0.149503414 | lipase, endothelial |
| 46 | AQP3 | 0.151534351 | aquaporin 3 (Gill blood group) |
| 47 | RASAL1 | 0.152332362 | RAS protein activator like 1 (GAP1 like) |
| 48 | CAMSAP3 | 0.152843564 | calmodulin regulated spectrin-associated protein family, member 3 |
| 49 | EHF | 0.153213829 | ets homologous factor |
| 50 | LAD1 | 0.153312724 | ladinin 1 |
| 51 | KRT14 | 0.153942955 | keratin 14, type I |
| 52 | LGALS7 | 0.15404238 | lectin, galactoside-binding, soluble, 7 |
| 53 | PADI2 | 0.156071582 | peptidyl arginine deiminase, type II |
| 54 | SUSD4 | 0.156225299 | sushi domain containing 4 |
| 55 | UNC13D | 0.156339417 | unc-13 homolog D (C. elegans) |
| 56 | LOC100128338 | 0.156403451 | |
| 57 | SLPI | 0.1564616 | secretory leukocyte peptidase inhibitor |
| 58 | VGLL1 | 0.158340308 | vestigial-like family member 1 |
| 59 | GUCY1B3 | 0.158370436 | guanylate cyclase 1, soluble, beta 3 |
| 60 | VTCN1 | 0.158429337 | V-set domain containing T cell activation inhibitor 1 |
| 61 | KRT15 | 0.160475562 | keratin 15, type I |
| 62 | DLX3 | 0.16236596 | distal-less homeobox 3 |
| 63 | JMJD7-PLA2G4B | 0.163768749 | JMJD7-PLA2G4B readthrough |
| 64 | TMEM54 | 0.166422176 | transmembrane protein 54 |
| 65 | ESRP1 | 0.167872286 | epithelial splicing regulatory protein 1 |
| 66 | B4GALNT3 | 0.167928138 | beta-1,4-N-acetyl-galactosaminyl transferase 3 |
| 67 | EFCAB4A | 0.167970125 | |
| 68 | PVRL4 | 0.168173739 | poliovirus receptor-related 4 |
| 69 | FMOD | 0.169938592 | fibromodulin |
| 70 | MUC5B | 0.170962449 | mucin 5B, oligomeric mucus/gel-forming |
| 71 | AGR2 | 0.171148032 | anterior gradient 2 |
| 72 | ATP2C2 | 0.172121778 | ATPase, Ca++ transporting, type 2C, member 2 |
| 73 | CALB2 | 0.172161605 | calbindin 2 |
| 74 | IGFL1 | 0.172251795 | IGF-like family member 1 |
| 75 | MUC1 | 0.172274959 | mucin 1, cell surface associated |
| 76 | IGFL2 | 0.174560115 | IGF-like family member 2 |
| 77 | PTPRU | 0.174636105 | protein tyrosine phosphatase, receptor type, U |
| 78 | FLVCR2 | 0.175418597 | feline leukemia virus subgroup C cellular receptor family, member 2 |
| 79 | RTN4RL1 | 0.176413331 | reticulon 4 receptor-like 1 |
| 80 | LOC728463 | 0.178605874 | |
| 81 | RBP1 | 0.178629336 | retinol binding protein 1, cellular |
| 82 | DSC3 | 0.179223577 | desmocollin 3 |
| 83 | KRT6B | 0.179990217 | keratin 6B, type II |
| 84 | FREM2 | 0.180121264 | FRAS1 related extracellular matrix protein 2 |
| 85 | KRT6C | 0.180811098 | keratin 6C, type II |
| 86 | SPINT1 | 0.180864618 | serine peptidase inhibitor, Kunitz type 1 |

| | | | |
|---|---|---|---|
| 87 | HIST1H4D | 0.181680765 | histone cluster 1, H4d |
| 88 | TMC6 | 0.181806274 | transmembrane channel-like 6 |
| 89 | C6orf15 | 0.183153963 | chromosome 6 open reading frame 15 |
| 90 | PDGFA | 0.183711689 | platelet-derived growth factor alpha polypeptide |
| 91 | ALDH3B2 | 0.184243618 | aldehyde dehydrogenase 3 family, member B2 |
| 92 | MAP7 | 0.184346294 | microtubule-associated protein 7 |
| 93 | CLDN23 | 0.18458237 | claudin 23 |
| 94 | NEBL | 0.187068659 | nebulette |
| 95 | TNNI2 | 0.187391417 | troponin I type 2 (skeletal, fast) |
| 96 | HSD11B2 | 0.188092237 | hydroxysteroid (11-beta) dehydrogenase 2 |
| 97 | CLIC3 | 0.188237351 | chloride intracellular channel 3 |
| 98 | S100A16 | 0.189391975 | S100 calcium binding protein A16 |
| 99 | KRT8 | 0.190759774 | keratin 8, type II |
| 100 | CTSL2 | 0.19103296 | |

Top 100 genes in $\beta4^-$ population

| **Rank** | **gene_id** | **fold.change.** $\beta4^-$ **/** $\beta4^+$ | **genename** |
|---|---|---|---|
| 1 | RPL31P11 | 39.56084 | ribosomal protein L31 pseudogene 11 |
| 2 | KRTAP20-2 | 25.62712 | keratin associated protein 20-2 |
| 3 | SUMO4 | 19.8716 | small ubiquitin-like modifier 4 |
| 4 | RAB11A | 19.22263546 | RAB11A, member RAS oncogene family |
| 5 | SNORA28 | 16.47679023 | small nucleolar RNA, H/ACA box 28 |
| 6 | ZEB2-AS1 | 14.29556367 | ZEB2 antisense RNA 1 |
| 7 | POTEM | 11.42675277 | POTE ankyrin domain family, member M |
| 8 | RMRP | 11.027 | RNA component of mitochondrial RNA processing endoribonuclease |
| 9 | SCARNA7 | 10.50824 | small Cajal body-specific RNA 7 |
| 10 | CXCL14 | 9.965201093 | chemokine (C-X-C motif) ligand 14 |
| 11 | POTEJ | 9.793759966 | POTE ankyrin domain family, member J |
| 12 | CSNK1A1L | 9.7768 | casein kinase 1, alpha 1-like |
| 13 | SPON2 | 9.673812413 | spondin 2, extracellular matrix protein |
| 14 | SNORA14B | 8.895367534 | small nucleolar RNA, H/ACA box 14B |
| 15 | FAM78B | 8.836337781 | family with sequence similarity 78, member B |
| 16 | POTEF | 8.173411643 | POTE ankyrin domain family, member F |
| 17 | HGF | 7.977776299 | hepatocyte growth factor (hepapoietin A; scatter factor) |
| 18 | SERPINE1 | 7.704713281 | serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 |
| 19 | MSC | 7.662533796 | musculin |
| 20 | ZEB2 | 7.239536869 | zinc finger E-box binding homeobox 2 |
| 21 | MIR3661 | 7.153655035 | microRNA 3661 |
| 22 | CXCL12 | 6.877907642 | chemokine (C-X-C motif) ligand 12 |
| 23 | ZNF804A | 6.832372958 | zinc finger protein 804A |

