FRIZZLED7 (FZD7) is a NOTCH3 Specific Target in Human Mammary Epithelial Cells

by

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Thesis Summary

Notch signaling pathway is an evolutionarily conserved signaling pathway that has distinct roles in proliferation and differentiation. Notch signaling is activated by the interaction of NOTCH ligands with the extracellular portion of the NOTCH receptors (NR), which leads to the release of the intracellular portion of the receptors (NICD). Once nucleated, the NICD binds to CSL protein releasing its suppressive effect and recruits co-activators to initiate the transcription of Notch target genes. There are four different Notch receptors and five ligands in mammals. Notch signaling has been shown to play a role in maintenance of an undifferentiated state and regulation of cell-fate in many cell types. In 2008, Raouf *et al.* demonstrated that the expression levels of Notch receptors vary in different subsets of human mammary epithelial cells and that the luminal cell differentiation capability of bipotential progenitors requires the expression of NR3. These observations indicate that NR3 plays an active role in luminal cell differentiation.

Recent publications from other groups have shown that different Notch receptors form receptor-specific complex, and that the binding of each Notch receptor does not alter the binding affinity or specificity to CSL. This finding suggests that the unique role in regulating luminal cell differentiation in mammary epithelial cells is likely through recruitment of different co-activators when forming the activating complex upon binding to CSL.

In this project I used non-malignant human breast epithelial cell lines, 184-hTert and HMEC as well as discarded tissues from breast reduction surgeries as sources of primary human breast epithelial cells that contain different subsets of epithelial progenitors to

determine if Notch receptors could regulate the expression of specific target genes.

Conclusion

Frizzled 7 (FZD7) was identified to be only regulated at both protein and mRNA level by NOTCH3 and not any other Notch receptors in HMECs. Furthermore, FZD7 protein and mRNA expression pattern in subsets of human mammary epithelial cells were similar of NOTCH3, suggesting that FZD7 may cooperate with NOTCH3 in regulating luminal lineage-determination process of bipotent progenitors.

Rational

Notch receptors have been shown to share redundant biological functions. This based on the fact that all four receptors regulate the expression of target genes through binding to CSL complex. As well, individual loss of Notch receptors show no mammary gland phenotype in the knockout animal models suggesting that these receptors exhibit overlapping biological functions in the mammary gland.

However, in human mammary gland Notch receptors, in particular NOTCH3 and NOTCH4 receptors where shown to be expressed in different cell types and that loss of signaling through NOTCH3 receptor diminished commitment of undifferentiated bipotential progenitors to the luminal cell fate. These observations suggest that Notch 3 may regulate expression of a unique set of target genes whose expression is not influenced by signaling through other Notch receptors.

Hypothesis

Based on the above rational, I hypothesized that NOTCH3 regulates luminal epithelial cell differentiation through activation of a unique set of target genes.

Abstract and Overview

The current paradigm of Notch signaling indicates that upon ligand binding each of the four NOTCH receptors (NRs) indiscriminately form complexes with a DNA binding complex and regulate the transcription of target genes. Knockout mouse models of individual Notch receptors have no mammary phenotype, suggesting that in mouse mammary glands Notch receptors likely have redundant biological functions. Recent evidence however, indicates that signaling through NR3 alone is essential for maintaining the luminal cell differentiation potential of the bipotential human mammary epithelial progenitors. This observation suggests that NRs play non-redundant roles in regulating the growth and differentiation of mammary epithelial cells and that they may have unique target genes. The focus of my thesis is to identify specific, non-redundant target(s) of Notch receptor 3, a member of the Notch signaling pathway. This project led to identification of FRIZZED-7 (FZD7), a trans-membrane receptor in the Wnt signaling pathway, as a specific gene target of Notch receptor 3. I further demonstrate that NOTCH3 and FZD7 are highly expressed in the luminal progenitors compared to the bipotent progenitors, suggesting that the cross-talk between the WNT and Notch signaling may be involved in the luminal cell fate determination in the bipotent progenitor cells. Since Notch and the Wnt signaling are potent mammary oncogenes, understanding their precise roles and mechanisms of action in regulating the normal mammary gland development provides an insight into how their altered expressions can lead to breast cancer or contribute to pre-malignancy lesion. Furthermore, since FZD7 blocking antibodies are currently in clinical trials for colorectal and intestinal cancers,

there might be potential therapeutic values for such antibodies in treating recurrent luminal-type breast cancers.

For clarity, the introduction and background sections were divided into three parts focusing on the interplay of signaling pathways during the mammary gland development, with particular focus on the two signaling pathways involved in this project.

I. Signaling pathways and mammary gland development
II. Notch signaling pathway
III. Wnt signaling pathway

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List of Abbreviations

CFC	Colony forming cell
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FZD7	Frizzled-7
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HMEC	Human mammary epithelial cells
JNK	Jun N-terminal kinase
MFI	Mean fluorescence intensity
NICD	Notch receptor intercellular domain
NR	Notch receptor
RPM	Revolutions per minute
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
shRNA	Short hairpin RNA
v/v	Volume/volume
WB	Western blot
qPCR	quantitative Polymerase Chain Reaction

I. Introduction and Background

I. Signaling pathways and mammary gland development

I.1 Mammary gland development and maturation

Much of what is known about the roles of signaling molecules in the mammary gland development is based on general interest in genes involved in breast cancer development and animal models. However, the knowledge on the roles of signaling molecules and pathways in normal human mammary gland development is minimal. Mammary gland is a dynamic tissue in that it goes through series of expansion and regression (involution) due to multiple pregnancy cycles throughout life. This regenerative property of the mammary gland indicates that there are populations of progenitor and stem cells that have the ability to go through multiple cycles of self-renewal, proliferation and differentiation, even in post-puberty adulthood. Also, recent evidence suggests that, abnormalities in the signaling pathways controlling these processes could lead to early disease progression (Raouf, *et al*., 2012). Therefore, the detailed study of these signaling molecules in the normal human breast could be important to understanding how altered functioning of these molecules could create and maintain breast tumors.

Our understanding of the human mammary gland development and maturation is based on a detailed study done by Howard and Gusterson in 2000. In this study they found that the human mammary gland developmental stages could be divided into prenatal, infant, pubertal, adult and postmenopausal. During prenatal to infant stages small rudimentary breast ducts are developed that shared between male and females. During puberty, ductal elongation and formation of lobular structures (small sacks) occur under the influence of hormones in females, while no further development occur at this stage for males.

Dichotomous branching with lateral bud formation occurs from previously blunt-ended ductal termini, and the terminal duct embedded in the intralobular stroma is considered as the functional unit of the breast. Same as mouse models, branching-morphogenesis is essential in the proper formation of the functional unit of the mammary gland.

In adulthood, nulliparous breast shows similar mature unit to the end of pubertal stage. The study of normal human mammary gland structure remains very difficult, as the limited samples are available from the different stages of mammary gland maturation. Few samples are available from surgeries removing solid tumors from the different stages of pregnancy and lactation. However, it is unclear whether these tissue samples that appear normal, have any pre-cancerous abnormalities. From the few samples that have been examined, it was observed that during lactation, the breast tissue showed similar structures to those in mouse with an increase in the numbers of lobules (Howard and Gusterson 2000). The lactating units of the gland are composed of dilated acini containing milk, which is produced by the inner luminal cells (now matured into functional milk-producing cells). And the outer myoepithelial or basal layer of cells facilitate in the contraction, excreting milk secreted. Following weaning, the breast tissue goes through involution, where the number of acini decreases, and the double layered epithelial of the breast is reformed. Ductal portions of the breast remain unchanged, as further branching morphogenesis is not required for subsequent pregnancies. Since mammals are capable of going through multiple pregnancy cycles, it is essential for the breast to have the ability to develop functional lactating units when needed which requires a large increase in the number of luminal and the myoepithelial cells. Recent data suggest that these expansion and involution cycles of the mammary gland could due

the special functions of stem cells with extensive self-renewal capacity. These stem cells are able to lay dormant during other stages while maintaining the self-renewal and differentiation properties. Upon differentiation breast stem cells produce bipotent progenitors that are able to create the mature luminal and myoepithelial cells through generation of lineage-restricted progenitors (Raouf *et al.,* 2012). These properties also increase the chances of abnormal growth and possible malignancies, because errors are made with each cycle of cell division and differentiation. Changes to the carefully regulated proliferation and differentiation programing of the breast stem and progenitor cells could bestow a cancer stem cell phenotype on these cells. Cancer stem cells are thought to share many properties of the normal stem cells and are responsible for maintaining and regeneration breast tumors.

I.2 Signaling pathways known to regulate mammary gland development

I.2A Hormones

Hormones and other endocrine factors play important roles in mammary gland and other developmental processies during puberty and adulthood. In mouse, the loss of estrogen signaling resulted in the loss of ductal elongation in mouse. In human, there was no experimental evidence eradicating hormone signaling, however, comparing male and female development during puberty, it was evident that estrogen as progesterone surges was important in developing secondary, functional mammary glands (Raouf, *et al*., 2012). Molecular signaling pathways such as Wnt signaling also coordinate with both estrogen and progesterone signals in development of mammary gland.

I.2B Molecular signals

Many evolutionarily conserved signaling pathways have important roles in mammary gland development. Signaling pathways such as the Wnt, Notch, and the Hedgehog pathways have been extensively studied in the context of mouse mammary gland development. The Wnt pathway mostly coordinates with estrogen signaling in ductal elongation. Interestingly Wnt signaling also coordinates with progesterone as part of branching morphogenesis during early development of the mammary gland. On the other hand, the Hedgehog signaling network was essential mainly in early embryonic mammary gland development (Lee, *et al*., 2013).

As one of the more extensively studied signaling pathways controlling mammary gland development in human, Notch signaling receptor 3 (NOTCH3) was shown to be essential in luminal cell differentiation. Interestingly, while Notch signaling regulates luminal cell fate, there seem to be extensive overlap in the functioning of the 4 different Notch receptors in this context. Since luminal cells originate from bipotent progenitors through a NOTCH3-dependent fate determination step (Raouf *et al.,* 2008), understanding this biological process and the molecular mechanisms involved could potentially provide valuable insights into developing new molecular therapeutical targets, and/or add powerful new biomarkers.

II. Notch Signaling Pathway

The Notch signaling pathway is an evolutionarily conserved developmental pathway, which plays critical roles throughout early development and tissue maintenance in adulthood (Han *et al*., 2011). Figure 1 summarizes the Notch signaling pathway proteins

and their respective cellular compartments. Notch receptors are single-pass transmembrane proteins that can act as surface receptors and transcriptional regulators with their domains. There are four Notch receptors in mammals, NOTCH1 - 4 (NR1 to NR4). Each Notch receptor has an extracellular domain, which upon binding with ligands expressing on the neighboring cell, goes through two successive protein cleavages by metalloprotease ADAM17 at the extracellular domain S3, and then the second cleavage at the transmembrane domain S3 by γ-secretase. There are five ligands in Notch signaling pathway, JAGGED 1, JAGGED 2, Delta-like 1 to Delta-like 3 (DLL1 to DLL3). Direct contact between two neighboring cells are required for signal activation, and although soluble ligands in certain cases had been shown to be able to activate the Notch signaling pathway, the results were inconclusive, and may not apply to all types of cells.

Upon two proteolytic cleavages, the active part of Notch receptors, the Notch intracellular domain (NICD's) is released to the cytoplasm, which then enters the nucleus to activate transcription of target genes. NICD's do not directly bind to DNA, instead, they bind to a transcriptional repressor Core binding factor-1/suppressor of Hairless/Lag-1 (CSL), displace the inactive protein complex, and then recruit co-activators such as mastermind-like (MAML) and P300 to the complex to active the transcription of target genes. The target gens can be divided in two families of transcriptional factors, Hairy and enhance of split (HES), and Hes-related repressor proteins (HEY). The sequence to which CSL bind to is evolutionarily conserved, TGGGAA (Bianco *et al*., 2010). The number of CSL-binding sequence in a gene's promoter region does not always associate with the strength of activation by NICD, and that not all sites are available or active for CSL-binding in a given cell. Notch signaling has many important roles in adult life, such as

maintaining the self-renewal and repopulation capacity of hematopoietic stem cells (Butler J.M. *et al.*, 2010), and thus non-discriminatively targeting the entire pathway could induce significant side effects in the body.

Since all Notch receptors bind to CSL and not directly to DNA sequences, it is generally believed that all Notch receptors have the same target genes, and that their functions are overlapping. Indeed, loss of Notch signaling attained through CSL-null mice show severe decrease in luminal cell production in the mouse mammary gland while the individual knockdown of the NRs show no phenotype, suggesting NRs have mostly redundant functions in mice. In human mammary gland development however, recent evidence suggest that NR3 plays a non-redundant roll in regulating luminal cell fate. Given the overlapping functions of the Notch receptors; much of the research efforts have been focused on NOTCH1, potentially due to its abundance in many cell types.

In recent years, accumulating evidence suggests that different Notch receptors have unique targets. In 2011, Han and colleagues had discovered that the size of activating complex formed by each Notch receptors was very different in breast cancer cell line MCF-7 in that NOTCH3 and NOTCH4 each showed very distinct protein fraction profiles compared to NOTCH1 and NOTCH2. Interestingly, in 2010 Bianco and colleagues had shown that binding of Notch receptors to co-activator MAML-1 does not change the DNA-binding specificity of the complex. Thus, the proposed difference in target genes activation of the different Notch receptors is not accomplished by changing the binding affinity of the activating complexes. These results combined suggested that

the differential activation of target genes were very likely through recruiting different sets of co-activators, and not by binding to different DNA sequences. This proposed regulatory system maximizes flexibility in that any Notch receptor can bind to a certain gene, and perhaps selectivity of target gene activation is governed through binding of various Notch receptor- co-activator complexes through developmental stages and during the adult life.

II.1, Role of Notch signaling in the normal mammary gland

In the more accessible mouse model, detailed studies could be done on the differential expression of Notch pathway genes in the mammary gland. In mouse, it was clear that Notch3 was required for the formation of functional lactation system, with its level peaking at day 5 post pregnancy, and gradually decreasing to its lowest at involution (Raafat *et al.*, 2010). In this study, it was found that the majority of the Notch3 protein was localized in the luminal, milk-producing region of the gland, while the Notch4 protein was most localized in the myoepithelial region, or basal portion of the gland. However compared to other Notch receptors, the mRNA levels of Notch4 was very low through the growth-involution cycle of the gland. Without comparable human studies, it was clear that Notch3 increase was needed for proper gland formation and lactation, but whether other Notch receptors could compensate for its function in this setting remains unknown.

Furthermore, it was demonstrated by Raouf and colleagues in 2008 that loss of NOTCH3 signaling specifically was sufficient to block luminal progenitor cells formation from the

bipotent progenitors at the fate-determination stage using the breast reduction samples and an *in vitro* colony cell forming (CFC) assay. This study also examined the mRNA expression of all Notch receptors in the hierarchy of human mammary epithelial cells. Interestingly, they showed that, NOTCH3 was highly expressed in luminal progenitors compared to bipotent progenitors, suggesting that the increase in its expression level was associated with luminal progenitors formation. On the contrary, the expression level of NOTCH4 was the highest in the bipotent progenitors compared to other populations in the hierarchy. NOTCH1 and NOTCH2 showed modest differences in expression and where highly expressed throughout the hierarchy. Based on these data it was concluded that at least NOTCH3 had a unique role in determining the luminal cell fate of the bipotent progenitors in human mammary epithelial cells and that its function could not be compensated by any other Notch receptors. Since Notch receptors carry out cellular functions by activating target genes, it was certain that there must be a specific set of target genes that were only activated by NOTCH3 and not any other Notch receptors in the process of luminal cell-fate determination. This study also showed that the CFC assay system, one could determine whether a particular gene has any effect on the fate-determination process by either knocking down or over expressing the said gene. Interestingly, Notch signaling pathway also contributes to hormone responsiveness in luminal breast cancers. It was recently reported (Haughian *et al*., 2011) that estrogen signaling increased NOTCH1 expression while no other Notch receptors were considered and based on these data they suggest that there is a potential for targeting Notch signaling in breast cancers that are resistant to endocrine therapies.

In another study by Dontu, G. *et al* in 2004 focused on activation of Notch receptors and examined the effect on the sphere-forming ability of the normal human mammary epithelial cells obtained from breast reduction samples. The ability to form spheres and grow as a non-adherent culture has been considered a sign for pre-cancerous lesion in many systems, mainly in the nervous system. However in the mammary gland, the content of these mammospheres formed was not well defined, and the method for generating these mammospheres had not been standardized across the field. In this study by Dontu and colleagues, the source of cells used for seeding the mammosphere culture was from the supernatant of an adherent culture, meaning that these cells were not able to grow as an adherent culture to start with. Although this method greatly increases the percentage of mammospheres formation, it was not completely conclusive to state that any manipulation done on these cells were done to "normally grown" human breast epithelial progenitors. The most notable conclusion from the study was that by blocking the NOTCH4 via extracellular neutralizing antibody, branching morphogenesis of the mammospheres in 3D Matrigel culture was diminished, while activating the Notch signaling pathway using extracellular ligands enhanced the branching morphogenesis process compared to control. Again the study did not examine whether other Notch receptors had the same effects as NOTCH4. More importantly, this study used unseparated bulk cells, which make the study of how Notch signaling affects each progenitor type very difficult to interpret. In a similar study done by Harrison and colleagues in 2010, the tumorigenic ability of breast cancer cell lines was examined *in vivo* upon Notch receptor inactivation. Using human breast cancer cell line MCF-7, it was shown that upon NOTCH4 inactivation but not NOTCH1 inactivation, leads to a drastic

decrease in tumor growth and tumor volume in immunodeficient mice. With NOTCH4 inhibition via shRNA knockdown, there was no tumor growth in the mouse model used whereas NOTCH1 knockdown caused only a slight decrease in tumor formation. The study also correlated the tumorigenicity of the cell lines to their ability to form mammospheres. Together these studies suggested that NOTCH4 plays a role in late stage tumor formation, and potentially has a role in pre-lesion transition phase of normal mammary epithelial progenitors as well.

