

ABSTRACT

Title of Thesis: **INVESTIGATING THE SPATIOTEMPORAL
WINDOW OF LINEAGE SPECIFICATION
DURING EARLY MAMMARY GLAND
DEVELOPMENT.**

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The mammary gland is derived from an epidermal placode that receives reciprocal induction from the underlying mesenchyme. The mammary mesenchyme is crucial for the specification of the mammary gland. Studies have reported that co-culture of non-mammary epithelial cells (for instance, salivary gland¹) with mammary mesenchyme/fat pad cells specify the epithelial cells into mammary epithelial cells. Even though mammary mesenchyme signaling is required for proper mammary specification and early development up to E14.5, subsequent growth and maintenance beyond E14.5 occurs independently of mesenchymal signaling. *In vitro* culture of mammary epithelium alone after E14.5 can give rise to all mammary cell types. Sexual dimorphism of the mouse mammary gland also begins around E14.5. The generation of an autonomous, transient mammary stem cell niche and new response to androgen signaling at E14.5 indicates a major developmental stage in the fetal mouse mammary gland. Therefore, we aim to understand the gene

networks expressed within fetal mouse mammary epithelial cells as they transition through this period. We also aim to identify different cell types within mammary epithelium and mammary mesenchyme. The mammary epithelial cells are not committed to either luminal or basal lineages until birth², although mammary epithelial cells of three different hybrid states of specification (luminal-like, basal-like, and hybrid cells) have been reported to present at E15.5^{2,3}. To obtain a more detailed gene expression map of early mammary lineage regulators, we performed multiplexed RNA-FISH on mouse mammary tissues from E13.5 to E15.5 (n=3 for each stage). We observed asymmetric expression patterns of *Notch1* (luminal specifier), *Lef1* (mammary mesenchymal marker), and *Pthlh* (basal specifier) between the peripheral and center cells of the mammary rudiments at E14.5 and E15.5, which suggests that the mammary epithelium is primed for lineage specification by E14.5. We observed the low expression of *Snail2* from E13.5-E15.5 where it was expressed both in the mammary epithelium and the mammary mesenchyme. We also observed the expression of *Sox10* as early as E13.5 which has been reported to not be expressed until E18.5. Our RNA-FISH data showed that *Sox9* and *Gata3* are exclusively expressed in the mammary rudiments, while *Lmo4* and *Sox10* were found to be expressed at similar levels in the mammary rudiments and the surrounding mesenchyme. The mammary epithelial specifier *Sox11* was observed to be upregulated at E15.5 as compared to E14.5 and E13.5. Qualitative analyses for *Notch1* and *Pthlh* suggest there are varied hybrid states of specification in the mouse mammary rudiments by E13.5. ScRNA-seq is an unbiased approach that can be used to identify cells at different hybrid states of specification. The collection of sufficient cells via the dissociation of tissues is a prerequisite of scRNA-seq. We optimized the previously published protocol for single-cell dissociation^{2,3} to collect cells from the mammary epithelium and mammary mesenchyme from both male and female embryos at E14.5. Our results and method optimizations serve as pioneering steps towards

performing quantitative, multiplexed gene expression analyses by coupling spatial techniques with single-cell-RNA-seq data. Together, our findings contribute towards the construction of an embryonic mouse mammary gene expression atlas as well as provide insight into fetal mammary reprogramming and dysfunction.

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by

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LIST OF ABBREVIATIONS

RNA	Ribonucleic acid
scRNA-Seq	Single-cell RNA sequencing
RNA-FISH	RNA fluorescent <i>in situ</i> hybridization
mRNA	messenger RNA
P0	Pups of zero days old
E13.5	Embryonic day 13.5
E14.5	Embryonic day 14.5
E15.5	Embryonic day 15.5
E18.5	Embryonic day 18.5
FBS	Fetal bovine serum
fMaSC	Fetal mammary stem cells
AR	Androgen receptor
Krt 8	Cytokeratin 8
Krt18	Cytokeratin 18
Tbx3	T-box transcription factor 3
Lef1	Lymphoid enhancer- binding factor 1
Pthlh	Parathyroid hormone like hormone
Gata3	GATA-binding protein 3
Sox9	SRY-box containing gene 9
Sox10	SRY-box containing gene 10
Sox11	SRY-box containing gene 11
Elf5	E74-like factor 5
Lmo4	LIM domain transcription factor 4
Trp63	Transformation related protein 63
RT-PCR	Reverse transcription Polymerase Chain Reaction
DEPC	Diethyl Pyrocarbonate
OCT	Optimal Cutting Temperature