| | | | |
|---|---|---|---|
| 24 | FAM90A2P | 6.813 | family with sequence similarity 90, member A2 pseudogene |
| 25 | CUZD1 | 6.469748699 | CUB and zona pellucida-like domains 1 |
| 26 | FLNC | 6.447612684 | filamin C, gamma |
| 27 | HIST1H4B | 6.4476 | histone cluster 1, H4b |
| 28 | HS3ST3A1 | 6.427331867 | heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1 |
| 29 | TGM2 | 6.396667265 | transglutaminase 2 |
| 30 | EBF1 | 6.37322519 | early B-cell factor 1 |
| 31 | TMEFF2 | 6.365964799 | transmembrane protein with EGF-like and two follistatin-like domains 2 |
| 32 | POTEE | 6.359760119 | POTE ankyrin domain family, member E |
| 33 | MIR3682 | 6.320083257 | microRNA 3682 |
| 34 | PIN4P1 | 6.307404114 | protein (peptidylprolyl cis/trans isomerase) NIMA-interacting, 4 pseudogene 1 |
| 35 | SERPINF1 | 6.196399774 | serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1 |
| 36 | PLAC8L1 | 6.089834984 | PLAC8-like 1 |
| 37 | CREB3L1 | 6.011548445 | cAMP responsive element binding protein 3-like 1 |
| 38 | TRPC6 | 5.880595105 | transient receptor potential cation channel, subfamily C, member 6 |
| 39 | PCDH7 | 5.857565254 | protocadherin 7 |
| 40 | MMP3 | 5.701584203 | matrix metallopeptidase 3 |
| 41 | PTENP1 | 5.67822539 | phosphatase and tensin homolog pseudogene 1 (functional) |
| 42 | SFRP2 | 5.671937194 | secreted frizzled-related protein 2 |
| 43 | DCN | 5.59704733 | decorin |
| 44 | TFPI2 | 5.457923828 | tissue factor pathway inhibitor 2 |
| 45 | FOXP1 | 5.434430939 | forkhead box P1 |
| 46 | COL5A2 | 5.394802843 | collagen, type V, alpha 2 |
| 47 | SNORA51 | 5.394565631 | small nucleolar RNA, H/ACA box 51 |
| 48 | DACT1 | 5.371712803 | dishevelled-binding antagonist of beta-catenin 1 |
| 49 | SCARNA11 | 5.314531105 | small Cajal body-specific RNA 11 |
| 50 | BMPER | 5.305486231 | BMP binding endothelial regulator |
| 51 | HEG1 | 5.304122148 | heart development protein with EGF-like domains 1 |
| 52 | PRAP1 | 5.24076 | proline-rich acidic protein 1 |
| 53 | GFAP | 5.189984324 | glial fibrillary acidic protein |
| 54 | LINC00235 | 5.051334656 | long intergenic non-protein coding RNA 235 |
| 55 | FEZ1 | 4.949002127 | fasciculation and elongation protein zeta 1 (zygin I) |
| 56 | PRPS1L1 | 4.934884 | phosphoribosyl pyrophosphate synthetase 1-like 1 |
| 57 | DENND5B-AS1 | 4.91194 | DENND5B antisense RNA 1 |
| 58 | CDRT15 | 4.88256 | CMT1A duplicated region transcript 15 |
| 59 | TFAP2B | 4.873186417 | transcription factor AP-2 beta (activating enhancer binding protein 2 beta) |
| 60 | GPR176 | 4.869509648 | G protein-coupled receptor 176 |
| 61 | INO80B-WBP1 | 4.842572365 | INO80B-WBP1 readthrough (NMD candidate) |
| 62 | MMP1 | 4.823947431 | matrix metallopeptidase 1 |

| | | | |
|---|---|---|---|
| 63 | LAYN | 4.813852191 | layilin |
| 64 | NNMT | 4.762956969 | nicotinamide N-methyltransferase |
| 65 | DDX3Y | 4.740717564 | DEAD (Asp-Glu-Ala-Asp) box helicase 3, Y-linked |
| 66 | COL3A1 | 4.661040209 | collagen, type III, alpha 1 |
| 67 | LOXL1 | 4.645667536 | lysyl oxidase-like 1 |
| 68 | DPT | 4.640355521 | dermatopontin |
| 69 | ADAM19 | 4.591757985 | ADAM metallopeptidase domain 19 |
| 70 | ESM1 | 4.590916332 | endothelial cell-specific molecule 1 |
| 71 | FHL1 | 4.549355217 | four and a half LIM domains 1 |
| 72 | PTPRN | 4.513944 | protein tyrosine phosphatase, receptor type, N |
| 73 | RPS10-NUDT3 | 4.485972758 | RPS10-NUDT3 readthrough |
| 74 | EXT1 | 4.475825975 | exostosin glycosyltransferase 1 |
| 75 | C9orf53 | 4.45826 | |
| 76 | MARCH4 | 4.456227523 | membrane-associated ring finger (C3HC4) 4, E3 ubiquitin protein ligase |
| 77 | LOC728875 | 4.417964747 | |
| 78 | HIC1 | 4.417683196 | hypermethylated in cancer 1 |
| 79 | DACT3 | 4.394819526 | dishevelled-binding antagonist of beta-catenin 3 |
| 80 | METRN | 4.354512 | meteorin, glial cell differentiation regulator |
| 81 | KCNE4 | 4.348465048 | potassium channel, voltage gated subfamily E regulatory beta subunit 4 |
| 82 | NPTX1 | 4.331684174 | neuronal pentraxin I |
| 83 | HBEGF | 4.277256745 | heparin-binding EGF-like growth factor |
| 84 | HAS2 | 4.242831962 | hyaluronan synthase 2 |
| 85 | MIR25 | 4.213524347 | microRNA 25 |
| 86 | ARL2-SNX15 | 4.206012874 | ARL2-SNX15 readthrough (NMD candidate) |
| 87 | NOVA1 | 4.205469071 | neuro-oncological ventral antigen 1 |
| 88 | PRG1 | 4.176908 | p53-responsive gene 1 |
| 89 | IL13RA2 | 4.158880367 | interleukin 13 receptor, alpha 2 |
| 90 | TSNAX-DISC1 | 4.158744 | TSNAX-DISC1 readthrough (NMD candidate) |
| 91 | NPPB | 4.141444389 | natriuretic peptide B |
| 92 | HS3ST3B1 | 4.136352538 | heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1 |
| 93 | PTEN | 4.114100491 | phosphatase and tensin homolog |
| 94 | RUNX1T1 | 4.103495281 | runt-related transcription factor 1; translocated to, 1 (cyclin D-related) |
| 95 | CNRIP1 | 4.103266866 | cannabinoid receptor interacting protein 1 |
| 96 | TOP1P1 | 4.100549285 | topoisomerase (DNA) I pseudogene 1 |
| 97 | STEAP1B | 4.076097027 | STEAP family member 1B |
| 98 | RNF216-IT1 | 4.040784 | RNF216 intronic transcript 1 |
| 99 | FMN2 | 4.025966549 | formin 2 |
| 100 | EEF1DP3 | 4.008811645 | eukaryotic translation elongation factor 1 delta pseudogene 3 |