In addition to NOTCH1 and NOTCH4, NOTCH3 has also been show to act as a potential regulator of tumorigenesis in the breast. In a study using T-cell acute lymphoblastic leukemia (T-ALL) cell lines, it was shown that while Notch1 intracellular domain was expressed in both growing tumors and dormant tumor cells at a constant level, Notch3 intracellular domain was only expressed in growing tumors but not in dormant tumors (Indraccolo *et al*., 2009). Interestingly, in the same study the authors found that the Notch ligand DLL4 was highly expressed in growing tumors but not in dormant tumors (Indraccolo *et al*., 2009). This expression profile not only suggests that Notch1 and Notch3 have different roles in tumor maintenance, but also suggests that the potential interaction between Dll4 and Notch3 due to the fact that they had similar expression patterns in dormant vs. growing tumors. By blocking Dll4 ligand with an extracellular antibody, it was found that the cleaved, active form of Notch3, Notch intracellular domain greatly decreased in the T-ALL cells, which suggests a ligand-receptor specific regulatory relationship in the system. In this particular system, activation of Notch3 signaling greatly increased angiogenesis when colorectal cancer cell lines where used in

immune-deficient mice to generate solid tumors, and blocking of the Dll4-Notch3 interaction resulted in reduced tumorigenicity and angiogenesis *in vivo* (Indraccolo *et al*., 2000). These findings suggest that Dll4 and/or Notch3 have the potential of becoming therapeutic targets.

Notch signaling pathway has also been shown to cooperate with other signaling pathways to carry out biological functions, such as the Wnt signaling pathway.

III. Wnt Signaling Pathway

Similar to the Notch signaling pathway, Wnt signaling is an evolutionarily conserved and an important signaling pathway that participate in maintaining adult stem cells in various tissues, as well as orchestrating early mammary gland development (Roarty and Rosen, 2010). Figure 2 summarizes the proteins involved in this pathway and their respective cellular compartments. The Wnt family of genes encodes for 19 secreted glycoproteins, WNT1, WNT2, etc, that initiate signal transduction by binding to Frizzled receptors on target cells. Depending on which WNT protein binds to the receptors, either canonical or non-canonical signaling cascade are activated. In the case of canonical pathway, binding of Wnt proteins to Frizzled (Fzd) and co-receptor Low Density Lipoprotein-receptor related protein family (Lrp5/6) activates Disheveled (DSH), which subsequently leads to the inactivation of glycogen synthase kinase-3β (GSK-3β). Active form of GSK-3 β phosphorylates β-catenin, slating it for protein degradation. However inactivation of GSK-3 β leads to the stabilization of β-catenin. The stabilized β-catenin enters the

nucleus and interacts with Lymphoid Enhancer Factor/T-Cell-Specific Transcription Factor (LEF/TCF), which activate downstream target genes. The canonical pathway therefore is also known as the β-catenin-dependent Wnt signaling pathway.

In the non-canonical, β-catenin independent pathway, secreted Wnt glycoprotein binds to Fzd receptors and glypican-4, which leads to activation of Rho and c-Jun N-terminal kinase (JNK) or the stimulation of calcium influx (Roarty and Rosen, 2010). The non-canonical Wnt pathway is often associated with planar cell polarity and calcium signaling. This pathway had also been shown to negatively regulate the canonical Wnt pathway. Furthermore, there are Fzd-independent Wnt membrane receptors of the tyrosine kinase-like orphan receptor (Ror) and related to receptor tyrosine kinase (Ryk) families, which can also bind to Wnt glycoproteins and initiate downstream signaling in a β-catenin independent manner (Roarty and Rosen, 2010). Due to the number of secreted proteins and receptors involved in the pathway, the exact interaction and specificity between Wnt glycoprotein and Fzd receptors remains unclear.

The planar cell polarity (PCP) pathway is poorly understood due to its complexity. It regulates the orientation and migration of polarized cells during early development (Fedon., *et al.*, 2012). Deficiencies and aberrations in this signaling pathway often lead to severe abnormalities. The PCP pathway mainly signals through the JNK pathway. Disheveled is activated upon interaction between Wnt ligand and its Frizzled receptor. JNK and Rho family GTPases are also activated. The activation of this pathway plays a part in directing asymmetric cytoskeletal organization and coordinated polarization of

cells in the same plane of epithelial sheets during embryonic development stages (Fedon., *et al.*, 2012).

III.1, Role of Wnt signaling in mammary gland development

A limited mouse study in 1992 by Gavin and McMahon showed that Wnt glycoproteins were differentially expressed during pregnancy and lactation, suggesting that Wnt genes were involved in mammary gland maintenance and development, much like the Notch signaling pathway. Out of the glycoproteins studied, Wnt4 was expressed from virgin to prior to lactation, Wnt5a was barely detectable, and Wnt7b was only expressed at the virgin stage. The differential expression of ligands strongly suggested the Wnt protein has different roles in mammary gland. Later on in 2000, Brisken and colleague in a mouse study showed that Wnt-4 was essential in side-branching in early pregnancy, in that it was needed to facilitate progesterone functions. It was unclear whether the signal was β-catenin dependent or independent.

There are other studies suggesting that within the developing mammary gland, estrogen receptor (ER) positive sensor cells secrete a specific set of soluble Wnt ligands, in response to reproductive hormones to activate and facilitate PR signaling (Roarty and Rosen, 2010). This could provide an explanation as to where the PR positive cells in the developing mammary gland come from.

In another study done in 1998, Uyttendaele and colleagues used a mouse mammary epithelial cell line to examine the role of Wnt signaling pathway. It was found that Wnt-1 could overcome the Notch4 mediated inhibition of branching morphogenesis when both

proteins were co-expressed. The data loosely suggested that Notch signaling pathway and Wnt signaling pathway may play opposite roles in regulating branching morphogenesis, however the data did not examine Wnt target genes to see whether the pathway was indeed active, or the phenomenon was not related to activation of either pathway, but a mere coincidence of other unrelated events. In order to provide more reliable, biologically relevant data for signaling pathways, one must consider whether the pathway under study is activated by examining the expression of target genes, and vice versa for inhibition of such pathways. Then and only then a conclusion regarding whether a biological event is due to activation or inhibition of a pathway can be drawn.

III.2, Wnt-FZD7 signaling axis

In skeletal muscle, mouse studies showed that Wnt7a-Fzd7 interaction activates distinct non-canonical pathways that directly stimulate hypertrophic growth of myofibres in muscle regeneration (Maltzahn *et al.*, 2011). In the same study, it was found Wnt7a-Fzd7 interaction activates the planar-cell-polarity pathway and drive the symmetric expansion of satellite stem cells in skeletal muscle repair. While the study did not show whether the interaction was specific to Fzd7 or from Wnt7a, it did however show the importance of Fzd7 in adult stem cell maintenance and regulation. In a more recent study, FZD7 was shown to be essential in maintaining the pluripotent state in human embryonic stem cells (Fernandez *et al*, 2014). It was shown that the signaling was through Wnt3a, hence it was through the canonical pathway. In the study, by either using blocking antibody or shRNA-mediated knockdown of FZD7, the human embryonic stem cells were no longer able to sustain the pluripotent state.

III.3, Study of Wnt signaling in the human mammary gland

Large majority of the published studies on the Wnt signaling pathway are done using animal models. While the mouse and human are very similar in many developmental aspects, there are key differences. With the ability to isolate populations of human mammary epithelial cells and the recent advances in mass sequencing (Eirew P., *et al*, 2008), studying Wnt signaling pathway in human mammary gland progenitor cells at both molecular and physiological levels became attainable without much technical difficulties.

II. Materials and Methods

Cell Culture

Human mammary epithelial cells (HMEC)

The cell strain was purchased from Lonza (Catalog Number CC-2551) and cultured in HMEC media (Mammary Epithelial Basal Medium supplemented with SingleQuots growth factor kit, CC-3151 and CC-3150, both from Lonza) at 4000 to 5000 cells per cm^2. These cells were passaged every 3-4 days using the standard trypsin method as described below.

184 h-TERT cultured in DMEM/F-12 medium (Sigma), supplemented with 10ng/mL EGF, 2.6ng/mL sodium selenite, 250ng/mL insulin, 1600ng/mL transferrin, G418 400μg/mL, 0.15U/mL prolactin, 10nM isoproterenol, and 500ng/mL hydrocortisone. These cells were passaged every 2-3 days or as needed following the standard trypsin method as described below.

MCF10A was cultured in 10% v/v fetal bovine serum (FBS, Gibco, Catalog no. 12483) supplemented Dulbecco's Modified Eagle Medium (DMEM, Gibco, Catalog no. 12430). These cells were passaged every 3-4 days or as needed following the standard trypsin method as described below.

Primary human mammary epithelial cells were cultured in EpiCult-B media (Stem cell technologies) supplemented with 5% v/v FBS along with irradiated 3T3 mouse

fibroblasts at 8 x 10^3 per cm^2. FBS was removed 1-2 days following seeding of these cells.

Cell passaging: The adherent culture was first gently washed with warm PBS solution, and then warm trypsin was applied. The cell culture with added trypsin was incubated for 5minutes in a 37°C air incubator with 5% CO_2. Hank's balanced salt solution with 2% v/v FBS was used at equal volume at room temperature was used to stop the trypsin reaction. The tissue culture container was then washed with 2% Hank's balanced salt solution to remove all cells. The cell suspension was transferred to a tube suitable for centrifugation. The cell suspension was centrifuged for 5minutes, and then the pellet was re-suspended in 2% Hank's balanced salt solution for counting. Tryphan blue was used to distinguish dead and live cells while counting. Cell suspension was then cultured into desired density with appropriate media.

Preparation and Isolation of Mammary Cell Subsets

Discarded non-cancerous reduction mammoplasty tissue was obtained through Manitoba Tumor Bank and Maples Surgery Centre with appropriate patient consent. Discarded tissue from breast reduction mammoplasty was transported from the operating room in sterile specimen cups in transport media by designated staff at either the surgical facility or Pathology cutting room. Upon transporting to the laboratory, the tissue samples were cut into approximately 0.5cm x 0.5cm x 0.5cm pieces to allow better digestion. Large chunks of fat tissue can be removed and discarded prior to processing. The tissue pieces were then shaken in dissociation flasks in dissociation media for 16-18 hours at 37°C at 105-110rpm. The shaking incubation time can be extended for another hour if the tissue

sample was not dissociated after 16-18 hours. The dissociate tissue was transfered from the dissociation flasks and walls of the flasks were washed with warm basic medium to wash and remove all tissue. The tissue suspensions were centrifuged at 75-80xg for 40 seconds to pellet the heavy organoid-enriched portion. The supernatant was transferred into another sterile tube for further processing. The organoid-enriched pellet was washed with warm basic medium and then re-pelleted at the 75-80x g for 40 sec.

The organoid-enriched pellet is further dissociated with 5% dispase for 5minutes at 37°C in water bath followed by 2% Hank's balanced salt solution (2% fetal calf serum) to stop the reaction. The cells were then pelleted via centrifugation (200 xg), then re-suspended in trypsin, followed by a 5minutes incubation in 37°C water bath. Repeated gentle pipetting using 10mL, 5mL then 2mL pipettes was applied to completely re-suspend the cell pellet at each step to ensure maximum re-suspension, and maximize enzyme action. 2% Hank's balanced salt solution was used to stop the trypsin action. Pellet the cells at 200xg for 5minutes. Re-suspend the cell pellet in warm dispase mixed with DNase, incubate for 5minutes at 37°C. 1 mL micropipette was used to further break down any remaining organoid pieces if needed. Subsequently cold 2% Hank's balanced salt solution was added to the dispase/DNase reaction at which point the cells were passed through a 40 μm cell strainer. The cell strainer was washed with cold 2% Hank's balanced salt solution to remove any leftover cells on the cell strainer. The cell suspension was then re-pelleted at 200xg for 5minutes. Cell pellet was re-suspended in 2% Hank's balanced salt solution, observe under the microscope, and cells were counted. These cells in single-cell suspension were then ready to be either cultured or labeled with antibodies for FACS procedures.

Molecular Cloning

Ligation of restriction-digested, purified DNA fragments was ligated into digested, purified KA391 vector in 1:6 vector: insert molecular ratio. The DNA fragments concentrations were determined using optical density method (Nanodrop instrument). Ligated plasmids were transformed into DH5α competent bacteria cells by mixing ligation reaction with cells followed by 30min incubation on ice. Subsequently cells were heat shocked for 50 seconds at 42°C and then incubated on ice for 2min. Then after, 300µL of S.O.C medium was added to the mixture, shaken horizontally at 225rpm for 1.5hr at 37°C. The cell mixture was then plated on warm L.B plates supplemented with ampicillin.

S.O.C medium composition: 2% tryptone, 0.5% yeast extract, 1% 1M NACl, 0.25% 1M KCl, 1% 2M sterile Mg^{2+}, 1% 2M sterile glucose.

2M Mg^{2+} solution: 20.33g $MgCl_2$ 5H2O, 24.65g MgSO4 7H2O for 100mL, filter sterilize with 0.2µL filter.

PCR

Intracellular domains of NOTCH3 and NOTCH2 were PCR cloned from non-malignant mammary epithelial cell line 184 h-Tert cells, using high fidelity polymerase, Phusion (Fermentas). The colonies were screened for positive clones with iTaq polymerase via end-point PCR. The primer sequences used for cloning were listed in Table 2.

Quantitative Real Time PCR

RNA was extracted using Agilent Microprep (for less than 0.5 x 10^6 cells) and Nanoprep (for less than 10 x 10^3 cells). Samples with cell numbers larger than 0.5 x 10^6 were extracted with Trizol. All extraction procedures were following manufacture's protocols.

In samples extracted using Trizol, an additional step of DNase, using RQ1 RNase-free DNase from Promega (M6101), following manufacture's protocol.

Primer sequences used for quantitative real time PCR were listed in Table 1.

Western Blot

Protein extraction was done by re-suspending the cells or scraping the cell culture plate with the lysis buffer (1% Triton X100, 0.5% NP40, 4% SDS, in PBS), and then followed by sonication at 70% power for 10 seconds to reduce viscosity.

Protein concentration was measured with BCA kit from Fisher Thermo Scientific at 25µL/ sample in 96-well plate following manufacture's protocol.

Protein samples were separated on 7.5-10% polyacrylamide gels, and transferred to PVDF membranes (0.2µm, Bio-Rad). The membranes were blocked for 1 hour with 5% v/v milk in TBST buffer, before being probed overnight at 4˚C with primary antibody in 5% milk – TBST solution. Antibodies used for Western Blot were listed in Table 4.

Intracellular FACS

Cells were fixed and stained using BD Cytofix/Cytoperm kits, following manufactures protocols, and then analyzed on Millipore Guava easyCyte flow cytometer. Isotype-specific antibodies were used as background controls. Antibodies used for intracellular FACS were listed in Table 4.

Lenti-Virus production

293T cells were plated at $5.5x10^6$ cells per 10cm plate in 7mL of 10% FBS supplemented DMEM. On the following day, media was changed 3 hours prior to transfection. Short hair-pin RNA (shRNA) vectors specific to the genes of interest were mixed with other packaging and envelope vectors in the following ratio: shRNA vector 70µg, deltaR 45.5µg, REV 17.5µg, VSVG 24.5µg (envelope) for every 7 10cm plates. Make the volume up to to 3150µL with H2O, add 350µL 2.5M CaCl2, then add the CaCl2-DNA mixture into equal volume 2xHBS buffer. Add 1mL of the mixture to each 10cm plate for the transfection process. The media was changed on the next day to 5% v/v FBS in DMEM. On the 4^{th} day, harvest the media to collect virus. 293T cell debris can be removed by centrifugation at 1500rpm for 5 minutes. The clarified media were used for infection of cells of interest as adherent culture. The shRNA vectors corresponding specifically to each gene were purchased from Open Biosystems.

III. Results

Result 1: HMEC (human mammary epithelial cells) were identified as the most suitable cell strain to study primary human epithelial cells

To study human mammary epithelial cells *in vitro*, a cell line or strain is needed since the number of cells available from each breast reduction sample is too few to run all the required screening experiments, and using different patient samples may result in data sets with large variations due to the nature of human sample variation, thus making identifying potential targets unnecessarily difficult. The main difference between a cell line and a cell strain is that cell lines are often immortalized, and consisted of a single type of cells, and cell strains often contain multiple types of cells, and have a finite number of passages. While both cell lines and cell strains were derived from isolated primary cells, cell lines often underwent either spontaneous or artificial alterations to give them immortality.