CHAPTER 1: INTRODUCTION

Mother's milk is crucial for the initial stages of neonatal life. Milk is the source of nutrition to newborns, and the mother also passes antibodies to her offspring whose immune systems are not immunocompetent to fight disease. Not all women are able to produce enough milk postpartum, and there could be various causes including congenital mammary defects. As mammary gland development starts *in utero*, issues occurring during lactation may be rooted in abnormal fetal mammary gland development. Fetuses are susceptible to both positive and negative influences from the maternal environment. Poor maternal nutrition and hormonal imbalance can affect fetal mammary gland development. In order to determine if adult mammary complications arise from problems originating during fetal development, it is imperative to understand how gene regulatory networks direct fetal mouse mammary organogenesis. Identifying the unique cell types within the fetal mammary gland, and characterizing their spatiotemporal gene expression patterns, can help to identify possible fetal mammary development issues. Our study aims to accomplish this objective by using innovative technologies like RNA Fluorescent *in situ* hybridization (RNA-FISH) and single-cell RNA sequencing (scRNA-seq).

The mouse is a relevant mammalian model to study human mammary gland development because of its genetic similarity. Mutations of many genes involved with human mammary gland development (*TBX3*, *PTHLH*, *EDA*) are associated with congenital breast defects, and mouse knockout models for these genes also exhibit impaired mammary development⁴. Among all the pairs, the third mammary gland was found to be most affected⁴. It suggests that the third pair in mice more resembles the human mammary gland. Mammary gland development in mice starts around E10.5 as a mammary line progressing towards placode formation around E12.5, mammary

buds around E13.5, and rudimentary mammary tree with primary and few secondary branches at E18.5. The female mammary rudiment resumes its development after mouse reaches the pubertal stage⁵.

The mammary gland consists of mammary epithelium and mammary mesenchyme. The known lineages of mammary epithelium are luminal and basal lineages. Mammary mesenchyme is comprised of primary mesenchyme and fat pad (also referred as secondary mesenchyme). Giraddi *et al.* 2018 reported that the mammary epithelium is segregated into luminal and basal lineages by postnatal day 4 (P4) but does not initiate until E18.5². Carabana *et al.* 2024 investigated earlier stages of development and observed different cell types in the mammary gland. They identified a single cell type within mammary epithelium at E13.5 and E14.5. At E15.5, three cell types were identified: luminal-primed cells, basal-primed cells, and hybrid cells expressing both luminal and basal cell markers³. Interestingly, all three cell types expressed both luminal markers (cytokeratins (Krt) 8 and 18) and basal markers (Krt5, Trp63). At P0, three different cell types were found: basal progenitor cells, luminal progenitor cells, and mature luminal cells. Carabana and colleagues also identified three distinct types of mammary mesenchyme: sub-epithelial, dermal, and proliferative mesenchyme cells at E13.5 and E14.5, while cells at P0 were Pi16⁺ fibroblasts, Col15a1⁺ fibroblasts and Eln⁺ fibroblasts³. A separate lineage tracing study revealed the presence of multipotent Notch1⁺ cells until E13.5, but then luminal-fated cells by E15.5, suggesting lineage segregation by E15.5 and the possibility of priming of mammary epithelial cells to differentiate around E14.5⁶. It was pointed out that the reason behind the different observations between Carabana and Giraddi findings could be due to differences in scRNA-seq number of reads and number of cells, variation in gene markers used for FACS sorting, differences in strains of mouse used, and the ways the data were analyzed. Both the research groups used

inbred yet different substrains of C57B6 mice. In contrast, we performed our study with outbred CD-1 mice. We performed RNA-FISH using three sets of four multiplexed probes, versus the previously published study using only two FISH probes. With this increase, we expect to better define the spatiotemporal window of specification. Furthermore, our study provides additional developmental context by investigating female and male mammary rudiments.