Table 3. Summary of RNA-seq of genes downregulated in β4 knockout cells.

| Rank | gene id | fold change sg3 / EV | fold change sg4 / EV | genename |
|---|---|---|---|---|
| 1 | TSTD1 | 0.04350126 | 0.069911745 | thiosulfate sulfurtransferase (rhodanese)-like domain containing 1 |
| 2 | SPANXA2 | 0.051349569 | 0.051349569 | SPANX family, member A2 |
| 3 | C16orf45 | 0.07342529 | 0.064272018 | chromosome 16 open reading frame 45 |
| 4 | FAM133DP | 0.073443954 | 0.827558717 | family with sequence similarity 133, member A pseudogene |
| 5 | CXCR4 | 0.073926139 | 0.057124818 | chemokine (C-X-C motif) receptor 4 |
| 6 | SPINK4 | 0.075410232 | 0.191924771 | serine peptidase inhibitor, Kazal type 4 |
| 7 | NGFR | 0.086140919 | 0.079623097 | nerve growth factor receptor |
| 8 | SPANXA2 | 0.092089193 | 0.092089014 | SPANX family, member A2 |
| 9 | EPCAM | 0.099203079 | 0.120440375 | epithelial cell adhesion molecule |
| 10 | CCBE1 | 0.116851125 | 0.111226787 | collagen and calcium binding EGF domains 1 |
| 11 | KISS1 | 0.121346758 | 0.257111317 | KiSS-1 metastasis-suppressor |
| 12 | SCARNA9 | 0.131348741 | 0.131348741 | small Cajal body-specific RNA 9 |
| 13 | HIST1H4A | 0.142589889 | 0.574498084 | histone cluster 1, H4a |
| 14 | OR2A20P | 0.145343836 | 0.302905078 | olfactory receptor, family 2, subfamily A, member 20 pseudogene |
| 15 | C10orf32-AS3MT | 0.151792632 | 0.151764504 | |
| 16 | LOC100129216 | 0.153774235 | 0.153774235 | beta-defensin 131-like |
| 17 | ZNF521 | 0.154848462 | 0.234063645 | zinc finger protein 521 |
| 18 | OR2A9P | 0.159888532 | 0.159876545 | olfactory receptor, family 2, subfamily A, member 9 pseudogene |
| 19 | SCARNA27 | 0.168141171 | 0.670204372 | small Cajal body-specific RNA 27 |
| 20 | NAP1L3 | 0.171950121 | 0.31324291 | nucleosome assembly protein 1-like 3 |
| 21 | RBPMS2 | 0.173485324 | 0.202433517 | RNA binding protein with multiple splicing 2 |
| 22 | TNFSF12-TNFSF13 | 0.174179488 | 1.623951512 | TNFSF12-TNFSF13 readthrough |
| 23 | DCN | 0.17713751 | 0.05923473 | decorin |
| 24 | IGFBP5 | 0.179743337 | 0.1656084 | insulin-like growth factor binding protein 5 |
| 25 | INHBB | 0.185578391 | 0.23093968 | inhibin, beta B |
| 26 | ZNF727 | 0.187631342 | 0.867948814 | zinc finger protein 727 |
| 27 | C2orf82 | 0.193909685 | 0.394747374 | chromosome 2 open reading frame 82 |
| 28 | SORCS2 | 0.19484684 | 0.433642666 | sortilin-related VPS10 domain containing receptor 2 |
| 29 | HIST1H3A | 0.19598313 | 0.396238692 | histone cluster 1, H3a |
| 30 | SNORA58 | 0.197098707 | 0.197098707 | small nucleolar RNA, H/ACA box 58 |
| 31 | SNORA55 | 0.204217229 | 1.013776963 | small nucleolar RNA, H/ACA box 55 |
| 32 | LGALS12 | 0.208078264 | 0.249252292 | lectin, galactoside-binding, soluble, 12 |
| 33 | FRMD3 | 0.210432574 | 1.261265508 | FERM domain containing 3 |
| 34 | MGMT | 0.213281099 | 0.272516171 | O-6-methylguanine-DNA methyltransferase |

| | | | | |
|---|---|---|---|---|
| 35 | LOC100130298 | 0.21811697 | 0.412259395 | hCG1816373-like |
| 36 | SCGB1B2P | 0.218850945 | 0.682335227 | secretoglobin, family 1B, member 2, pseudogene |
| 37 | C19orf73 | 0.221246658 | 0.450753757 | chromosome 19 open reading frame 73 |
| 38 | LOC400927 | 0.222905072 | 0.774445985 | TPTE and PTEN homologous inositol lipid phosphatase pseudogene |
| 39 | SHANK2 | 0.226461863 | 0.287905246 | SH3 and multiple ankyrin repeat domains 2 |
| 40 | DPYD-AS1 | 0.227782338 | 0.802344154 | DPYD antisense RNA 1 |
| 41 | RPS10-NUDT3 | 0.236315084 | 0.948134529 | RPS10-NUDT3 readthrough |
| 42 | LOC339535 | 0.239718369 | 0.195576834 | |
| 43 | C11orf94 | 0.245990115 | 0.245990115 | chromosome 11 open reading frame 94 |
| 44 | SCARNA6 | 0.250293093 | 1.028429291 | small Cajal body-specific RNA 6 |
| 45 | CRYAB | 0.250299439 | 0.342318396 | crystallin, alpha B |
| 46 | MCAM | 0.252200635 | 0.128516192 | melanoma cell adhesion molecule |
| 47 | ITGB4 | 0.255371167 | 0.275924966 | integrin, beta 4 |
| 48 | F2RL2 | 0.256479997 | 0.261992419 | coagulation factor II (thrombin) receptor-like 2 |
| 49 | GYPC | 0.257663691 | 0.266597922 | glycophorin C (Gerbich blood group) |
| 50 | CBWD5 | 0.261927415 | 1.013365068 | COBW domain containing 5 |
| 51 | PCSK1N | 0.262831201 | 0.413415047 | proprotein convertase subtilisin/kexin type 1 inhibitor |
| 52 | PDCL3P4 | 0.264976447 | 1.200811281 | phosducin-like 3 pseudogene 4 |
| 53 | GYG2 | 0.268174461 | 0.546234848 | glycogenin 2 |
| 54 | ASTN1 | 0.269359997 | 0.289423557 | astrotactin 1 |
| 55 | RPS4Y1 | 0.27005168 | 0.18999848 | ribosomal protein S4, Y-linked 1 |
| 56 | RPPH1 | 0.273742397 | 0.584716071 | ribonuclease P RNA component H1 |
| 57 | HLA-DRB4 | 0.275207359 | 0.124839581 | major histocompatibility complex, class II, DR beta 4 |
| 58 | PTGES3L | 0.277866041 | 0.361699879 | prostaglandin E synthase 3 (cytosolic)-like |
| 59 | PSG1 | 0.279738817 | 3.076952994 | pregnancy specific beta-1-glycoprotein 1 |
| 60 | C4orf22 | 0.280510889 | 0.516061492 | chromosome 4 open reading frame 22 |
| 61 | MT1JP | 0.280572907 | 0.641128172 | metallothionein 1J, pseudogene |
| 62 | CKLF-CMTM1 | 0.282259749 | 0.9087251 | CKLF-CMTM1 readthrough |
| 63 | IDH1-AS1 | 0.288881305 | 0.289558529 | IDH1 antisense RNA 1 |
| 64 | NCOR1P1 | 0.289551924 | 0.289551924 | nuclear receptor corepressor 1 pseudogene 1 |
| 65 | RAB3IL1 | 0.290038762 | 1.120807546 | RAB3A interacting protein (rabin3)-like 1 |
| 66 | IGLL3P | 0.29010229 | 0.640255754 | immunoglobulin lambda-like polypeptide 3, pseudogene |
| 67 | LOC100270746 | 0.294879562 | 0.612377396 | uncharacterized LOC100270746 |
| 68 | LRP3 | 0.29535187 | 0.956468679 | low density lipoprotein receptor-related protein 3 |