Comparing all three available non-malignant breast epithelial cell lines, 184 h-Tert, MCF10A and human mammary epithelial cells (HMEC), HMEC contain both the luminal and myoepithelial cells seen in the primary human epithelial cells, marked by surface marker MUCI and THYI, respectively. Comparing to the other two non-malignant cell line, neither of the non-malignant cells contained a substantial amount of luminal or myoepithelial cell markers (Figure 3). Furthermore, HMECs express consistently detectable levels of NOTCH4 at 0.029 relative to GAPDH, where as the expression level of NOTCH4 in the other two cell lines are much lower and often not detectable (Figure 4). Based on the level of NOTCH4 mRNA expression and the

presence of both cell types, HMECs were chosen as a model cell strain to study primary human epithelial cells *in vitro*.

Result 2: Real-time quantitative PCR (qPCR) array analysis identified FRIZZLED-7 (FZD7) as a NOTCH3 specific target

To knockdown the expression of each Notch receptor in the HMEC cells, lentiviral transduction system expressing short hairpin RNA sequences (Lenti-shRNA) against each Notch receptor was employed. The Lenti-shRNA vectors used express a puromycin-resistant gene, which facilitates the selection of infected cells. To determine the suitable puromycin concentration to be used in the selection process that would kill 95% of uninfected cells, a puromycin survival curve was obtained. HMECs were cultured in the presence of various concentrations of puromycin for a period of 48 hours, then Fluorescent Activated Cell Sorting (FACS) analysis was done to determine the percentage of live cells (Propidium Iodide, PI, negative cells) in each condition compared to the control, where only vehicle control (sterile H_2O) was added in place of puromycin. As shown, 3µg/mL was sufficient to result in < 5% survival (i.e. 95% killed) of HMECs (Figure 3). Therefore, this concentration was chosen as the puromycin selection concentration for subsequent experiments with Lenti-shRNA infected cells (Figure 5).

To identify specific targets of NOTCH3 using a pathway-focused qPCR array, each Notch receptor was individually knocked down by infecting with Lenti-shNOTCH1, Lenti-shNOTCH2, Lenti-shNOTCH3, Lenti-shNOTCH4 produced in 293T cells. The efficiency of the knockdowns at mRNA level was measured by qPCR using primers

designed in our laboratory. There were three different shRNA plasmids used to prepare the Lentivirus, all were purchased from a commercial source (Open Biosystems). Each shRNA plasmid has a complementary sequence against a different region of the same gene (i.e. NOTCH1, NOTCH2, NOTCH3 and NOTCH4), to ensure maximum knockdown was achieved. The mRNA expression levels were obtained relative to GAPDH expression in the transduced cells compared to the Scramble control (Scramble Ct.) infected cells. As shown in Figure 6a, the lenti-shNOTCH1 infected cells show a 92% decrease in the NOTCH1 expression (1.97 compared to 24.91), NOTCH2 shows a 90% knockdown (2.04 compared to 20.69), NOTCH3 shows 90% knockdown of the receptor (0.24 compared to 2.36), and finally, the lenit-shNOTCH4 infected cells show a 97% knockdown of the receptor (1.67 compared to 61.04). Average of 3 knockdown experiments are shown in Figure 6a.

To ensure the protein levels of the Notch receptors were also knocked down, intracellular FACS analysis was done to determine the levels of each Notch receptor in the knockdown cell lines compared to the scramble control. Figure 6b shows the relative level of each Notch receptor in their corresponding knockdown lines compared to the scramble control. Other than NOTCH4, all Lenti-shRNA targeting individual Notch receptors showed a significant decrease in protein level, which is defined as 2 fold or higher. Since the level of NOTCH4 did not show a decrease in the knockdown cell line via intracellular FACS analysis, Western blots were used to examine NOTCH4 protein level in the lenti-shNOTCH4 infected cells. To standardize the Western blot conditions and protein detection, increasing amounts of protein were run on acrylamide gels,

transferred onto a nylon membrane and expression of NOTCH4 and the beta actin antibody was determined using specific antibodies. As shown in Figure 6c, 35µg, 70µg, 105µg and 140µg protein were loaded to each lane, and blotted with each antibody. It is clear that within this loading range, the protein detection is still in the linear range (R^2 value of 0.99 for NOTCH4 and beta actin). The subsequent western blots were done using 75µg of protein per lane, as shown in Figure 6d. Based on densitometry analysis, compared to beta actin, shNOTCH4 infected HMECs showed nearly a 2-fold decrease in protein level compared to scramble control. The reason that a decreased in the NOTCH4 protein levels could not be detected using intracellular FACS could be attributed to high background staining of the antibody. Taken together, Figure 6 shows that the lenti-shRNA targeting each Notch receptor was able to knockdown the corresponding receptor at both mRNA and the protein levels in the HMECs.

RNA extracts from the shNOTCH3 and shNOTCH4 transduced cells were made into cDNA and used as templates for the Notch pathway-specific qPCR array plates. Because NOTCH3 and NOTCH4 are the most differentially expressed Notch receptors in bipotent and luminal progenitors (Raouf *et al*, 2008), qPCR array plates were run with these two samples first. Three independent cDNA samples where used to generate a heat map using the online software provided by SA BioSciences. Each spot on the heat map represent a separate gene, and its corresponding increased or decreased expression compared to the lenti-shScrmble control transduced cells (the transcript expression of each gene was first normalized to internal control, GAPDH that was included on the plate, Figure 7a). The qPCR array data were analyzed to identify Notch target genes whose transcript

expression were only affected by the loss of NOTCH3 expression and not NOTCH4. Such analysis yielded 7 genes namely, HR, HEY1, MGNG, DLL1, FZD7, JAG2, and MMP7, which were differentially up- or down- regulated in knockdowns of NOTCH3 compared to knockdowns of NOTCH4. To further validate the differential expression of these 7 genes in the knockdown samples of Notch receptors, independent primer sets were designed and the specific changes to the transcript expression of the shortlisted genes in the new set of lenti-shNOTCH1, lenti-shNOTCH2, lenti-shNOTCH3, lenti-shNOTCH4 and Lenti-shScrambled control transduced cells were examined (Figure 7b).

Interestingly, only 3 out of the 7 candidate genes (DLL1, KRT1, JAG2, MMP7, and FZD7) were specifically down regulated only in samples that when NOTCH3 signaling deficient (Figure 7b). These experiments suggest that some of these Notch target genes (in particular FZD7) could indeed be specifically regulated by the NOTCH3 receptor only.

To examine whether these three NOTCH3-specific targets are indeed regulated by the Notch signaling, γ secretase blocker N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was used to block Notch signaling in HMECs. Initially, the optimum concentration of DAPT was obtained through a set of dose response experiments (repeated in triplicate, Figure 8a) using increasing concentrations of DAPT ranging from 5 μM to 25 μM. In these experiments equal molar concentrations of DMSO were used as vehicle control. After 24 hr, RNA was extracted from the cells and expression of a classical Notch-responsive gene HES1 transcript expression was assessed

(via qPCR) as a marker of Notch signaling activation. These experiments revealed that 10μM DAPT resulted the largest suppression of Notch activity and therefore, this concentration was used in the subsequent experiments. Subsequently, HMECs were treated for 24 hr using 10μM DAPT or DMSO and the mRNA expression of 3 candidate NOTCH3-specific targets was examined (Figure 8b). As it can be seen, DAPT treatment decreased the transcript expression of DLL1, FZD7 and MMP7. These data suggests that DLL1, FZD7 and MMP7 are bona fide Notch signaling targets that are exclusively regulated by the NOTCH3 signaling.

Previous transcriptome profiling suggested that FZD7 might be more significantly expressed in luminal progenitors compared to the bipotent progenitors (Raouf, *et al* 2008). Because luminal progenitors express NOTCH3 at significantly higher levels compared to the any other cell type in the human mammary gland, FZD7 was chosen for further study.

To determine whether FZD7 protein level was also decreased as a result of NOTCH3 knockdown, intracellular FACS was used to quantify the FZD7 and NOTCH receptor expression in HMECs transduced with lenti-shNOTCH virus. These lentiviral vectors express GFP protein as well as the shRNA fragment, allowing the study of the transduced cells via FACS. Therefore, to ensure that only the infected cells were considered, the FZD7 protein expression was measured in the GFP+ cells. Results in Figure 9 shows that compared to scramble control, only shNOTCH3 expressing cells showed a 2 fold decrease in FZD7 protein level. To compare the effects of loss of each Notch receptor on

the FZD7 expression, ANOVA analysis was employed (Table 6). Interestingly, loss of NOTCH3 decreased FZD7 protein expression most significantly than loss of other Notch receptors. Also ANOVA analysis revealed that NOTCH2 and NOTCH4 loss do not alter the expression of FZD7 transcript while loss of NOTCH1 led to a modest 1.8X decrease in FZD7 protein levels. Together these results show that FZD7 is a potential NOTCH3 target based on knockdown of Notch receptors at protein and mRNA level.

Result 3: Over expression studies identified FZD7 as a direct NOTCH3 specific target

To firmly establish that the Notch-dependent expression of FZD7 is directly regulated by NOTCH3 and not through other Notch receptors, the constitutively active form (the intracellular domain [ICD]) of NOTCH2 and NOTCH3 were PCR-cloned into Lentivirus over expression vector and Lentivirus was prepared with each vector using the 293-Tcells. The over expression vector of NOTCH1 and NOTCH4 were made possible through expression vectors provided by Dr. Aly Karsan (NOTCH1 expressing vector) and Dr. Andrew Wang (NOTCH4 expressing vector). The transgenes from these vectors were also cloned into the Lentiviral vector and used in virus production.

The Lentiviral preparations were used to infect the HMECs along with a Lentivirus expressing GFP only as a control. The GFP expressing cells were sorted via FACS and transcript expression of each Notch receptor was quantified using qPCR (Figure 10a). Compared to KA391 vehicle control, the expression of the corresponding Notch receptor was increased anywhere from 3.2 to >100 fold. Because NOTCH4 is expressed at low levels in the HMECs, the overexpression of NOTCH4 produces an exaggerated fold

increase (i.e. >100 fold increase, Figure 10a). When the transcript expression of FZD7 was considered in these Lenti-NOTCH-ICD transduced cells, the NOTCH3 overexpressing cells showed a 252 fold increase in the transcript expression of FZD7 (compared to the KA391 empty control levels, as determined by qPCR). Interestingly, overexpression of the active form of the other Notch receptors failed to enhance FZD7 expression (Figure 10c) These data provide further support to the notion that FZD7 could be a non-redundant gene target of NOTCH3 signaling.

Next the FZD7 protein expression was determined in the lenti-NOTCH-ICD transduced cells using intracellular FACS. Compared to the empty vector control, the percentage of FZD7 positive cells was only increased in NOTCH3 over expressing HMECs (1.13% to 7.66%), whereas the percentage of FZD7 positive cells in other Notch receptor over expressing HMEC was not significantly changed (Figure 10b). Also, the the NOTCH3 overexpressing cells showed stronger expression of FZD7 as measure by the median fluorescent intensity through FACS analysis.

Together these results suggest that activation of FZD7 by Notch signaling is regulated by NOTCH3 and not the other receptors.

Result 4: FZD7 shows similar expression profile as NOTCH3 in separated subsets of primary human mammary epithelial cells

Our observations so far suggest that a FZD7 expression could be regulated by the Notch signaling and that FZD7 represents a non-redundant gene target of the NOTCH3. Since signaling through NOTCH3 has been shown to be essential to the luminal cell fate, we

investigated potential involvement of FZD7 in luminal fate commitment of the bipotent progenitors. To this end, the expression of FZD7 was examined in the different subsets of human primary mammary epithelial cells. Previously it was shown that cell surface markers EpCAM (Epithelial Cell Adhesion) and CD449f (alpha 6 integrin), can be used to isolate the bipotent progenitors ($EpCAM^{low}CD49f^{high}$), and luminal progenitor cells ($EpCAM^{high}CD49f^{low}$) via FACS (Figure 11a). For the purposes of this aim, breast reduction samples were first dissociated and pre-cultured for 3 days as this short culturing of the cells enriches for the bipotent and luminal progenitors (Raouf, *et al*, 2008). The relative mRNA expression of NOTCH3, NOTCH4 and FZD7 were determined in the FACS-sorted subpopulations of human breast cells (obtained from breast reduction samples) using qPCR. This analysis revealed that FZD7 has the similar expression pattern as NOTCH3, in that it was expressed at higher level in the luminal progenitors compared to bipotent progenitors (Figure 11b).

To determine whether the protein level of FZD7 was also differentially expressed in the subpopulations of primary human mammary epithelial cells, 3 different human samples were dissociated and FACS analyzed based on the EpCAM, CD49f and FZD7 expression surface marker expression. In this case, the dissociated breast reduction samples were not pre-cultured as before since we found that short-term cultures of the primary cells produced an inferior FZD7 cell surface expression profile compared to non-cultured cells (data not shown). It was previously shown that in non-cultured human breast cells the $EpCAM^{high}CD49f^{high}$ cells are enriched in the luminal progenitors while the $EpCAM^{low}CD49f^{high}$ cells are enriched in the bipotent progenitors (Eirew P, *et al*, 2008).

FZD7 expression in these experiments was measured using an antibody raised against the extracellular portion of FZD7. This antibody was titrated to find a dilution that provided the best signal above the isotype control (Figure 9a). Figure 10 shows that the luminal progenitors contained significantly more FZD7+ cells and that FZD7 expression was brighter in these cells compared to the bipotent progenitors, which corresponds well with the mRNA expression data.

Taken together these data suggested that NOTCH3 might regulate luminal cell fate through increased expression of FZD7. Therefore a potential cross-talk between the Notch and Wnt signaling pathway could be important to the lineage restriction of the bipotent progenitors during the normal development and maturation of the adult human mammary gland.

IV. Discussion

While some evidence points to possible non-redundant roles of each NOTCH receptor, the current paradigm suggests that all four Notch receptors exhibit overlapping biological functions through activation of similar Notch target gene. Therefore much of the research that is being done currently only focuses on one of the receptors (mostly NOTCH1 due to its abundant expression), with the assumption that the other Notch receptors would act in the same manner in experimental and biological conditions. The rational for this assumption stems from the fact that all four Notch receptors exert their biological function by binding to a DNA-binding complex, and not through direct binding DNA itself. The binding process recruits a set of protein activators, initiating the transcription of the target genes (e.g. Hes1).

The interactions between the Notch signaling and other signaling pathways has been described before, in particular in malignant tissue. However, the cooperation of Notch with other signaling pathways has not been described in development or maturation of the adult mammary gland.

Similar to other biological systems, much of what is known about the mammary glands development is extrapolated the mouse and rat experimental models. Although there are many similarities between mouse and human biology, there are key differences that need to be considered. For example, while the human mammary gland consists of extracellular matrix, the mouse mammary gland mostly consists of adipose tissue. To study the early

breast development and tissue maintenance in the adult throughout pregnancy cycles of human mammary gland, a suitable cell line or cell system is needed for in vitro studies. Since one of the identifying features of non-malignant cells is the ability to regulate growth, cells have inherit control and check points for a finite number of divisions (Dawson, *et al*., 1996). This property makes maintaining a normal, non-malignant cell line or cell strain technically challenging. As the result, all currently available non-malignant human mammary epithelial cell lines have genetic abnormalities that enables them to be passaged in culture indefinitely. These cells typically only consists of luminal-like cells (i.e. Cytokeratin 18 positive cells).

Since using patient samples would generate data with a large variation, and that only a small number of cells can be obtained from a single breast reduction sample, identifying an *in vitro* cell system was crucial to this project. The choices of non-malignant human breast epithelial cell lines is limited to the MCF7, 184-hTert, and a Human Mammary Epithelial Cell (HMEC) strain. For the purposes of the experiments performed toward this Thesis, HMECs were used because these cells express the desired cell surface marker expression profile. As seen in Figure 3, HMECs were the only cell strain expressing both luminal (MUCI+) and myoepithelial (THYI+) cell markers. HMECs were obtained from a breast reduction sample, and can be passaged *in vitro* 7 to 10 times. In our experience, slowing of cell division, enlargement of nuclei and poor survival upon passaging started showing around passage 8.

Cells used in this study were cultured on 2D adherent tissue culture plates, while as in biological systems; these cells would be constituted in a 3D environment. A few studies had looked at gene expression patterns of cells grown in 2D vs. 3D cultures, and found startling differences Suggesting that 3D systems such as Matrigel are more suitable to simulate the *in vivo* environment of the cells. Conventional 2D cultures were used for this study for many reasons. Firstly, 2D culturing techniques are much more developed and standardized, whereas the growth conditions of these cells in 3D cultures are not well defined. We used 2D cultures to to identify potential unique gene targets of NOTCH3. Subsequently these potential targets are to be validated in primary human breast epithelial cells from a functional point of view. Therefore the potential difference in 2D vs. 3D culturing systems was not critical.