Paracrine signaling from mammary mesenchyme is crucial for mammary epithelium development. A study was performed where mammary rudiments from various embryonic stages were *in vitro* cultured in Matrigel without mesenchyme. It was found out that mammary rudiments from E15.5 can undergo branching and development during ex-vivo culture on Matrigel without mesenchyme while the mammary rudiment culture prior to E15.5 required mammary mesenchyme co-culture¹. This suggests that after E14.5, mammary rudiments start to lose dependency on the mammary mesenchyme. It would be interesting to study genetic changes that happen in the mammary epithelium during this transition from mammary mesenchyme dependence to independence. We aim to define the spatiotemporal gene expression profiles of mammary epithelial cells in the mouse embryonic mammary rudiment from E13.5 to E15.5.

The sexual dimorphism of mammary gland also starts by E14.5. The mesenchyme surrounding the mammary placodes differentiates into mammary mesenchyme around E11.5-E12.5. The mammary mesenchyme begins to express androgen receptors. Around E12.5, the male fetal testes start making testosterone. The testosterone activates the androgen receptors leading to the condensation of mammary mesenchyme surrounding the mammary bud which becomes apparent by E14.5. The condensed mammary mesenchyme compresses the mammary bud stalk and causes detachment from the epidermis. Hence the mammary glands degenerate in male mice⁷.

Since female fetuses do not produce testosterone, their mammary gland continues development⁸ . The exposure of male embryonic mammary rudiments and mammary mesenchyme to the testosterone should cause deviation in some gene networks and variation in the cells at different hybrid states of specification in comparison to the female embryonic mammary tissue. It has been reported that in male mammary tissue, Pthlh signaling from the mammary epithelium suppresses wnt signaling to the mammary mesenchyme due to its regulation of Lef1. Lef1 expression gets upregulated in the mammary epithelium and downregulates in the mammary mesenchyme which is opposite to normal mammary gland development in females. Hence, this alternate expression of Lef1 leads to the degeneration of the mammary rudiments in male. Beside this mechanism, there could be proteases involved which aid in detaching the male mammary rudiments. During the degeneration of male mammary rudiments, there could be apoptosis of the mammary epithelial cells, leading to different hybrid cell types in the male mammary epithelium at E14.5. Condensed male mammary mesenchyme could also have some genetic differences versus female mammary mesenchyme at the same embryonic stage. Single-cell RNA-sequencing (scRNA-seq) is an advance technique that can be used to observe the hybrid cell types of the male and female mammary epithelium and the mammary mesenchyme. It may identify proteases involved in the degeneration of the male mammary epithelium, indicating the degree of degeneration/apoptosis of the male mammary epithelium at E14.5. ScRNA-seq data depends on various factors including number of cells sequenced/read. We aim to optimize protocols to come up with a single protocol that can be used for both mammary epithelium and mammary mesenchyme dissociation male and female embryos at E14.5.

Because E14.5 is one of the major transition stages in the mammary gland development, we seek to understand the changes in gene expression from E13.5 to E15.5. Our study will help to