| | | | | |
|---|---|---|---|---|
| 69 | HIGD1C | 0.295529234 | 0.295529234 | HIG1 hypoxia inducible domain family, member 1C |
| 70 | DIP2A-IT1 | 0.295945896 | 1.000113643 | DIP2A intronic transcript 1 |
| 71 | PTGES3L-AARSD1 | 0.296339847 | 0.47612212 | PTGES3L-AARSD1 readthrough |
| 72 | ZG16B | 0.298211566 | 0.298211566 | zymogen granule protein 16B |
| 73 | HIST1H2BB | 0.299100664 | 1.35017629 | histone cluster 1, H2bb |
| 74 | LOC100505806 | 0.30304665 | 0.415919052 | |
| 75 | SPANXE | 0.30687487 | 0.306874152 | |
| 76 | HTRA3 | 0.311659042 | 0.311219353 | HtrA serine peptidase 3 |
| 77 | THY1 | 0.313556509 | 0.23963804 | Thy-1 cell surface antigen |
| 78 | TERC | 0.314465804 | 1.682530418 | telomerase RNA component |
| 79 | TP73-AS1 | 0.315250251 | 0.397491492 | TP73 antisense RNA 1 |
| 80 | SYNGR1 | 0.316266361 | 0.555500139 | synaptogyrin 1 |
| 81 | DAPK1 | 0.31656718 | 0.316370303 | death-associated protein kinase 1 |
| 82 | LINC00173 | 0.320532503 | 0.57799114 | long intergenic non-protein coding RNA 173 |
| 83 | DACT3-AS1 | 0.321700638 | 0.321700638 | DACT3 antisense RNA 1 |
| 84 | COL3A1 | 0.322228195 | 0.11756305 | collagen, type III, alpha 1 |
| 85 | MIR186 | 0.324137158 | 0.000175196 | microRNA 186 |
| 86 | SCN5A | 0.327542966 | 0.531201419 | sodium channel, voltage gated, type V alpha subunit |
| 87 | LINC00707 | 0.329058609 | 0.921487632 | long intergenic non-protein coding RNA 707 |
| 88 | SNORD23 | 0.329967959 | 1.476360074 | small nucleolar RNA, C/D box 23 |
| 89 | MMP2 | 0.332096761 | 0.082891432 | matrix metallopeptidase 2 |
| 90 | SNORA76 | 0.334330762 | 1.183125653 | |
| 91 | NPM2 | 0.335076625 | 0.335076625 | nucleophosmin/nucleoplasmin 2 |
| 92 | TAS2R14 | 0.335832374 | 1.248389684 | taste receptor, type 2, member 14 |
| 93 | ENG | 0.336152969 | 0.439583302 | endoglin |
| 94 | SNORA66 | 0.337839813 | 0.434350617 | small nucleolar RNA, H/ACA box 66 |
| 95 | ARHGAP30 | 0.338493546 | 0.463041349 | Rho GTPase activating protein 30 |
| 96 | SOST | 0.339875277 | 0.609284823 | sclerostin |
| 97 | HIST1H4C | 0.34467218 | 0.348488932 | histone cluster 1, H4c |
| 98 | COL1A2 | 0.346016848 | 0.066684619 | collagen, type I, alpha 2 |
| 99 | LINC00516 | 0.349443687 | 0.713864264 | |
| 100 | LOC101101776 | 0.349443687 | 0.531330429 | |

Table 4. Sequences of the primers used in this study.

| qPCR | Forward | Reverse |
|---|---|---|
| BMP4 | 5'-AAAGTCGCCGAGATTCAGGG-3' | 5'-GACGGCACTCTTGCTAGGC-3' |
| CD24 | 5'-CTCCTACCCACGCAGATTTATTC-3' | 5'-AGAGTGAGACCACGAAGAGAC-3' |
| E-cad | 5’-TTCCAGGAACCTCTGTGATG-3’ | 5’-TCTTGGCTGAGGATGGTGTA-3’ |
| ETS1 | 5'-TACACAGGCAGTGGACCAATC-3' | 5'-CCCCGCTGTCTTGTGGATG-3' |
| GAPDH | 5'-CTGGGCTACACTGAGCACC-3' | 5'-AAGTGGTCGTTGAGGGCAATG-3' |
| HEY1 | 5'-ATCTGCTAAGCTAGAAAAAGCCG-3' | 5'-GTGCGCGTCAAAGTAACCT-3' |
| ITGB4 | 5'-CTCCACCGAGTCAGCCTTC-3' | 5'-CGGGTAGTCCTGTGTCCTGTA-3' |
| JAG1 | 5'-GGGGCAACACCTTCAACCTC-3' | 5'-CCAGGCGAAACTGAAAGGC-3' |
| KRT5 | 5’-ACATGAACAAGGTGGAGCTG-3’ | 5’-AGCTCCGCATCAAAGAACAT-3’ |
| KRT14 | 5’-AACGAGATGCGTGACCAGTA-3’ | 5’-TCAGCTCCTCTGTCTTGGTG-3’ |
| NOTCH3 | 5'-CGTGGCTTCTTTCTACTGTGC-3' | 5'-CGTTCACCGGATTTGTGTCAC-3' |
| OCT4 | 5’-GAGAAGGATGTGGTCCGAGT-3’ | 5’-GTGCATAGTCGCTGCTTGAT-3’ |
| P-cad | 5'-TGGAGATCCTTGATGCCAATGA-3' | 5'-GCGTCCAGATCAGTGACCG-3' |
| PTH1R | 5'-AGTGCGAAAAACGGCTCAAG-3' | 5'-GATGCCTTATCTTTCCTGGGC-3' |
| PTHrP | 5-AAGGTGGAGACGTACAAAGAGC-3' | 5'-CAGAGCGAGTTCGCCGTTT-3' |
| TP63 | 5'-GGACCAGCAGATTCAGAACGG-3' | 5'-AGGACACGTCGAAACTGTGC-3' |
| VEGF-A | 5'-AGGGCAGAATCATCACGAAGT-3' | 5'-AGGGTCTCGATTGGATGGCA-3' |
| | | |
| CRISPR | Oligo 1 | Oligo 2 |
| sg3 | CACCGTTGTCCAGATCATCGGACA | AAACTGTCCGATGATCTGGACAAC |
| sg4 | CACCGAAATCCAATAGTGTAGTCGC | AAACGCGACTACACTATTGGATTTC |