The colony forming cell assays (CFC assay) is a challenging assay in that poor colony formation and even cell death are common with fluctuation CO_2 levels in the incubator. HMEC survival heavily depends on the CO_2 levels as well, and if the levels were less than 3% for extended periods of time, the cells experience irreversible damage, and the majority of the cells lose the ability to further multiply. Compared to the non-malignant human mammary epithelial cell lines, this property of HMEC was unique, as no observable signs of stress were found in other cell lines with fluctuating CO_2 levels in the incubators.

The MCF10A, a non-malignant cell line, was obtained from spontaneous immortalization of human breast epithelial cells from a patient with fibrocystic breast disease.

Immunohistochemical (IHC) staining and FACS analysis of these cells revealed that nearly all MCF10A cells express markers of luminal type cells and lack expression of markers for the myoepithelial cells. These cells are nearly all positive for Cytokeratin 18, which is a luminal cell marker (data not shown, Eirew *et al*., 2008), and nearly all cells were positive for MUCI, the luminal marker, while none of the cells were positive for the myoepithelial THY1 (Figure 3). As well the transcript expression of Notch receptors were not desirable, since NOTCH4 was only observed at limit detection (Figure 4). The low expression of NOTCH4 is also seen in the luminal cells in primary human mammary tissue, as shown by Raouf *et al* in 2008.

The other cell line, 184 h-Tert was generated by forcing the expression of human telomerase enzyme in a population of spontaneously transformed mammary epithelial cells from a patient breast reduction sample. Upon serial passaging, it was found that these cells gradually obtained a P16 mutation (Stampfer, 2001). Contrary to the MCF10A cell line, the 184 h-Tert cells had an expression profile more closely related to the myoepithelial cell type, rather than the luminal cell type. These cells have much lower mRNA expression of NOTCH3, and a slightly higher expression of NOTCH4 compared to MCF10A (Figure 4). Unlike MCF10A cells, the 184 h-Tert cell line shows no detectable expression of the luminal cell marker MUCI or the myoepithelial cell marker THYI (Figure 3). Overall, the 184 h-Tert cell line was not an ideal model to study normal human mammary gland biology, as these cells were largely composed of myoepithelial cells, and the transcript expression level of NOTCH4 was very low.

Comparing all three cell systems, HMECs represented the most desirable cell strain and therefore this cell strain was used as the *in vitro* model system to identify specific target of NOTCH3 in this study.

In order to identify the specific targets of NOTCH3, we must identify genes whose transcript level changes in response to decreased NOTCH3 signaling and not any other Notch receptors. With the recent advances in bioinformatics, various mass-sequencing techniques such as RNA-Seq have gained much popularity, even though storing and interpreting the data require special data storage facilities and bioinformatics specialists. Interpreting the data coming out from these sequencing process and giving them biological relevance becomes the new challenge for researchers. Microarrays such as the ones offered by Affymatrix is another option. The data obtained from the microarrays are more shallow compared to sequencing techniques, and the genes available for study are biased based on the availability of primer sequences included on the microarrays. With either technique, a "cut off" value for signal strength is required to eliminate false-positives and background noise. If the cut off value is set too high, genes with very low expression levels will not be identified (i.e. slightly above the background signal) and would be deemed as false negatives. Choosing a low cut off valued would result in increased number of false positive targets genes. Independent quantitative PCR assays are required to validate the accuracy of the detections by the microarrays. Furthermore, analysis of the large data sets that will be obtained through RNA-Seq or Affymetrix methods will make the identification of Notch target genes very difficult as the

application of a secondary filter to identify demonstrated notch targets and/or Notch signaling associated genes will be necessary.

With respect to the present study, the analysis of RNA-seq or microarray data sets to obtain Notch signaling target genes whose expression is specifically regulated by NOTCH3 would have been very challenging. Therefore using a gene expression analysis method that is focused on the Notch signaling targets and related genes was deemed to be a more prudent approach. The Pathways-focused, quantitative-PCR (qPCR) plates pre-loaded with primers representing known targets of Notch signaling or genes associated with the Notch signaling pathway were chosen as the method to identify differentially expressed genes between Notch receptor knockdowns and the scramble control. These pathway-focused qPCR array sets are however limited in that target gene identification is limited to the available primer sets within the array and that low abundant genes may not amplify well-enough to be used in the analysis. As well, RNA quality and genomic DNA contamination could lead to erroneous conclusions.

With the said limitations and caveats in mind, these qPCR-based plates were still very useful, as they are convenient, easy to use, and data analysis could be done without the need for bioinformatics specialists or additional data storage. Because the main focus for this project was to identify NOTCH3-specific target genes, the use of a qPCR-based technique focused on Notch signaling related genes is the most suitable.

To identify unique targets for NOTCH3, we first systematically knocked down the expression of the individual Notch receptors (NOTCH1 to NOTCH4) using lentivirus expressing shRNA fragments against each receptor. Lentiviral infection was chosen as the transduction mechanisms since it offered the best transduction efficiency (upwards of 90% of cells were transduced) and that the lentiviral gene cassette including the shRNA fragment could be incorporated into the host cell's genomic DNA, leading to possible generation of Notch receptor-deficient cell lines. Transfection as a method of gene transduction was not used because tranfection is much harsher on cells and since the HMECs were already relatively delicate to maintain, the transfection of vectors method was not considered.

Other than the shRNA portion, these commercially available lentiviral constructs also contain two mammalian selection markers, a turbo green fluorescent protein (tGFP) and a puromycin resistant gene, hence selection for infected cells can be done through tGFP expression and/or puromycin resistance. For the purposes of the experiments described here puromycin selection was chosen because the HMECs grow very slowly in culture and therefore obtaining large numbers of transduced cells needed for this study proved to be cumbersome. Since puromycin affect non-resistant cells by inhibiting protein synthesis, sufficient time was needed for selection to take place. To determine the optimal puromycin concentration for selection, kill curve experiments were done. Puromycin was added to normally grown HMECs at different concentrations, with final concentration in growth media ranging from 0.5µg/mL to 10µg/mL. The number of live or dead cells were quantified using propidium iodide (PI) where the percentage of PI+

cells (i.e. dead cells) was determined via FACS. At 3µg/mL, 95% of the nonresistant cells were killed by puromycin (Figure 3). This concentration was chosen as the ideal concentration for selection. The efficiency of puromycin selection was also verified by FACS, the aim was to ensure that all cells were GFP positive after selection. With random sampling of selected, infected cells, it was found that more than 95% of survival cells were tGFP positive (data not shown), proving that puromycin selection was an effective method of selection.

Different shRNA species were obtained based on their sequence specificity for different regions of the Notch receptors. Depending on the sequence, each construct could lead to different level of gene knockdown. To maximize the levels of knockdowns, three different constructs against each Notch receptor were used. In principal each cell could only accept one round of insertion, however we had found that using the collective of several effective shRNA constructs maximized the levels of knockdowns consistently to 90% or more, while individual shRNA construct alone can knockdown a gene anywhere between 5% to 90%, based on qPCR analysis on mRNA expression levels. Only HMECs that showed Notch receptor knockdown by at least 90% at mRNA level (Figure 4a) were used. Intracellular FACS was used to determine the levels of protein knockdown, with the exception of NOTCH4, all other Notch receptors was knocked down by at least 50% (Figure 4b). the intracellular dectection of NOTCH4 via FACS proved difficult based on the high background associated with this antibody. Therefore, Western blots were done to determine the level of protein knockdown in the shNOTCH4 expressing cells. As

shown in Figures 4c and 4d, the level of NOTCH4 protein was knocked down by 50% in the knockdown cells.

Depending on the half-life of the protein, the decrease in protein level was not as significant as that of mRNA levels. This observation could be due to the residual protein that is still inside the transduced cells (depending on the protein half-life). In our case, all analysis were done after 48 hours of puromycin selection. Technique wise, mRNA measurements are far more quantitative compared to protein measurements. All commonly used protein measurements are antibody-based, so affinity of antibody binding, efficiency in detection, whether it is biochemical (Western Blots) or via FACS, could all affect the accuracy in measurements. Whereas the PCR-based mRNA detection and measurements are much more precise. Because the protein levels decreased by at least 2 folds (Figures 6b, d) for all Notch receptors, the decrease in potential unique targets should be detectable.

As can be seen in Figure 4, the mRNA levels of Notch receptors in HMEC were considerably higher once the cells were treated with the Lentivirus carrying shRNA constructs against each Notch receptors and the Scramble control in Figure 4a. This could be one of the side effects of the Lentivirus infection process and the subsequent culturing. Since the objective for this project was to identify the unique targets of NOTCH3, and later verify the targets for their biological function, as long as comparing the shNOTCH3 treated cells to the Scramble control showing at least 90% decrease in expression level, the results are considered valid.

Since in the primary human mammary epithelial cells, the levels of NOTCH1 and NOTCH2 were not dramatically different between the bipotential and luminal progenitors, and the levels of NOTCH3 and NOTCH4 mRNA expression were opposite in these two populations of interest, NOTCH3 and NOTCH4 knockdown samples were used for the qPCR array, with the intention to get more noticeable results. cDNA samples prepared from shScrambled, shNOTCH3 and snNOTCH4 were used to examine changes in the expression of Notch signaling associated genes using the SABioscience's Notch pathway qPCR array. These experiments revealed 3 genes whose expression were only changed in the shNOTCH3 infected cells. These results were verified using sets of independently designed primers. Also, the expression of these 7 genes in shNOTCH1 or shNOTCH2 infected cells were also examined. Not surprisingly, some of these 7 genes were found to be also regulated by NOTCH1 and/or NOTCH2 receptors and were therefore removed from the study (Figure 5b).

Out of all 7 selected differentially regulated genes from the qPCR array (Figure 7a), only 3 genes were uniquely regulated by NOTCH3, and not by any other Notch receptors (Figure 7b). The cut off for significant change was set to be at 2 folds, so genes whose transcription level either decreased by 2 folds (at 0.5 or lower) or increased by 2 folds were considered changed. The 3 genes were delta-like 1 (DLL1), metalloproteinase 7 (MMP7), and Frizzled 7 (FZD7).

Since the focus of this project was on canonical Notch signaling, and canonical Notch signaling requires the cleavage of extracellular part of the Notch receptor by γ-secretase to initiate the signaling pathway, any target gene of the receptors would also be sensitive to γ-secretase blockage. To test whether the 3 identified potential unique NOTCH3 targets were γ-secretase dependent, a conventional γ-secretase blocker, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was used. The typical concentration of DAPT used in literatures was 10μM in human cell lines, and primary human mammary epithelial cells, but there was no study using DAPT in HMECs. Therefore the effective DAPT dose was determined by treating HMEC with DAPT or DMSO (vehicle control) for 24 hours at 5μM, 10μM and 20μM. The transcript expression of Hairy and Enhancer of Split-1 (HES1, a Notch target gene), was used as a measure Notch signaling. In these experiments 10μM produced the greatest decrease in HES1 expression and therefore this concentration was chosen as the ideal DAPT dose. Interestingly, upon DAPT treatment, all 3 genes of interest showed a decrease in mRNA expression in HMECs compared to the DMSO treated cells. There had been some studies showing the Notch signaling pathway ligand DLL1 as a target for Notch signaling itself, while there were very little known about the relationship between Notch signaling pathway and FZD7. Since MMP7 is closely related to a notch activating membrane cleavage enzyme ADAM (A Disintegrin And Mettaloproteinase), there had been one study suggesting that in a pre-cancerous lesion state, MMP7 could be controlled by Notch signaling as a feedback mechanism. None of the 3 genes had been shown to have involvement in human mammary gland development.

Although it is often used as a γ-secretase blocker, DAPT is not specific, as it blocks the actions of other enzymes as well. For more sensitive and comprehensive studies, other Notch signaling pathway blocker should be used to complement the DAPT data, just for a small chance that the changes in transcript levels of selected target genes were off-target effects.

Interestingly, MMP7 and DLL1 transcript levels were increased in cells expressing a constitutively active form of NOTCH4. While this regulatory relationship could be interesting, it was outside the scope of this study, and thus FZD7 was selected as the unique NOTCH3 target for further experiments. Upon knocking down of each Notch receptor, the protein levels of FZD7 only significantly decreased with knockdown of NOTCH3 (Figure 7). Also, FZD7 protein level increased by 7 folds in cells expressing a constitutively active form of NOTCH3 (NOTCH3ICD) compared to the vector control KA391 while activation of other receptors had no effect (Figure 8). The delivery system for these overexpression vectors was also Lentivirus-based, since this method generates progenies carrying the overexpressing gene, provided that the overexpressing gene was not toxic for the cells. The selection marker available through KA391 vector was GFP. The strategy to use constitutively active forms of each Notch receptor is based on the lack of knowledge about which Notch ligands would activate each Notch receptor specifically. For the purposes of this study it was important to measure molecular changes that are associated with the increased or decreased signaling through each individual Notch receptor separately.

If FZD7 were truly a unique NOTCH3 target in human mammary epithelial cells, it would have similar expression patterns in the different subpopulations of primary human mammary epithelial cells. Toward this idea, 3 discarded human mammoplasties were made into single cell suspension, and FACS separated into the bipotent and luminal progenitors. NOTCH3 expression showed some variation. Compared to the bipotent progenitors, luminal progenitors expressed higher levels of NOTCH3 and FZD7 (Figure 9a-b). Furthermore, the protein levels of FZD7 was also significantly higher in luminal progenitors compared to bipotent progenitors in the same patient samples based on mean fluorescence intensity (MFI) of FZD7 positive cells measured via intracellular FACS (Figure 10). In combination with the mRNA expression data, it is very likely that FZD7 represents a unique NOTCH3 target gene in human mammary epithelial cells.

It is noteworthy that FZD7 is expressed in cells that show very little expression of NOTCH3 (e.g. the bipotent progenitors). Therefore, the conclusion of this study is that the Notch signaling regulation of FZD7 is governed through NOTCH3 specifically. Whether NOTCH3 regulation of luminal cell fate requires signaling through FZD7 is interesting and requires further studies. Our observations suggest that there might be a cooperation (cross-talk) between the Notch and Wnt signaling during restriction of bipotent progenitors to the luminal cell fate.

To determine whether FZD7 plays a role in bipotent progenitor restriction to the luminal cell fate, the colony forming cell (CFC) assay would be the first step. The CFC assay is a highly quantifiable *in vitro* assay to quantify progenitor cell numbers (i.e colony

numbers) and their differentiation potential (i.e. colony type). If luminal cell fate requires FZD7, the bipotent progenitors lacking FZD7 would not be able to develop into luminal colonies compared to the control cells of the same patient sample. The knocking down of FZD7 could be achieved using shRNA technique. By comparing the efficiency of luminal colony formation of lenti-shFZD7 infected bipotent progenitors to the lenti-shScramble control, one could determine whether or not FZD7 plays a role in the process.

Currently, there is little known about the potential role of FZD7 in mammary gland development or breast carcinogenesis. As a member of the Wnt signaling pathway, the distinct role of FZD7 compared to other FZD proteins is not well studied. Similar to the Notch signaling pathway, the Wnt signaling pathway also plays important roles in early development and tissue maintenance. Many Wnt signaling components were shown to have involvements in various cancerous states, particularly in the brain tumor development. There had been a few studies linking the Notch signaling pathway with the Wnt signaling pathway, which then makes this new finding even more exciting and also plausible.

Anti-FZD7 antibody therapy is currently in clinical trials for treating gastrointestinal cancers. Therefore, if FZD7 plays a role in mammary carcinogenesis, the antibody therapy could be investigated as a potentially new treatments for breast cancer. With the tumor bank resources available, one could easily examine whether FZD7 is differentially expressed in various types and stages of breast cancer, thus making it a possible prognosis marker for the disease. This finding also confirms the current, emerging

evidences indicating that Notch receptors have overlapping as well as non-redundant functions even though they activate transcription of target genes by binding to a DNA-binding complex. The mechanisms through which NOTCH3 could specifically regulate FZD7 transcription are not known. However it is possible that NOTCH3 could form a different DNA binding and activating complex than the other Notch receptors.

Combining data obtained in this project and previous research done by other groups, one proposed mechanism of action was summarized in Figure 13. Although Wnt ligands are expressed and very liked excreted by all types of cells in the mammary epithelial microenvironment, because luminal progenitors and luminal cells have enhanced expression of FZD7 protein on their cell membrane, these cells would be more responsive to Wnt ligands, in order to develop into and maintain sufficient amount of luminal cells.

V. Conclusion

The process of lineage-determination in the human mammary gland is seminal to generating the two functional types of cells (i.e. the luminal and the myoepithelial cells) carrying out the biological function in the gland. This process is crucial in the maintaining and regeneration of the gland during puberty and subsequent pregnancy cycles through out the reproductive life of a woman. This process relies on carefully orchestrated interaction between different signaling pathways and if improperly regulated could lead to pre-cancerous lesions.

It was previously found that signaling through Notch receptor 3 (NOTCH3) alone was required for the development of the luminal cell type. Interestingly, overexpression of NOTCH3 in mouse mammary gland leads to breast cancer development. Therefore, identifying specific non-redundant targets of NOTCH3 would provide significant new knowledge about the critical lineage-determination step in mammary gland biology, and it could potentially provide alternative (i.e. blocking specific targets of NOTCH3 as oppose to blocking NOTCH3) therapeutic target for breast cancer treatments. This is important since Notch signaling blockers have been considered in clinical trials as a potential therapy for breast cancer and other malignancies with little success (Radtke, 2005). The lack of success has been due to the serious side-effects of blocking Notch signaling in the patients (Radtke, 2005).