# Chapter III

## Generate β4 Reporter Mouse Using CRISPR


### Abstract

To better study the role of integrin β4 in tumor formation and track the $\beta4^+$ population during tumor progression, I made a β4 reporter mouse using CRISPR. I inserted a p2A-mCherry cassette before the stop codon of the ITGB4 gene, and first tested the design strategy in vitro in NMuMG cells.


### Introduction

To better study the role of the $\beta4^+$ population in breast tumors, it will be helpful if we can track the $\beta4^+$ population during tumor progression. Previous models suggest that tumor cells need to undergo EMT to drive tumor invasion and break away from the primary tumor site. Cells will then undergo MET at the secondary site and form metastasis (Thiery et al., 2009). However, there is no clear evidence showing that EMT and MET are real and necessary during metastasis. Studies from the Ewald group show that the leader cells that drive tumor invasion are a basal epithelial population, not the cells that undergo EMT (Cheung et al., 2013; Shamir et al., 2014). Recently, two back-to-back studies showed that EMT is not required for metastasis in lung and pancreatic cancer (Fischer et al., 2015; Zheng et al., 2015). These studies show that there is a basal epithelial population that drive tumor invasion, and epithelial tumor cells can intravasate into the blood

system and form metastasis at the secondary site without undergoing EMT and MET. To show how β4 is involved in this process and localize the $\beta4^+$ population, I wanted to make a β4 reporter mouse and cross it with breast tumor model so that I can track the $\beta4^+$ population during tumor progression.

Homozygous β4 deletion mice die shortly after birth (Dowling et al., 1996; van der Neut et al., 1996; 1999b). Our lab showed that β4 is important for mammary gland development, and even heterozygotes have defects in the mammary gland (Li et al., 2015). Thus, this reporter mouse design strategy has to keep the ITGB4 gene intact after inserting mCherry. Moreover, to avoid blocking the ligand-binding site of β4, it is better to add the reporter gene at the C-terminus of β4. However, our lab showed that fusing GFP to the C-terminus of β4 drives β4 into nucleus. Thus, I added a p2A self-cleaved peptide between ITGB4 and mCherry. After translation, p2A will be cleaved automatically so that β4 and mCherry will be separated with β4 going to the cell surface while mCherry stays in the cytoplasm (Kim et al., 2011b). Thus, my design is to insert the p2A-mCherry cassette right before the TGA stop codon of ITGB4 without affecting the sequence of ITGB4.

## Results and Discussion

Initially I designed 4 sgRNAs near the TGA stop codon of the ITGB4 gene. To test the efficiency of the 4 sgRNAs, I cloned the 4 sgRNAs into PX458 vector and

transfected them into NMuMG cells. The transfection efficiency was about 10%. I sorted $GFP^+$ cells and did a SURVEYOR Assay (Fig 5B). I chose the sgRNA #2 that cut 3bp after the stop codon of ITGB4 since it would give me higher homology recombination (HR) efficiency. To make the plasmid donor for HR, I PCR amplified ITGB4 2kb left homology arm and ITGB4 3kb right homology arm from the genomic DNA of NMuMG cells, and cloned them flanking the p2A-mCherry cassette (Fig 5A).

I transfected PX330-sgRNA and plasmid donor together into NMuMG cells, and used PX330 vector alone with plasmid donor as negative control. I got 0.3% mCherry-positive cells without any selection 5 days post-transfection (Fig 5C). To confirm the correct insertion of the p2A-mCherry cassette, I designed a pair of primers with the Forward primer outside of the 2kb ITGB4 left homology arm and the Reverse primer inside of the mCherry sequence (Fig 5A). I did PCR with genomic DNA extracted from post-transfected NMuMG cells before mCherry sorting. I saw the correct size band of about 2.5kb in the sgRNA cells but not in the negative control cells (Fig 5D). I cut the 2.5kb band and did sequencing to make sure p2A-mCherry was inserted before TGA stop codon correctly (Fig 5D).

Western blot showed that the size and expression of integrin β4 is not affected by mCherry insertion in sorted mCherry-positive cells, and mCherry mRNA is also detected (Fig 5E). I also tested my design strategy and HR frequency in E14

cells which is a normal mouse embryonic stem cell line, and saw the similar results (data not shown). After I confirmed the CRISPR design successfully in vitro with cell lines, I made the mouse in BL6 background at Jackson Lab, collaborating with Dr. Steve Murray. There is one potentially correct mouse, and we'll do further examination to confirm it.

## Materials and Methods

**Generating sgRNAs Expressing Vector and plasmid donor.** Bicistronic expression vector (PX458 or PX330) expressing Cas9 and sgRNA (Cong et al., 2013) were digested with BbsI and treated with Antarctic Phosphatase, and the linearized vector was gel purified. A pair of oligos for each targeting site (Table 5) was annealed, phosphorylated, and ligated to linearized vector. For plamid donor, Nanog-2A-mCherry plasmid (Addgene) was cut at BamHI/NheI on the left and PacI/NotI on the right. ITGB4 2kb left homologous arm and ITGB4 3kb right homologous arm was PCR amplified from the genomic DNA of NMuMG cells and cloned into digested Nanog-2A-mCherry on each side, respectively.

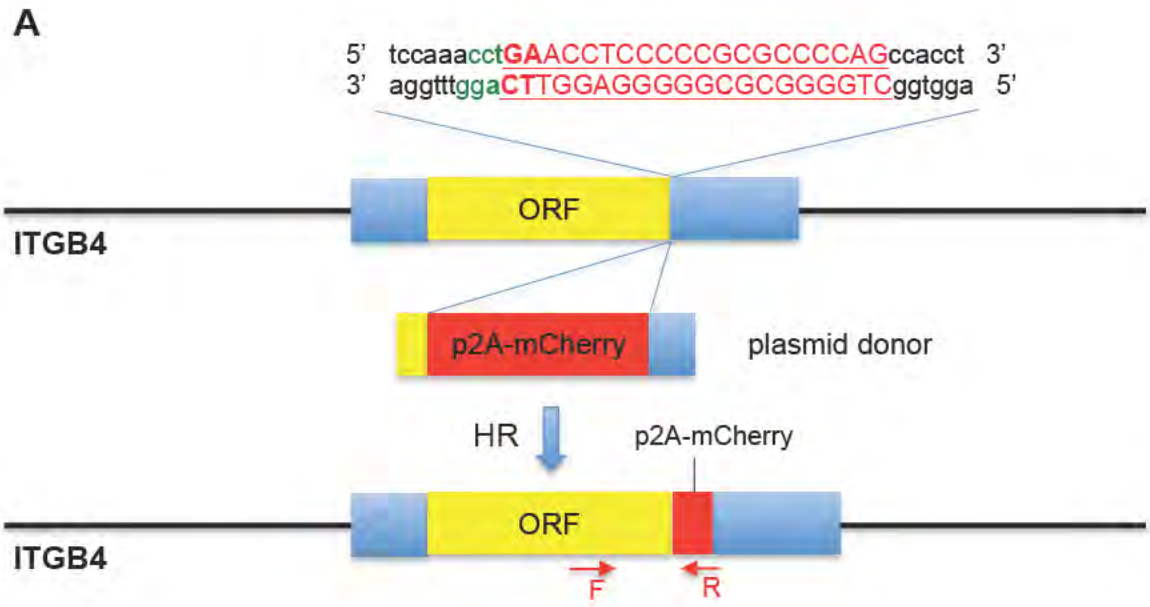
**Cell Culture and Transfection.** NMuMG cells were purchased from ATCC and maintained as described (Chang et al., 2013). PX458 vector (Addgene) cloned with indicated sgRNAs were transfected into NMuMG cells to examine to efficiency of sgRNAs. PX330 vector (Addgene) cloned with indicated sgRNAs

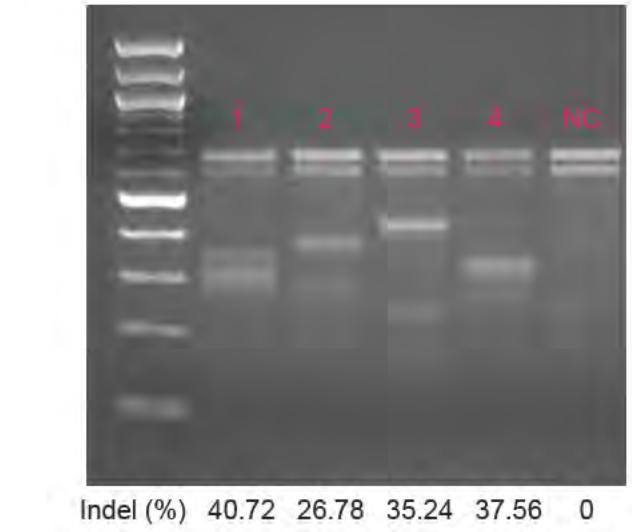
and plasmid donor were transfected in NMuMG cells to test the homology recombination in vitro.

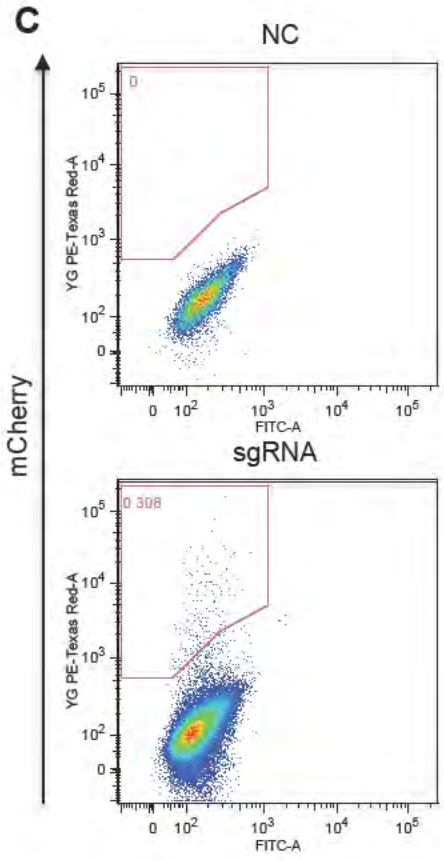
**Surveyor Assay.** Genomic DNA from NMuMG cells transfected with indicated sgRNAs in the PX458 vector were extracted and surveyor assay was performed as described by (Guschin et al., 2010). Sequences of the primers used for suveryor assay were provided in Table 5.

**Immunoblotting and qPCR.** Cells were extracted in RIPA (50mM Tris-HCL [pH 7.4], 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1mM PMSF, and protease inhibitors). The proteins were separated by SDS-PAGE and immunoblotted using antibodies for actin (Sigma-Aldrich) and β4 (505) (Rabinovitz et al., 1999). For qPCR, RNA isolation was done with the NucleopSpin RNA kit (Macherey-Nagel) and 1ug of total RNA was used to produce cDNAs using Roche First strand synthesis kit (Roche). qPCR was performed using a SYBR green (Applied Biosystems) master mix as described by the manufacturer. Sequences for qPCR primers used are provided in Table 5.

## Figures


A
5' tccaaacctGAACCTCCCCCGCGCCCCAGccacct 3'
3' aggtttggaCTTGGAGGGGGCGCGGGGTCggtgga 5'
ORF
ITGB4
p2A-mCherry
plasmid donor
HR
p2A-mCherry
ORF
ITGB4
F
R