Using quantitative PCR (qPCR) based array in combination with qPCR and non-malignant human mammary cell strain HMECs, five genes were found to be specifically

up or down regulated as a result of decreased NOTCH3 expression. Our studies identified FRIZZLED-7 (FZD7) as a specific, non-redundant NOTCH3 target gene. It was found previously that NOTCH3 expression is necessary for the luminal cell commitment of the biopotent progenitors. Combined with the observations reported in this study, it is inviting to suggest that NOTCH3-FZD7 signaling axis could be important in luminal cell fate determination in the human mammary gland.

The paradigm of Notch signaling is changing from one which assumes that all 4 Notch receptors perform overlapping biological function to one which is embracing that different Notch receptor could perform non-redundant functions through activation of genes unique to each receptor. The work presented here provides proof of principle that NOTCH3 could perform its unique biological functions (i.e. luminal cell fate determination) through activation of non-redundant target genes (i.e. FZD7).

Since FZD7 is a trans-membrane protein, antibody-based therapies against FZD7 can be considered. Currently blocking antibodies against FZD7 are being considered in the clinical trials for gastroenteric malignancies.

Perhaps future studies will ascertain the exact nature of NOTCH3-FZD7 signaling axis in the normal human mammary gland development and if this signaling plays a role in breast carcinogenesis. Based on such knowledge perhaps blocking FZD7 in breast cancers can be considered as a potential therapy against breast cancer, in particular against luminal-type breast cancers.

VI. Figures

Figure 1: Brief overview of Notch signaling pathway (Cell Signaling Technologies)

Figure 2: Overview of Wnt signaling pathway (Fedon et al., 2012)

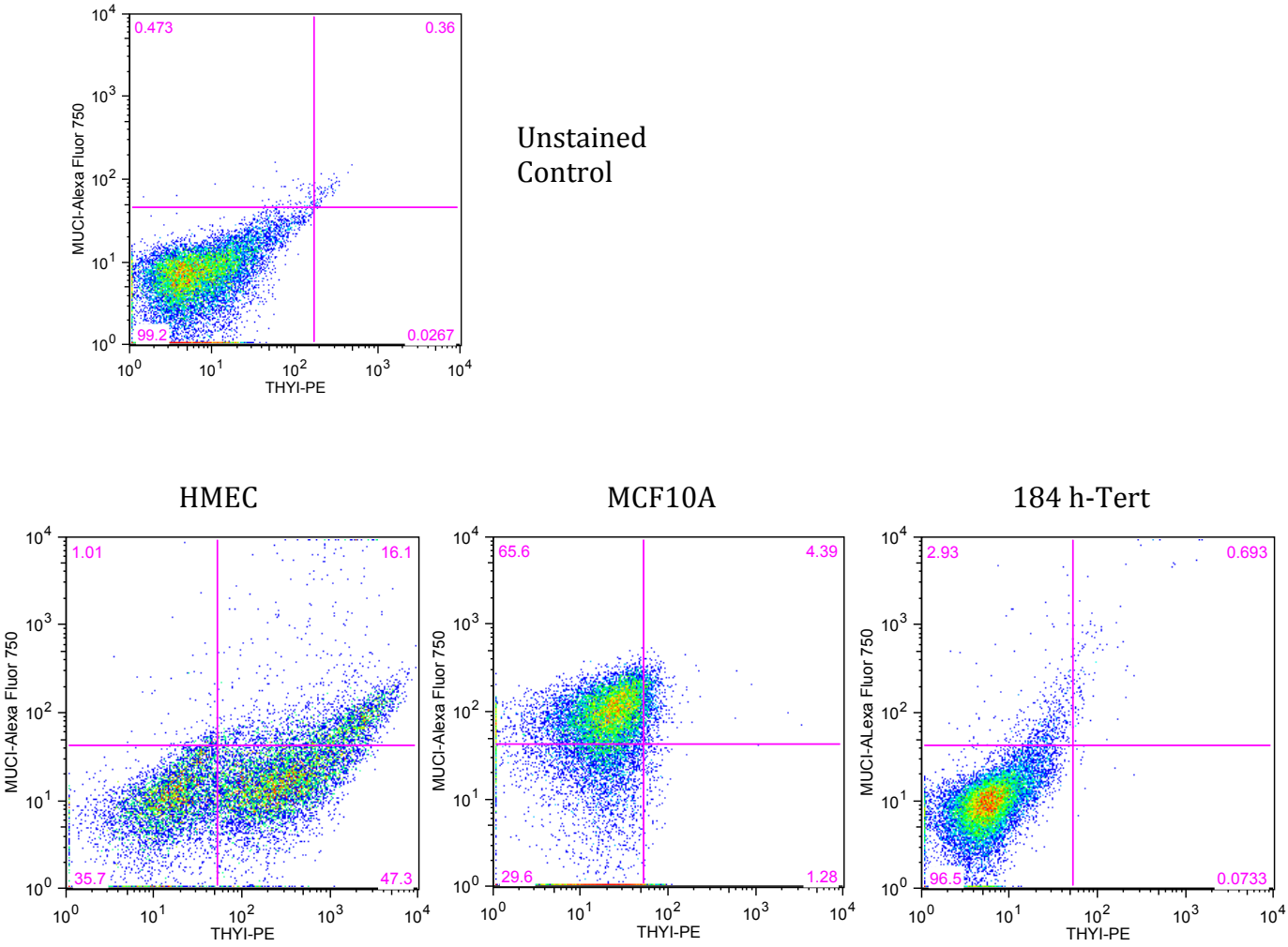


Figure 3: Comparing mammary epithelial surface markers in non-malignant human breast epithelial cell lines and strain. FACS analysis comparing HMEC, MCF10A and 184 h-Tert showing that HMEC express luminal and myoepithelial surface markers MUCI and THY1, seen in primary reduction samples; whereas 184 h-Tert and MCF10A do not have THY1 expression.

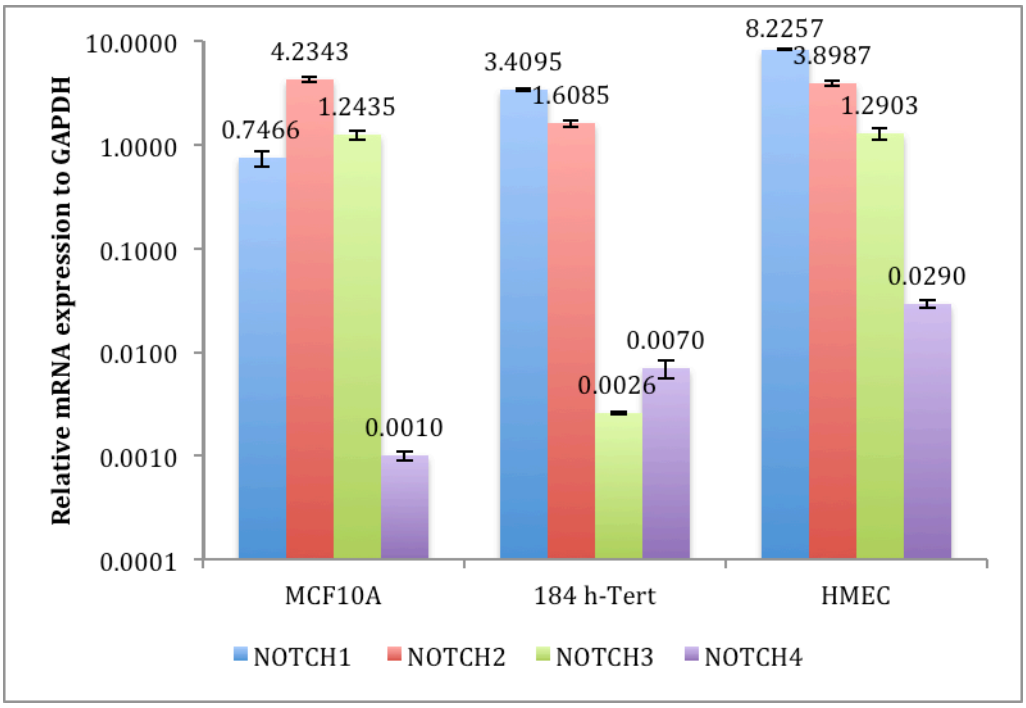


Figure 4. qPCR analysis of normally cultured non-malignant cell lines and cell strain showing cell strain HMEC express detectable levels of 4 Notch receptors relative to GAPDH in each cell line/strain, compared to other non-malignant cell line 184 h-Tert and

MCF10A, where NOTCH4 levels are near the detection limit. N=3, ± Standard Deviation

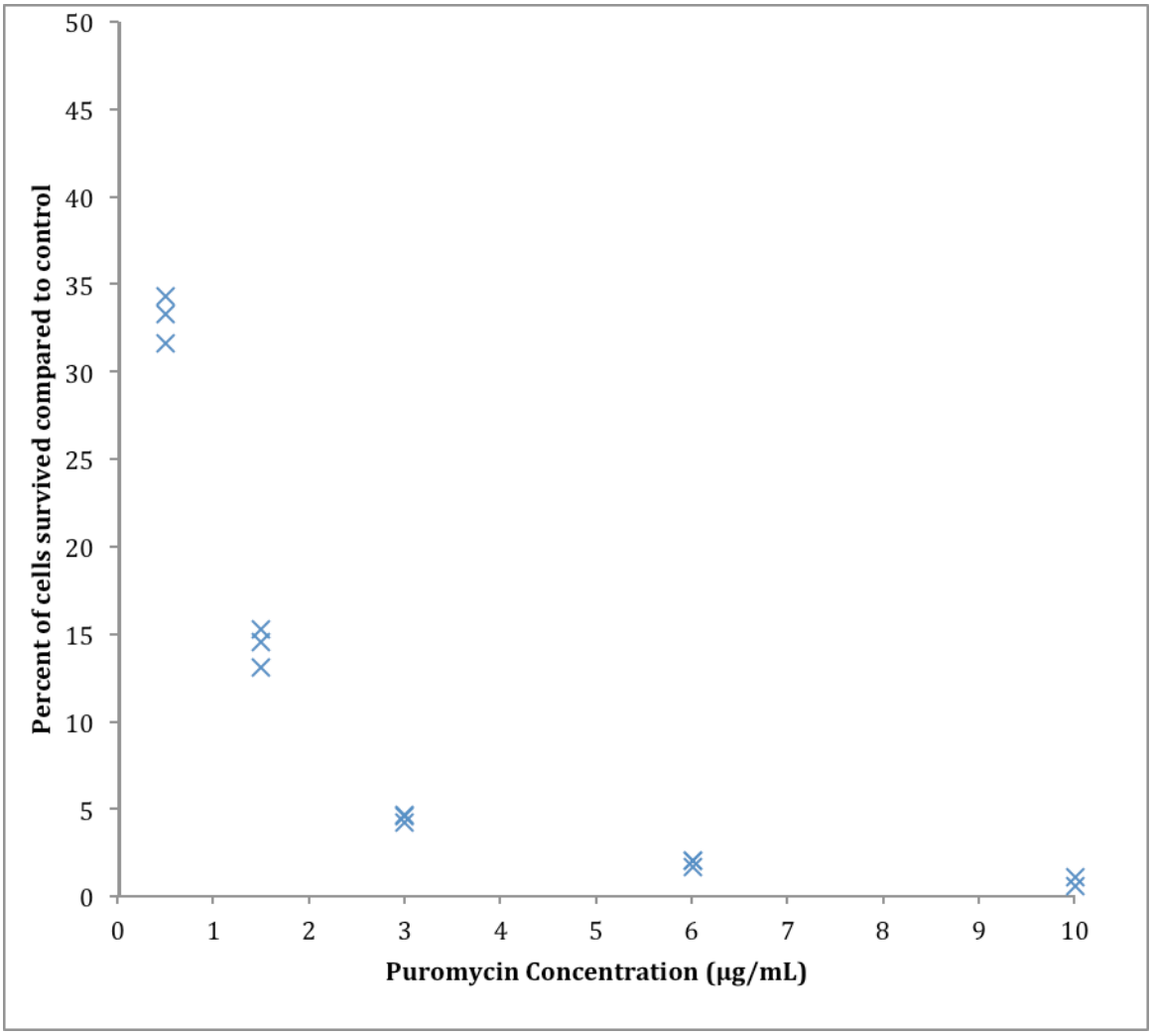
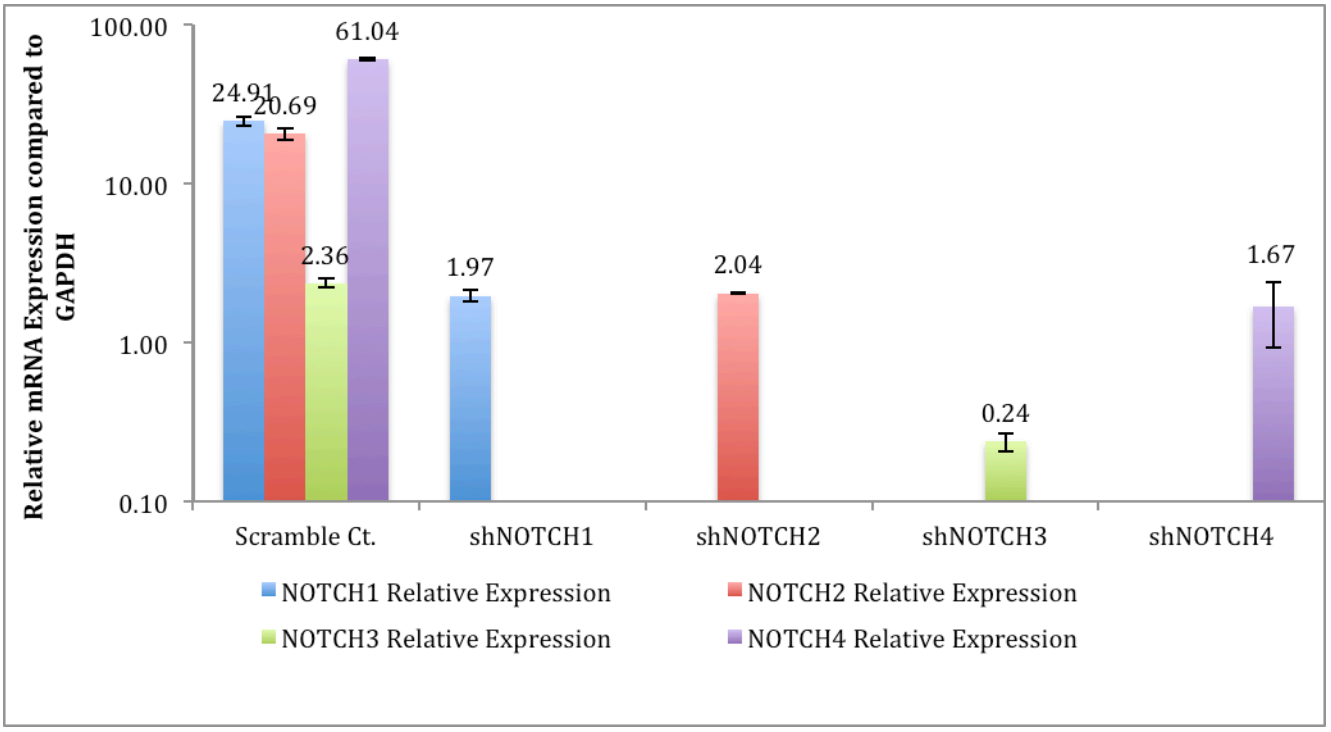


Figure 5: Kill curve determining the optimal puromycin selection concentration in cell strain HMEC. Media containing varying concentration of puromycin was added to growing culture for a selection period of 48 hours. FACS analysis was done to determine what percentage of PI negative cells was in each sample compared to media containing no puromycin. At 3µg/mL around 95% of cells were killed, this concentration was determined as the puromycin selection concentration for subsequent experiments. N=3, ±Standard Deviation

(a)



(b)