B
1
2
3
4
NC
Indel (%) 40.72 26.78 35.24 37.56 0


C
NC
mCherry
YG PE-Texas Red-A
FITC-A
sgRNA
0.398

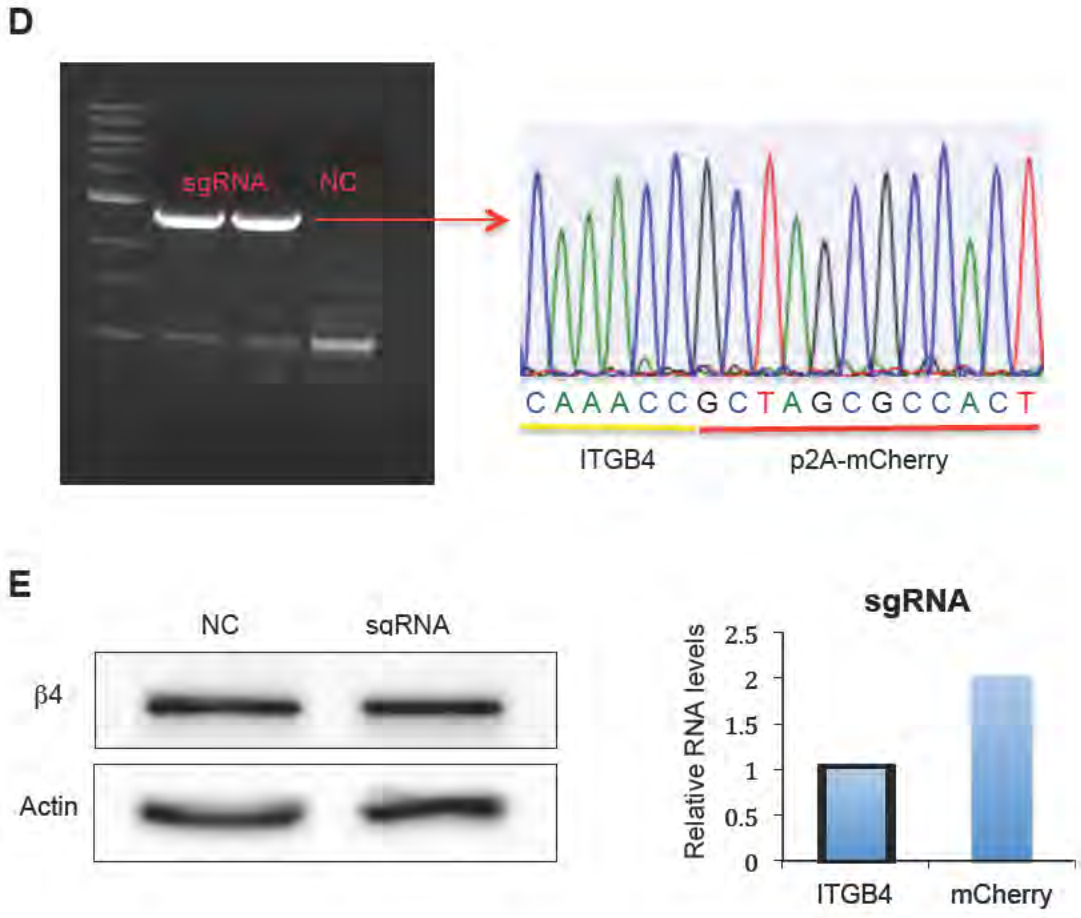



**Figure 5. Generate integrin β4 reporter mouse using CRISPR.**

(A) Schematic overview of strategy to generate a ITGB4-p2A-mCherry knockin allele. The sgRNA coding sequence is underlined, capitalized, and labeled in red. The protospacer-adjacent motif (PAM) sequence is labeled in green. The stop codon of ITGB4 is in bold. The 2kb ITGB4 left homologous arm and 3kb ITGB4 right homologous arm are indicated in yellow and blue, respectively flanking the p2A-mCherry cassette in red in plasmid donor. (B) Surveyor assay of 4 sgRNAs cloned in PX458 vectors transfected to NMuMG cells. Indel percentages are

shown at the bottom. (C) mCherry expression in NMuMG cells transfected with PX330-sgRNA2 and plasmid donor. (D) PCR checking the correct insertion of p2A-mCherry. Primers are shown in (A). Sequencing result of the 2.5kb band is shown on the right. (E) Western blot and qPCR results checking β4 and mCherry expression.

Table 5. Sequences of the primers used in this study.

| qPCR | Forward | Reverse |
|---|---|---|
| GAPDH | 5'-CTGGGCTACACTGAGCACC-3' | 5'-AAGTGGTCGTTGAGGGCAATG-3' |
| ITGB4 | 5'-CTCCACCGAGTCAGCCTTC-3' | 5'-CGGGTAGTCCTGTGTCCTGTA-3' |
| mCherry | 5'-AACGGCCACGAGTTCGAGAT-3' | 5'-GTAGGCCTTGGAGCCGTACA-3' |
| | | |
| | Forward | Reverse |
| Surveyor | 5'-ATCCTGACTGACGGATCTGCC-3' | 5'-AAGTGCAGAACAAAAGGCTGGG-3' |
| mCherry Seq | 5'-TAGGCCTCCCTCCTATCTGGGAAG-3' | 5'-TCCTCGAAGTTCATCACGCGCT-3' |
| | | |
| CRISPR | Oligo 1 | Oligo 2 |
| sgRNA1 | 5'-CACCGTGACCCAGGAATTCGTGACC-3' | 5'-AAACGGTCACGAATTCCTGGGTCAC-3' |
| sgRNA2 | 5'-CACCGCTGGGGCGCGGGGGAGGTTC-3' | 5'-CACCGTGACCCAGGAATTCGTGACC-3' |
| sgRNA3 | 5'-CACCGAGGAAGAAGGCGCTAGGAG-3' | 5'-AAACCTCCTAGCGCCTTCTTCCTC-3' |
| sgRNA4 | 5'-CACCGAGAGAGCCACTGGCCGTTA-3' | 5'-AAACTAACGGCCAGTGGCTCTCTC-3' |
| | | |
| Cloning | Sequence | |
| BamHI-Fw | 5'-CGCGG TCCCATGGCTGTCACGCTCCATTT-3' | |
| NheI-Rv | 5'-CTAGCTAGCGGTTTGGAAGAACTGTTGGTCCAT-3' | |
| PacI-Fw | 5'-TTAATTAATTAATGAACCTCCCCCGCGCCCCAG-3' | |
| NotI-Rv | 5'-AAGGAAAAAAGCGGCCGCAGTGGTCCTTGGAGCCTGGAATCCCACAG-3' | |

# Chapter IV

# General Discussion

## EMT and CSC

A $CD24^{-}CD44^{+}$ population has been identified as CSCs in breast cancer by showing that this population alone is sufficient to form new tumors in vivo (Al-Hajj et al., 2003). EMT generates cells with stem-cell properties that express mesenchymal markers and are $CD24^{-}CD44^{+}$ (Mani et al., 2008). In the past decade, this EMT model has received lots of interest in the field. At the primary tumor site, normal epithelial cells undergo EMT and become mesenchymal-like with CSC properties, which is important for tumor initiation. According to this model, cells that undergo EMT can intravasate into the blood system and extravasate to arrive at the secondary site. Upon arrival, these cells need to undergo MET to form metastasis (Thiery et al., 2009). However, no clear evidence shows that EMT and MET processes are real and necessary for tumor progression and metastasis.

There are two CSC models during tumor metastasis. Both models propose that cells need to undergo EMT for invasion and MET to form colonization at the secondary site. In the first model, CSCs are motile and circulate through blood system (Tsai et al., 2012). In the second model, EMT factors need to be repressed for cells to gain CSC properties. It proposes that CSCs are stationary and exist in both primary and secondary site all the time (Ocaña et al., 2012).

However, recently there are two back-to-back papers indicating that EMT is not required for metastasis in lung cancer and pancreatic cancer, respectively (Fischer et al., 2015; Zheng et al., 2015). Studies also show that it is a basal epithelial population that drives tumor invasion, which contradicts the idea that cells need to undergo EMT to break away from the primary site (Thiery et al., 2009).

**Intratumor heterogeneity and cell cooperation**

Cancer genome sequencing studies indicate that there are multiple subpopulations that have distinct molecular properties within a single breast cancer (Ding et al., 2010; Navin et al., 2011; Nik-Zainal et al., 2012; Shah et al., 2009). It is known that tumor cells need to interact with the microenvironment and communicate with the surrounding cells of various types, such as endothelial cells, mesenchymal stem cells, cancer-associated fibroblasts and immune cells (Ungefroren et al., 2011).

However, within the tumor cells, different subpopulations also need to interact with each other. In the view of clonal evolution model, subpopulations are considered as competitors to others that the certain subpopulation will gain growth advantage over others after certain mutations. At some times, tumor cells also need to cooperate. By injecting EMT cells and non-EMT cells separately or

together, Tsuji et al. show that both EMT and non-EMT breast tumor cells are needed for lung metastasis and they work together (Tsuji et al., 2009). Cleary et al. show that in Wnt-driven mammary cancers, both basal Hras mutant and luminal Hras wild-type subpopulations are required for tumor formation (Cleary et al., 2014). Yamamoto et al. also showed that CSCs and non-CSCs interact with each other through paracrine signaling (Yamamoto et al., 2013). In this study, they found that non-CSCs produce JAG1 while CSCs express Notch3 receptor so that non-CSCs can regulate CSCs through paracrine Notch signaling by providing JAG1 ligand.

**Integrin β4 in breast tumor metastasis**

In breast cancer, β4 has been shown to switch from linking cytokeratin to actin and redistribute to cell protrusions (Lipscomb and Mercurio, 2005; Rabinovitz and Mercurio, 1997; Rabinovitz et al., 1999). In addition LM332, the ligand of β4, is enriched at the invading front of several carcinomas (Kim et al., 2011a; Pyke et al., 1995; Xue et al., 2011). Given the important role that β4 plays for tumor invasion, I think it is very likely that $\beta4^+$ cells associated with a basal epithelial program in tumor are the cells that at the invading front to drive tumor invasion. These cells don't undergo EMT or MET, and they stay epithelial through tumor metastasis. To examine this hypothesis, I made a β4 reporter mouse so that I can cross it with breast tumor models and track $\beta4^+$ cells during tumor progression and metastasis.

Expression of integrin β4 has been found to correlate with highly metastatic cells {Dedhar:1993ww, Cimino:1997uv}. Periostin, an integrin β4 ligand, is an extracellular matrix protein induced in the lung stroma and provides a niche to support metastatic colonization (Baril et al., 2006; Malanchi et al., 2012). This could be the way that $\beta4^+$ tumor cells migrate to the metastatic site and form metastasis. Interestingly, a recent study linked integrin β4 to the exosomes in tumor metastasis (Hoshino et al., 2015). Exosomes are released from tumor cells into the microenvironment of the primary site and have been found to play roles in tumor invasion and metastasis (Lowry et al., 2015). This study shows that exosomes have distinct integrin expression patterns, in which the integrin α6β4 and α6β1 are associated with lung metastasis (Hoshino et al., 2015). The data suggests that exosomes expressing integrins will first travel to secondary site and secrete various proteins they bring to create a metastasis niche. Tumor cells will then migrate to that secondary site to form metastasis. These studies suggest a role for integrin β4 in creating the metastatic niche at the secondary site and supporting metastatic colonization.

**Integrin β4 and PTHrP**

The role of PTHrP as a mediator of osteolytic bone metastasis in breast cancer has been extensively studied in bone metastasis. PTHrP is a paracrine factor that is expressed by tumor cells and is secreted to the bone microenvironment,

which together with cytokines and growth factors play a negative impact on osteroblast function and lead to the destruction of bone. PTHrP produced by breast cancer cells enhaces osteoclastic activity via the expression of osteoblastic receptor activator of nuclear factor kappa B ligand (RANKL), vascular endothelial growth factor (VEGF) and matrix metalloproteinase-13 (MMP13) (Chen et al., 2010; Ibaragi et al., 2010; Isowa et al., 2010; Zhang et al., 2010). PTHrP is also important for mammary tumor formation and invasion (Lowry et al., 2015). This thesis study also discussed the possibility that β4 regulation of PTHrP is important for tumor formation by regulating CSCs in a non-cell-autonomous manner. We showed in a couple of different systems that PTHrP is expressed by $\beta4^{+}CD24^{+}CD44^{+}$ non-CSCs that have basal epithelial markers while its receptor PTH1R is associated with $\beta4^{-}CD24^{-}CD44^{+}$ CSCs. These two populations interact with each other through PTH1R paracrine signaling. This work also shows that integrin β4 can regulate PTHrP expression which can be directly affected by the ligation of integrin α6β4 when culturing cells on laminins.

## Conclusion Remarks and Perspectives

This study investigates the role of integrin β4 in breast tumor formation and the mechanism involved. It shows that β4 is heterogeneously expressed in breast tumors and it is important for tumor formation though it is not directly expressed by CSCs. I characterized $\beta4^{+}$ and $\beta4^{-}$ subpopulations in breast tumors and

investigated how they may cooperate with each other during tumor progression. My data shows that β4 is expressed by a basal epithelial $CD24^{+}CD44^{+}$ population which are likely to be the leader cells at the invading front to drive tumor invasion. $\beta4^{+}$ non-CSCs can regulate $\beta4^{-}$ CSC functions through paracrine signaling, such as Notch signaling and PTH1R signaling. Further experiments needed to be done to demonstrate the interactions between $\beta4^{+}$ and $\beta4^{-}$ populations and show that these interactions are essential for stem cell properties. $\beta4^{+}$ and $\beta4^{-}$ populations can be isolated and labeled before co-culture to examine the stem cell properties as well as signalings and downstream genes in $\beta4^{-}$ population. $\beta4^{+}$ and $\beta4^{-}$ populations can also be cultured in transwell plates to see if the physical interactions between the two populations are needed to maintain the CSC properties of $\beta4^{-}$ population.

I also investigated the possibility of PTH1R signaling involved in β4 regulation on CSCs. More experiments need to be done to confirm the regulation of integrin β4 on PTHrP and study the mechanism involved. It is also important to show that PTH1R signaling is critical for CSC functions. This can be studied by knocking down PTH1R in breast cancer cell lines and examine CSC properties either by mammosphere formation assay or tumor initiation assay in vivo. I also showed that β4 deletion in SUM-159 cells affected the CSC function of $CD24^{-}CD44^{+}$ population to give rise to $CD24^{+}CD44^{+}$ population. Further examination is needed to check the changes in $CD24^{-}CD44^{+}$ population after β4 deletion at the

molecular level. Finally, to better study the role of integrin β4 in tumor progression and metastasis, the β4 reporter mouse I'm making will be a very useful tool. The β4 reporter mouse can be crossed with breast tumor model so that the role of $\beta4^+$ population and the interactions between $\beta4^+$ and $\beta4^-$ populations can be better studied. Moreover, $\beta4^+$ population can also be tracked during tumor progression from the primary tumor site to metastatic site to study the role of β4 in tumor metastasis. One important question is that whether $\beta4^+$ population travel to the metastatic site or the $\beta4^-$ tumor cells from primary tumor site undergo MET and gain β4 expression at the metastatic site.

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