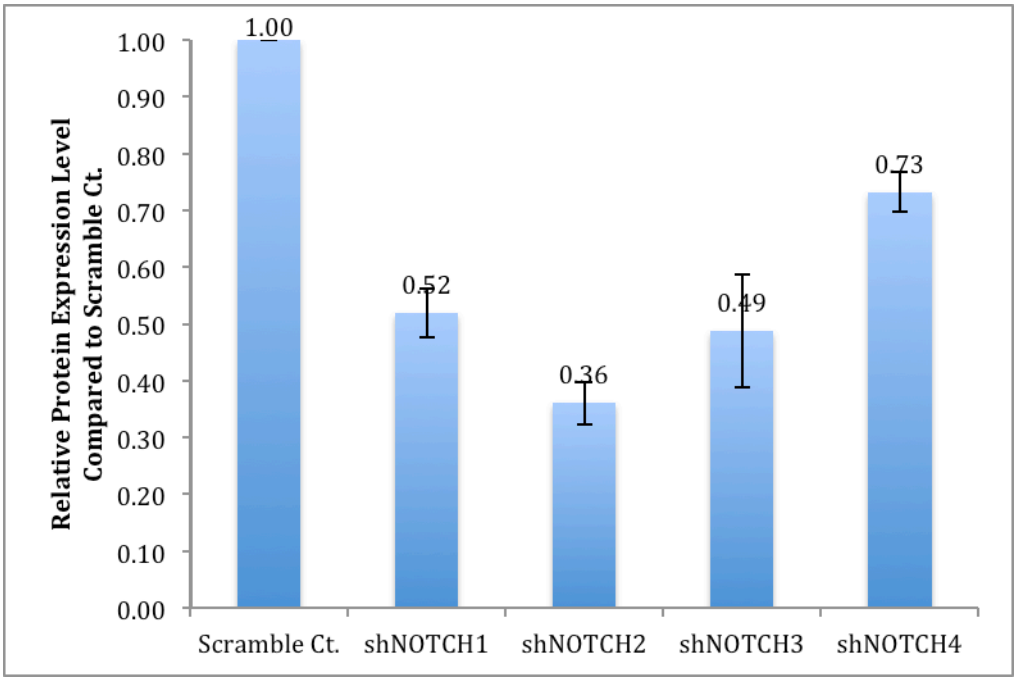
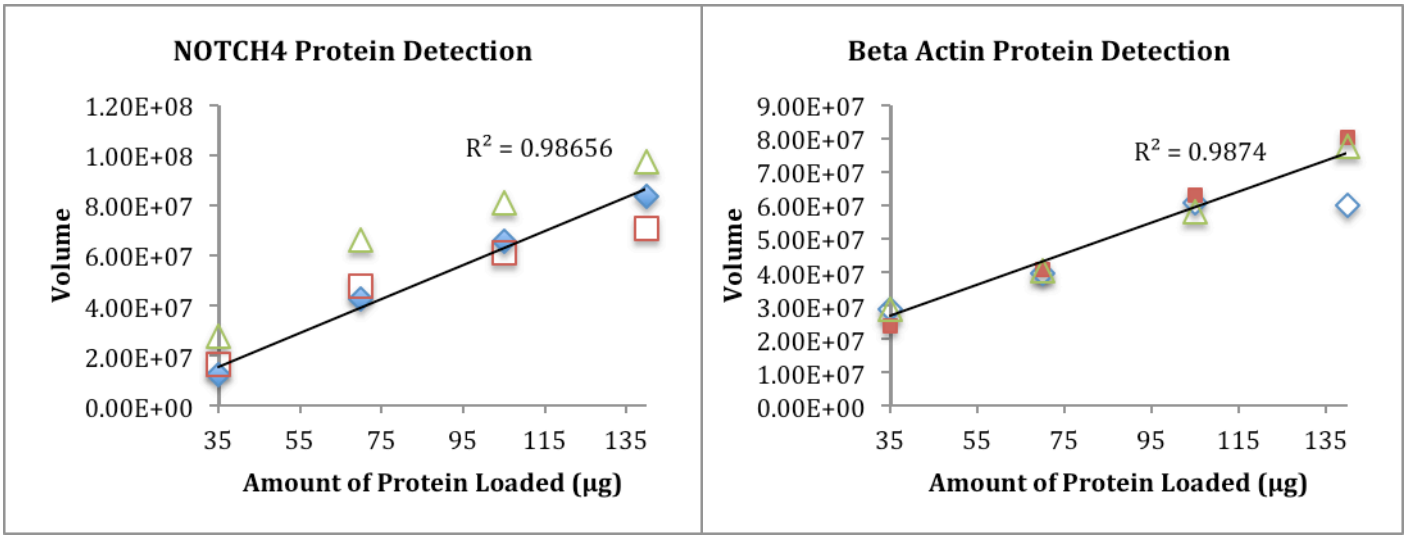


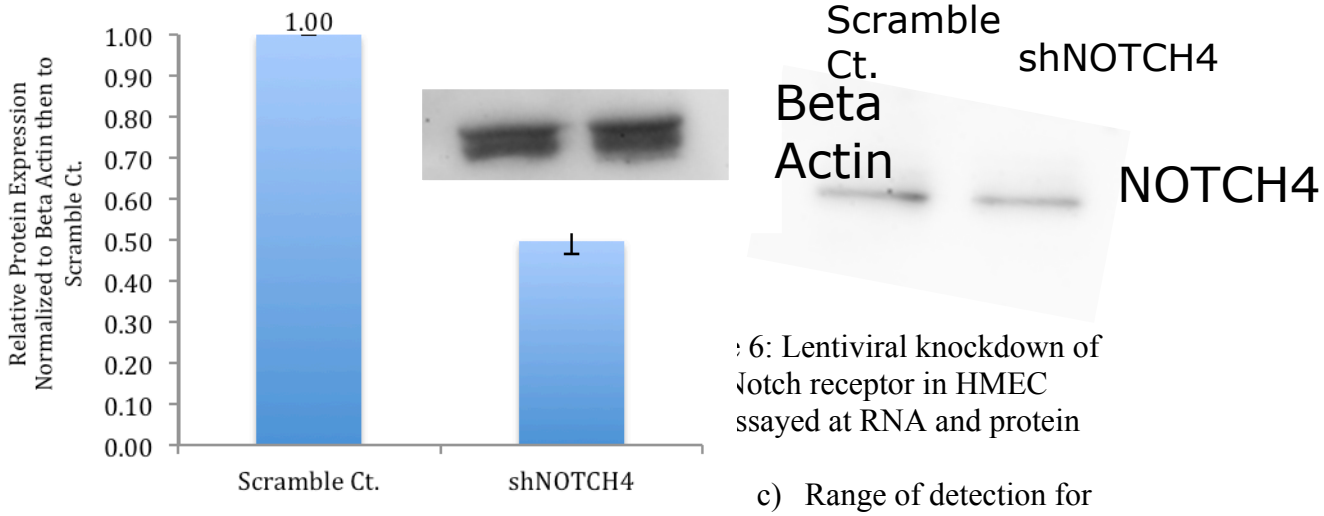
Figure 6: Lentiviral knockdown of each Notch receptor in HMEC assayed at RNA and protein levels

a) Relative expression to GAPDH of each Notch receptor in knock down HMEC compared to Scramble control via qPCR, N=3, ±Standard Deviation
b) Relative protein expression of each Notch receptor in knock down HMEC compared to Scramble control via intracellular FACS, N=3, ±Standard Deviation

(c)



(d)

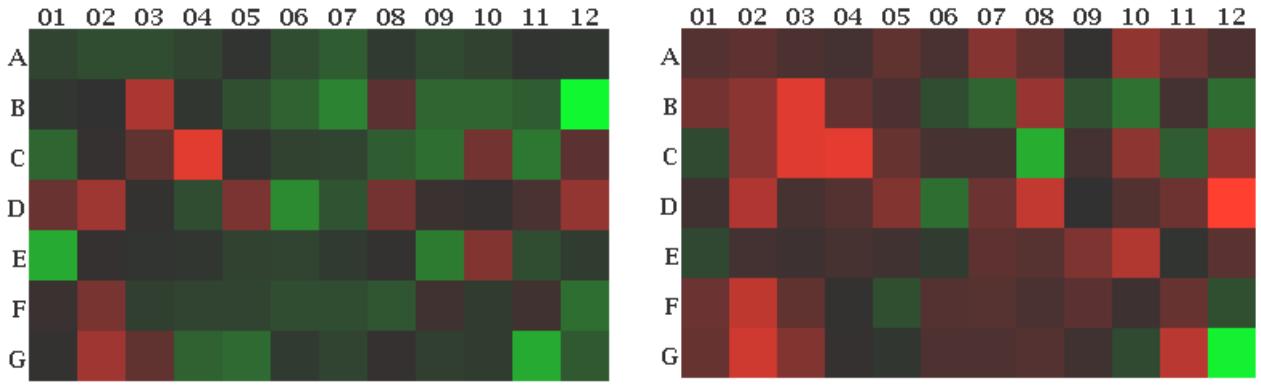


: 6: Lentiviral knockdown of
Notch receptor in HMEC
ssayed at RNA and protein

c) Range of detection for Western Blot analysis using NOTCH4 and ACTIN antibodies for protein quantification, each symbol represents one separate trial, sample blot is a typical blot from one of the trials, N=3.

d) Relative protein expression to ACTIN of NOTCH4 in knock down HMEC compared to Scramble control via Western blot, N=3

(a)



(b)

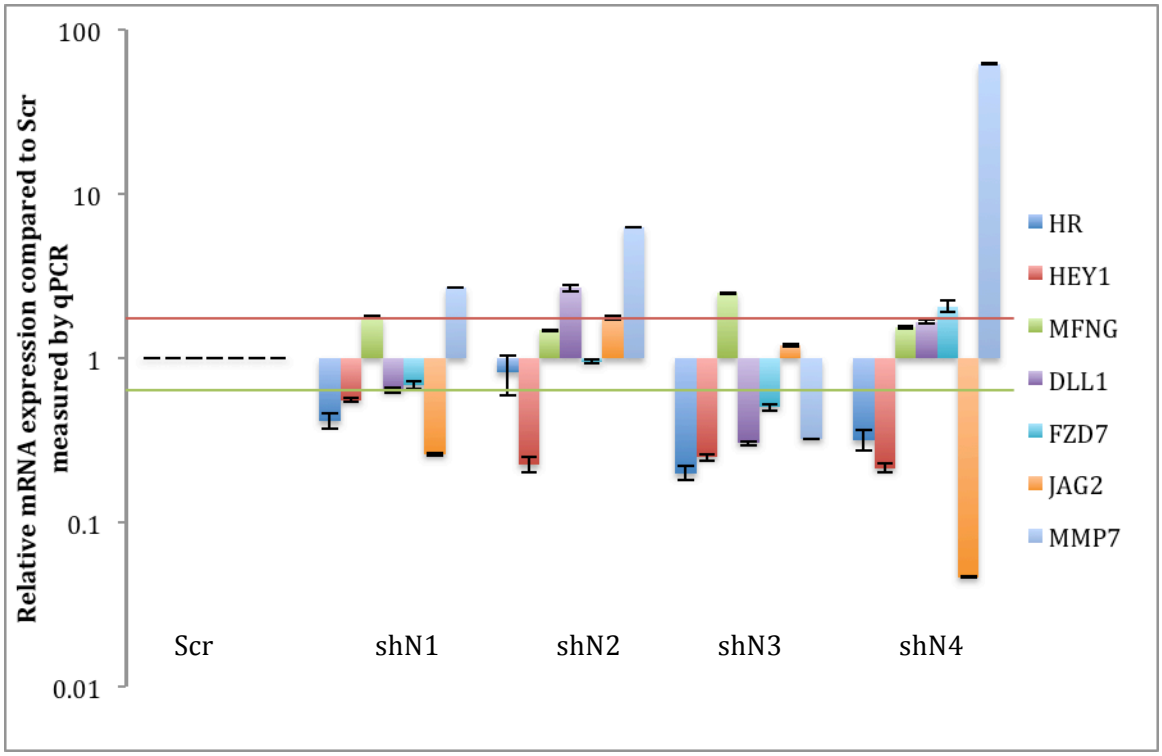
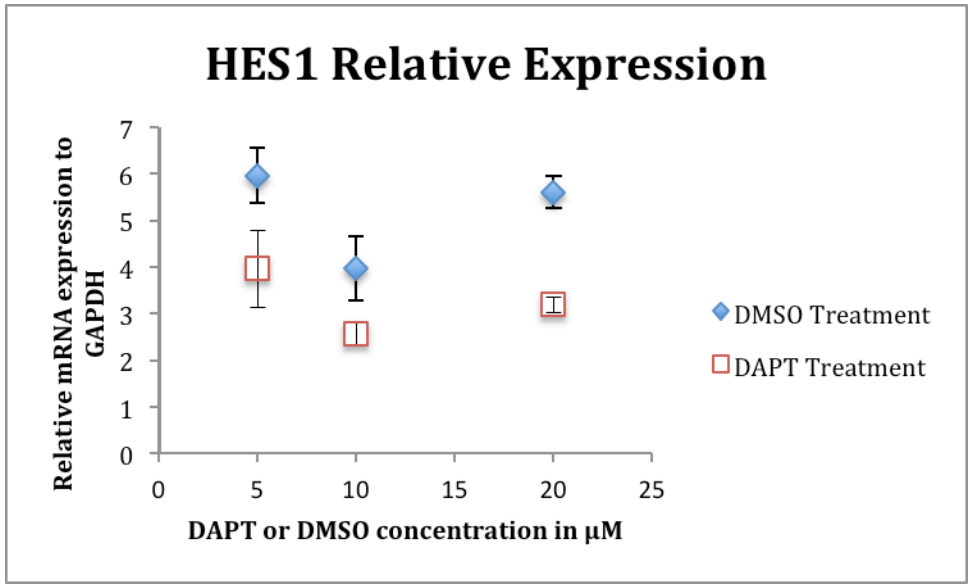


Figure 7: Heat map generated from qPCR array analysis revealed that there are a set of genes specifically down regulated by a) NOTCH3 and NOTCH4 compared to Scramble control. HMEC were infected with shNOTCH3, shNOTCH4 and shScrambleCt, then puromycin-selected prior to qPCR analysis using qPCR array. Each spot represent a gene, red shows the genes which expression levels are increased and green shows the genes which expression levels are decreased, b) Target genes identified on qPCR array were further validated using independently designed primers in shNotch Receptor (shNR) infected HMEC, showing relative mRNA expression compared to scramble control (Scr) normalized to GAPDH. Genes up or down regulated more than 2 folds (red, green lines) are identified as significantly regulated, N=3, ±Standard Deviation

a)



b)

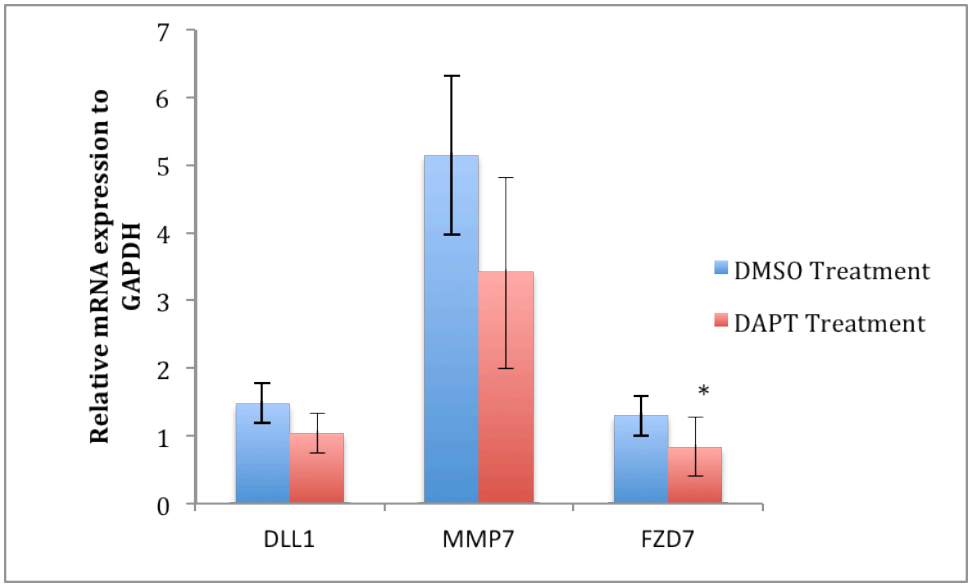


Figure 8: γ-secretase blocker DAPT treated HMEC compared to vehicle control DMSO. Cells were treated for 24hrs.

a) 10µM DAPT or DMSO in normal growth media was determined to be the optimal concentration based on HES1 relative mRNA expression compared to GAPDH at different concentrations, N=3, ±Standard Deviation
b) RNA was collected at the end of treatment, and qPCR was done to determine the relative expression of each gene upon DAPT treatment compared to DMSO vehicle control, N=3, ±Standard Deviation, *$p \leq 0.03$

c)

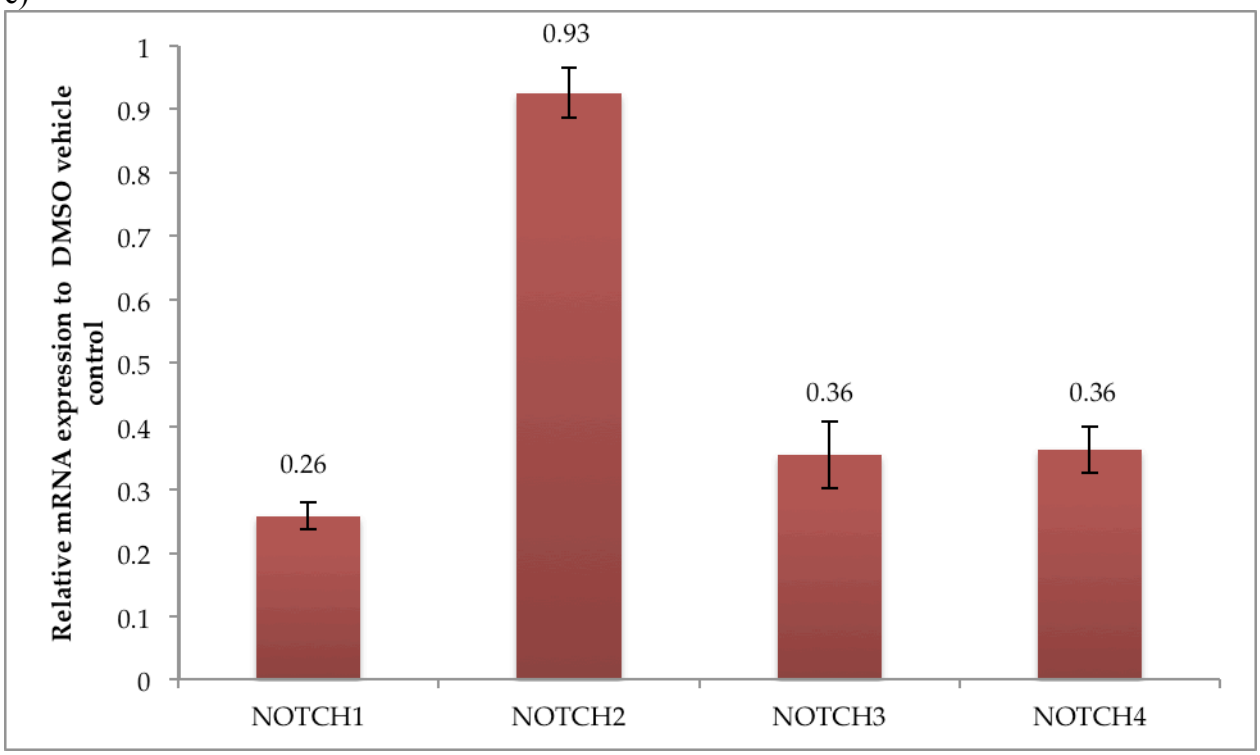
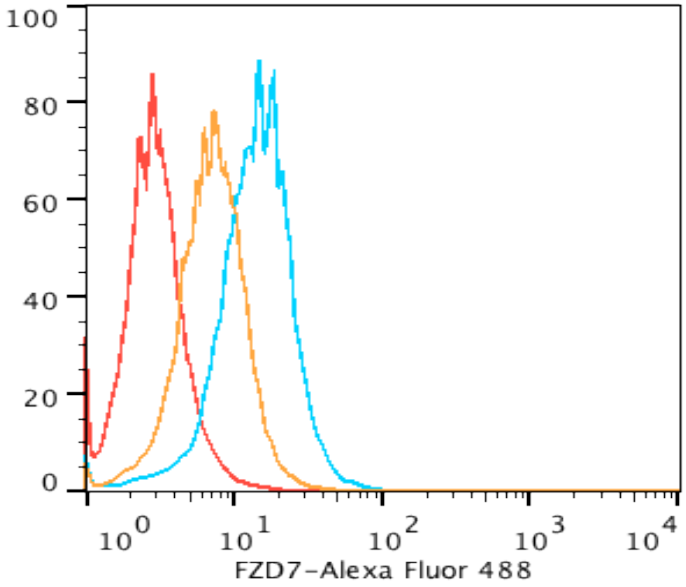


Figure 8: γ-secretase blocker DAPT treated HMEC compared to vehicle control DMSO. Cells were treated for 24hrs.

c) RNA was collected at the end of treatment, and qPCR was done to determine the relative expression of each Notch receptor gene upon DAPT treatment compared to DMSO vehicle control, N=3, ±Standard Deviation

a)



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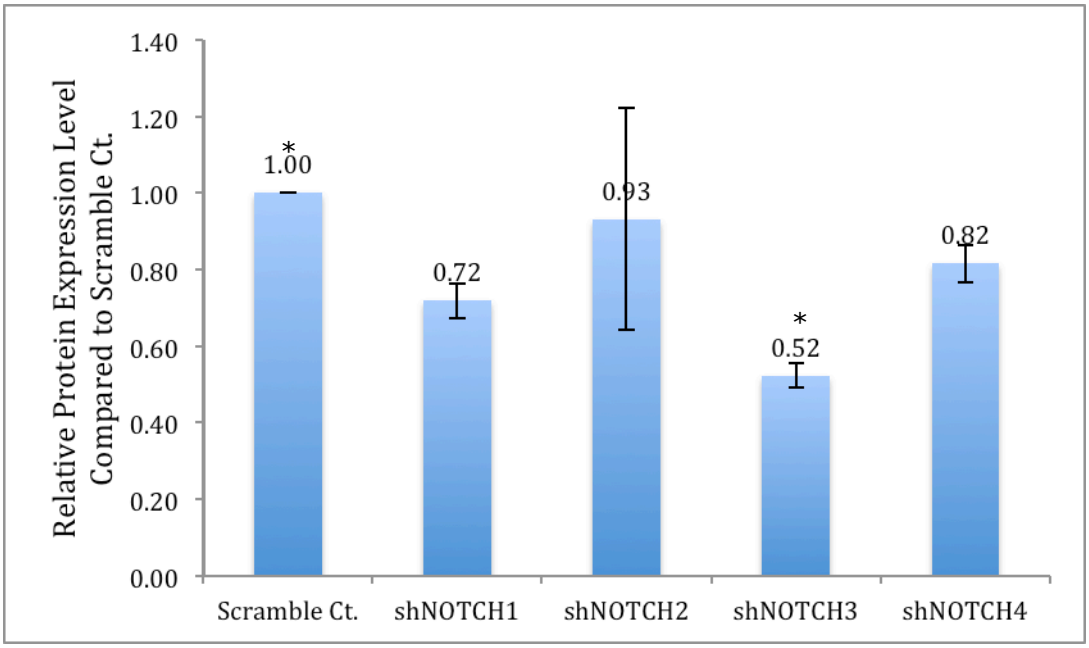


Figure 9: Intracellular FACS analysis showing FZD7 protein levels in Notch receptor knockdown lines. a) FZD7 antibody titration, red – isotype control, yellow – 1/2000 dilution, blue – 1/500 dilution. b) FZD7 protein level decreases in knockdown of NOTCH3, not in knockdown of other Notch receptors, N=3, *p≤0.002, ±Standard Deviation

a)

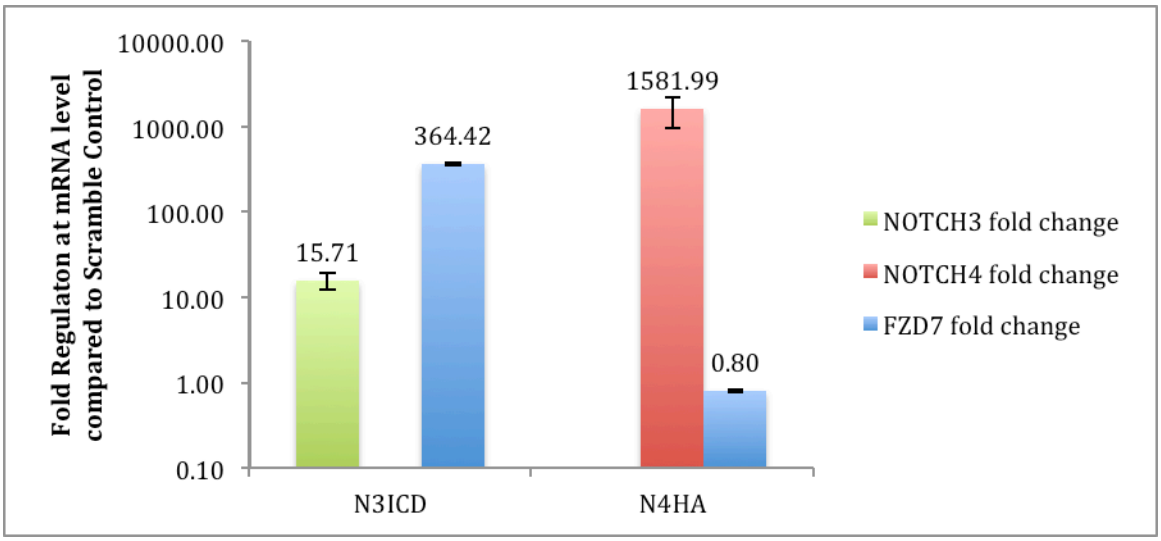
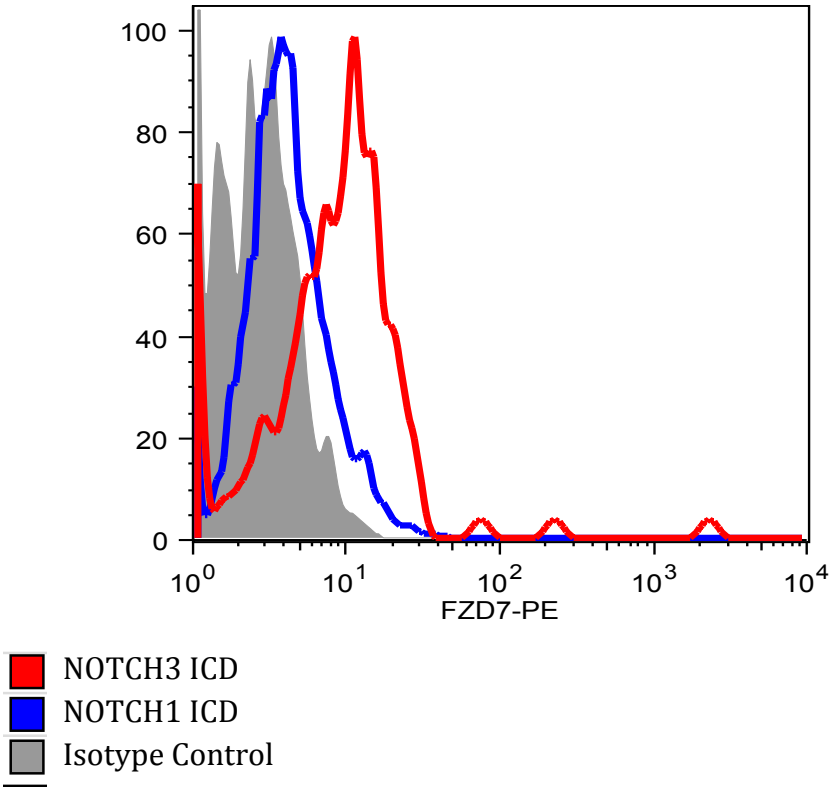


Figure 10: FZD7 mRNA and protein level increase in cells over express N3ICD. a) FZD7 mRNA level increases in cells over expressing N3ICD but not in cells over express active form of N4ICD measured by qPCR in HMEC over expressing the active form of each receptor, N=3, ±Standard Deviation

b)



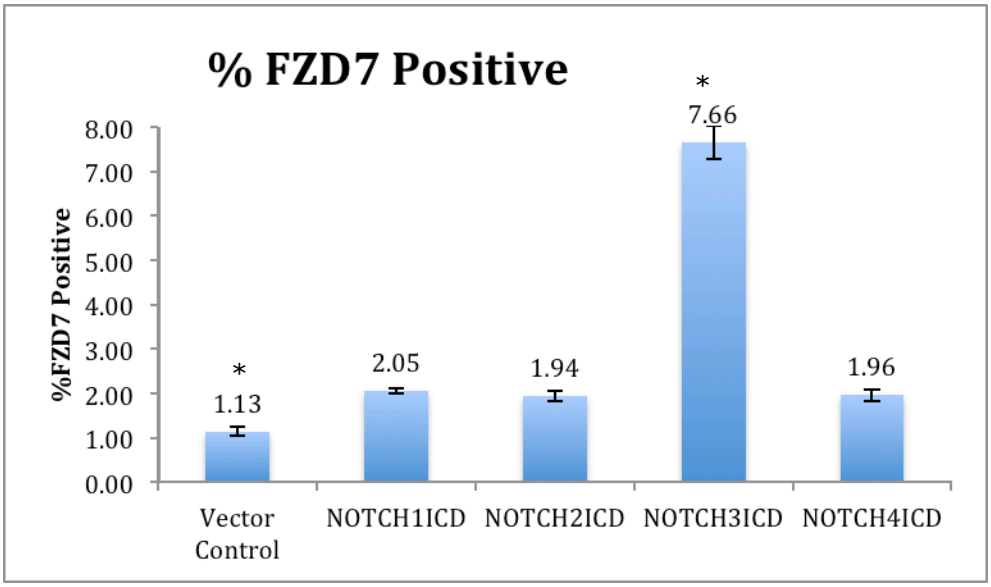


Figure 10: FZD7 RNA and protein level increase in cells over express N3ICD. b) FZD7 protein level increases in cells over express N3ICD but not in cells over express active forms of other Notch receptors N1ICD, N2ICD, N4ICD measured by intracellular FACS in HMEC over expressing the active form of each receptor, N=3, ±Standard Deviation, *p≤0.0008

a)

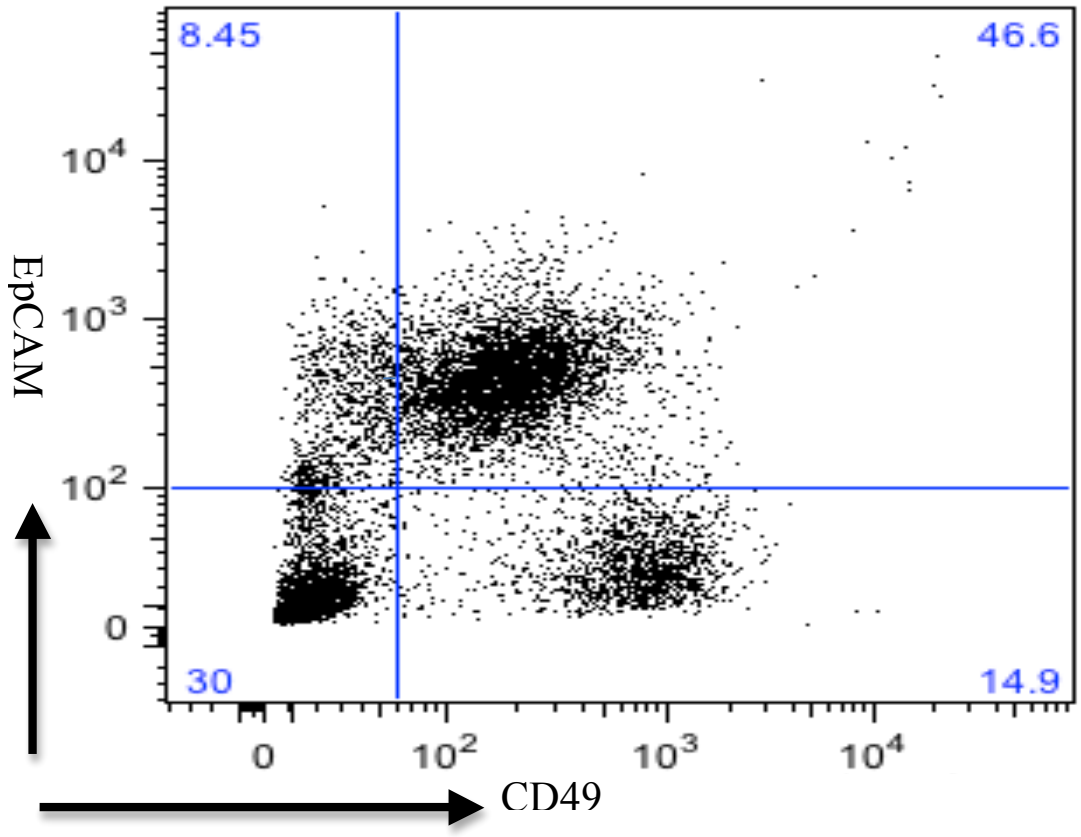


Figure 11: FZD7 has similar expression profile as NOTCH3 in subsets of human mammary epithelial cells at RNA level relative to GAPDH.

a) Sample FACS profile showing non-precultured human mammary epithelial cells can be separated into luminal and bipotent CFC's based on surface marker expression, EpCAM and CD49f

b)

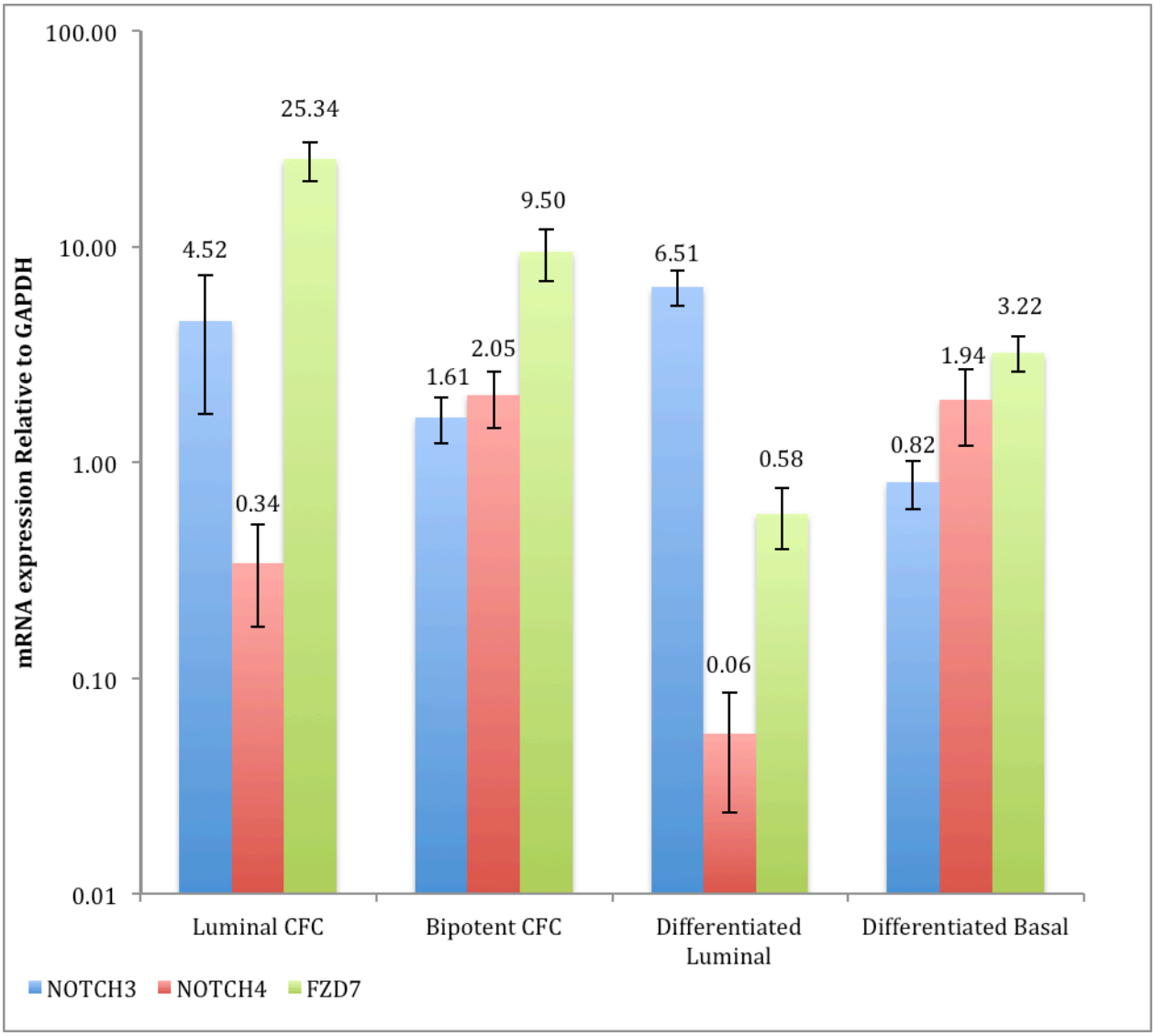


Figure 11: FZD7 has similar expression profile as NOTCH3 in subsets of human mammary epithelial cells at mRNA level relative to GAPDH.

b) FZD7 transcription level is higher in luminal CFC compared to bipotent CFC, N=4 human samples.

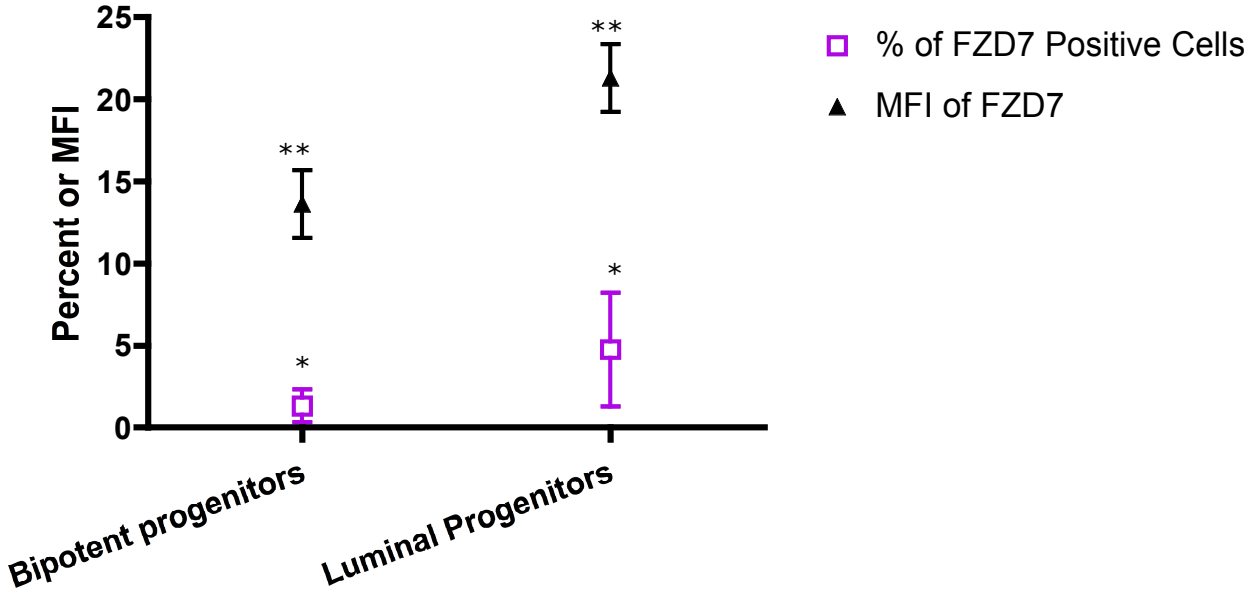


Figure 12: FZD7 has significantly higher expression in the luminal progenitor population compared to bipotent progenitors in non-precultured primary human mammary epithelial cells measured by extracellular FACS, N=3 human samples, ±Standard Deviation.
* p=0.01, **p=0.1

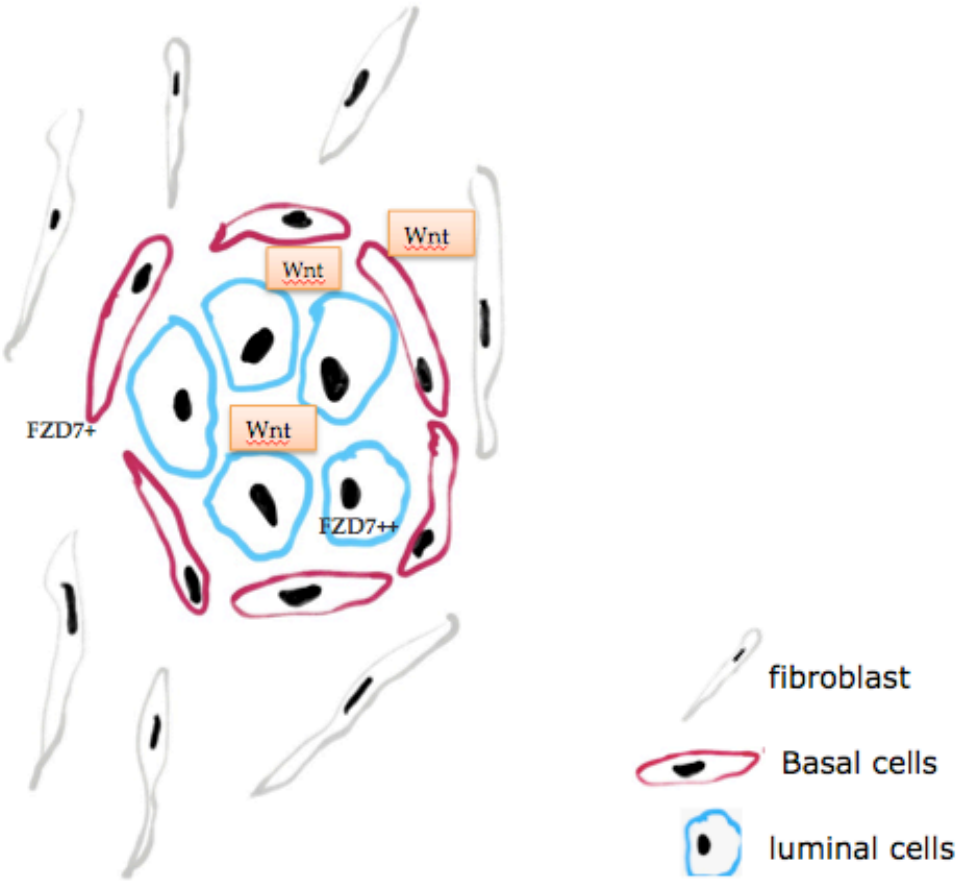


Figure 13: Proposed mechanism of mammary epithelial regulation and maintenance. In the breast epithelial microenvironment, while Wnt ligands are secreted by all types of cells (fibroblast, basal cells, luminal cells), because the luminal cells express enhanced levels of FZD7, these cells are more susceptible to Wnt signaling activation.

VII. Tables

Table 1: List of primer sequences used for quantitative PCR

Gene name – orientation	Accession number	Primer sequence
GAPDH-Forward	NM_002046.4	gcctcccgcttcgctctc
GAPDH-Reverse	NM_002046.4	ccgttgactccgaccttcacc
HES1-Forward	NM_005524.3	ggaagcacctccggaacct
HES1-Reverse	NM_005524.3	ggtcacctcgttcatgcactc
NOTCH1-Forward	NM_017617.3	gcggggctaacaaagatatgc
NOTCH1-Reverse	NM_017617.3	gcaccttggcggtctcgta
NOTCH2-Forward	NM_024408.3	gatgcccaggacaacatgg
NOTCH2-Reverse	NM_024408.3	gactcggttgcgaatcagaa
NOTCH3-Forward	NM_000435.2	cgtggtgtctgccagagtt
NOTCH3-Reverse	NM_000435.2	ctggcagggagcagtcag
NOTCH4-Forward	NM_004557.3	tccccaggaatctgagatgga
NOTCH4-Reverse	NM_004557.3	ggactgtacttccccacagcaaac
JAG2-Forward	NM_002226.4	gctgctggtgttgctttgc
JAG2-Reverse	NM_002226.4	ggctgctgtcaggcaggtc
FZD7-Forward	NM_003507	tctcccatttggatcctttg
FZD7-Reverse	NM_003507	ggacaaaatggctctttgct
KRT1-Forward	NM_006121.3	gcctccttcattgacaaggt
KRT1-Reverse	NM_006121.3	gctcccattttgtttgcagt
MMP7-Forward	NM_002423	ggctttgcgcgaggag
MMP7-Reverse	NM_002423	ggcgcaaaggcatgag
DLL1-Forward	NM_005618	cttccccttcggcttcac
DLL1-Reverse	NM_005618	gggttttctgttgcgaggt
HR-Forward	NM_005144.4	ggacagcatgatgagcagaa
HR-Reverse	NM_005144.4	gcatggtatgtcctgaagtcc
GSK3B-Forward	NM_002093	gacatttcacctcaggagtgc
GSK3B-Reverse	NM_002093	gttagtcgggcagttggtgt
HEY1-Forward	NM_012258.3	cttccatgtccccaactacatc
HEY1-Reverse	NM_012258.3	ctgttattgatccggtctcgtc
MFNG-Forward	NM_001166343.1	ctctttcactcccacctgga
MFNG-Reverse	NM_001166343.1	tcccctcaaagacaccgtag
PPARG-Forward	NM_015869	tccatgctgttatgggtgaa
PPARG-Reverse	NM_015869	tgtgtcaaccatggtcatttc

Table 2: List of primers used for PCR cloning of NOTCH2 and NOTCH3 intracellular domain

Primer name – orientation	Restriction Enzyme Overhang Sequence	Primer sequence
NOTCH2 AscI – Forward	aataggcgcgccg	aaacgaaagcgtaagcatggctct ctc
NOTCH2 PacI – Reverse	aaattaattaa	cgcataaacctgcatgttgttgtgtg
NOTCH3 AscI - Forward	aataggcgcgccgg	tcattctcgtcctgggtgtcat
NOTCH3 PacI - Reverse	aaattaattaa	gagtgttaactattccttttattaggtg gtgagg

Table 3: List of shRNA sequence encoded in Thermo Scientific pGIPZ vectors used for making Lenti-shRNA virus

Gene Name	Mature sense sequence	Mature antisense sequence	Binding region on cDNA
NOTCH2	CAAGAATTGTCAGACA GTA	TACTGTCTGACAATTCT TG	2778-2796
NOTCH2	GAGCACCTGTGAGAGG AAT	ATCCTCTCACAGGTGCT C	1059-1075
NOTCH2	CACATCCTCTCCAATGA TT	AATCATTGGAGAGGAT GTG	6789－6807
NOTCH4	CATGGAACCTGTACTCC CA	TGGGAGTACAGGTTCC ATG	3077－3095
NOTCH4	CTCCCTCCTTCTGTTCC AA	TTGGAACAGAAGGAGG GAG	507-525
NOTCH4	CGCTATTTAAGAACCCT AA	TTAGGGTTCTTAAATAG CG	6691-6709
NOTCH1	CGGCCCAACCCGTGTCA CA	TGTGACACGGGTTGGG CCG	2734－2752
NOTCH1	GGGACCAACTGTGACA TCA	TGATGTCACAGTTGGTC CC	2239-2257
NOTCH1	CGATGCGAGATCGACG TCA	TGACGTCGATCTCGCAT CG	1360-1342
NOTCH1	GGGACCAACTGTGACA TCA	TGATGTCACAGTTGGTC CC	2239-2257
NOTCH3	CCAGTTCACCTGTATCT GT	ACAGATACAGGTGAAC TGG	1429-1447
NOTCH3	CCAATAAGGACATGCA GGA	TCCTGCATGTCCTTATT GG	5964-5982

Table 4: List of antibodies used for western blot, FACS and concentrations of each antibody

Gene	Supplier	Clone Name	Catalog Number	Application	Final Concentration (μg/mL) or dilution
NOTCH1	Bethyl Laboratories	N/A	A301-894A	Western Blot, Intracellular FACS	FACS: 5 Western Blot: 0.4
NOTCH2	Bethyl Laboratories	N/A	A301-083A	Western Blot, Intracellular FACS	FACS: 5 Western Blot: 1
NOTCH3	Cell Signaling	N/A	2889	Western Blot, Intracellular FACS	FACS: 5 Western Blot: 1
NOTCH4	Aviva Systems Biology	N/A	ARP32726	Western Blot, Intracellular FACS	FACS: Western Blot: 0.25
Beta Actin	Sigma-Aldrich	AC-15	A5441	Western Blot	1 in 10000
FZD7	R & D Research	151143	MAB1981	Extracellular FACS	20
FZD7	Abcam	N/A	Ab51049	Intracellular FACS	1
MUCI	Millipore	214D4	05-062	Extracellular FACS	1 in 100
CD49f	BioLegend	GoH3	313610	Extracellular FACS	1 in 100
THY1	BioLegend	5E10	328110	Extracellular FACS	1 in 100
EpCAM	StemCell Technologies	VU-1D9	01420	Extracellular FACS	10
EpCAM-FITC	StemCell Technologies	VU-1D9	10109	Extracellular FACS	1 in 5
Anti-Rabbit HRP	Sigma-Aldrich	N/A	A6154	Western Blot	1 in 10000
Anti-Rat PE	Jackson ImmunoResearch	N/A	112-116-072	Intracellular and extracellular FACS	40
Anti-Rat FITC	Sigma-Aldrich	N/A	F1763	Extracellular FACS	40
Anti-Rabbit PE	Jackson ImmunoResearch	N/A	111-116-144	Intracellular FACS	40
Anti-mouse PE	BioLegend	Poly4053	405307	Extracellular FACS	40

N/A: information not available from supplier

Table 5: List of reagents and supplier information

Reagent	Supplier	Catalog Number	Solute	Stock Concentration
Dulbecco's Modified Eagle Medium (DMEM), high glucose	Invitrogen	12430	NA	NA
DMEM/F-12	Thermo Scientific	SH30126	NA	NA
Fetal bovine serum (FBS)	Invitrogen	12483	NA	NA
Dispase	StemCell Technologies	07913	NA	NA
Adult bovine serum (BS)	Sigma	B9433	NA	NA
L-glutamine 200mM	Invitrogen	25030	NA	NA
Rat IgG	Sigma-Aldrich	14131	PBS	0.25 mg/mL
Phosphate Buffered Saline (PBS)	Thermo Scientific	SH30256	NA	NA
Hank's Balanced Salt Solution (HBSS)	Thermo Scientific	SH30268	NA	NA
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A7979	NA	NA

NA: not applicable

Table 6: ANOVA analysis of Figure 9b – FZD7 protein level decreases in knockdown of Notch receptors

Newman Keuls (Compare all pairs of columns)			
Table Analyzed	Fig 9b		
One-way analysis of variance			
P value	0.0013		
P value summary	**		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	5		
F	7.867		
R square	0.6772		
ANOVA Table	SS	df	MS
Treatment (between columns)	0.5637	4	0.1409
Residual (within columns)	0.2687	15	0.01791
Total	0.8324	19	

Newman-Keuls Multiple Comparison Test	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**
shNOTCH3 vs Scramble ct.	-0.477	7.127	Yes	**
shNOTCH3 vs shNOTCH2	-0.4086	6.106	Yes	**
shNOTCH3 vs shNOTCH4	-0.2924	4.37	Yes	*
shNOTCH3 vs shNOTCH1	-0.1952	2.917	No	ns
shNOTCH1 vs Scramble ct.	-0.2818	4.21	Yes	*
shNOTCH1 vs shNOTCH2	-0.2134	3.189	No	ns
shNOTCH1 vs shNOTCH4	-0.09726	---	No	ns
shNOTCH4 vs Scramble ct.	-0.1845	2.757	No	ns
shNOTCH4 vs shNOTCH3	-0.1161	---	No	ns
shNOTCH2 vs Scramble ct.	-0.06836	---	No	ns

ns: not significant

VIII. References

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