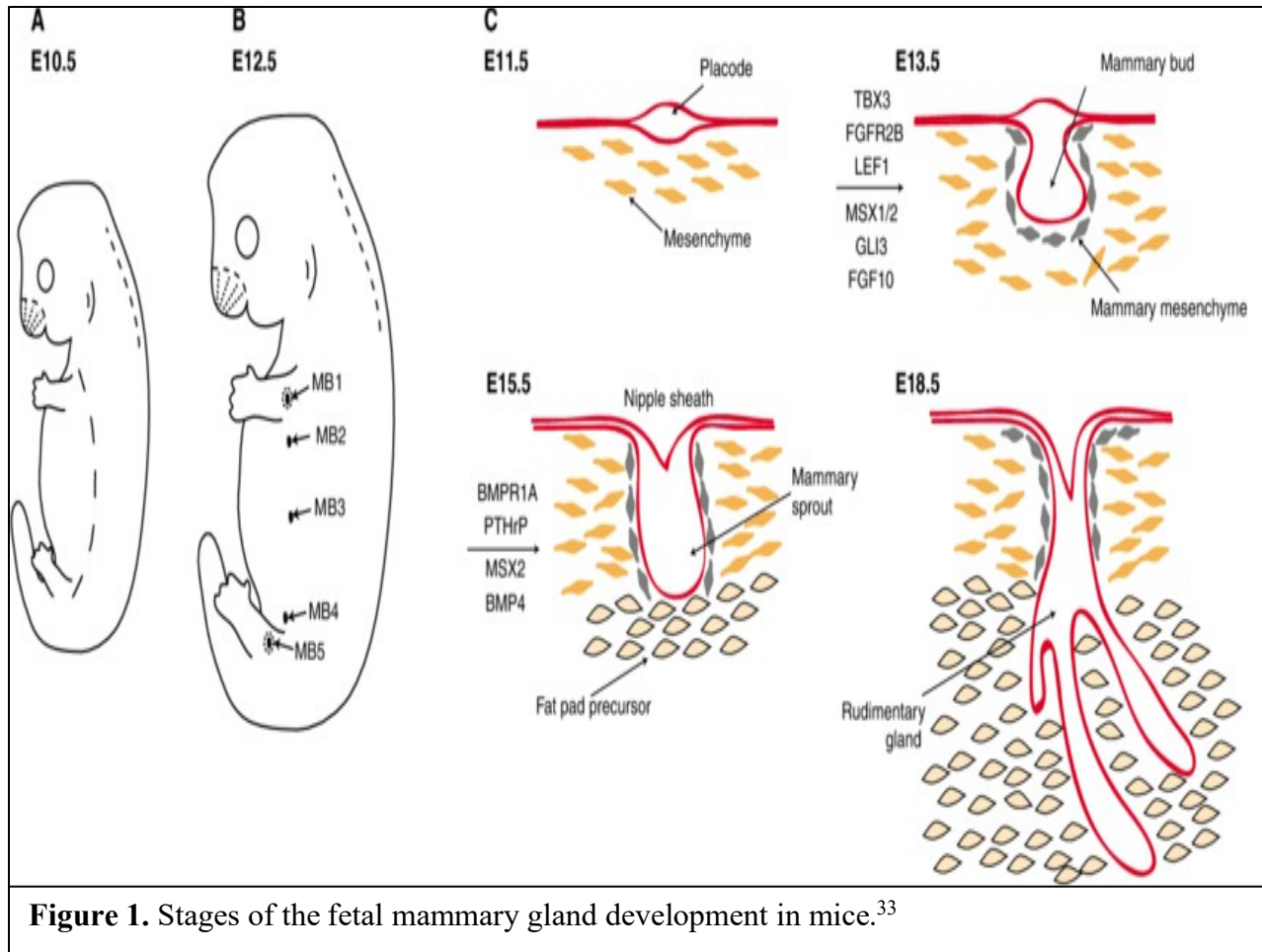
identify unique gene expression signatures in the mammary epithelium and mammary mesenchyme of male and female fetuses, determine the stage at which segregation of fetal mouse mammary epithelium occurs, and lastly determine the spatial gene expression pattern of different cell types in the mammary gland. We aim to integrate these data into a gene atlas of fetal mouse mammary gland development.

CHAPTER 2: LITERATURE REVIEW

Class Mammalia of Kingdom Animalia possesses specific features including the presence of mammary glands and hairs on the body. Mammals give birth and the first source of nutrition for newborns is mother's milk. Interestingly, milk not only functions to fulfill the offspring's nutritional requirement but also protects newborns from various fatal diseases via antibody-containing colostrum. Unfortunately, not every mother can lactate after childbirth. Formula milk can act as a replacement; however, it might not be accessible in low socioeconomic communities. The protein, fat, and minerals in cow milk may not be suitable for newborn infants. According to the report of Centers for Disease Control and Prevention 2022, children fed with cow's milk before 12 months of age are at greater risk of developing iron deficiency anemia as cow's milk is low in iron as its composition of high casein and calcium has been reported to reduce iron absorption from the gastrointestinal tract⁹.

Mammary gland biology is an extensively studied field, but most of the research has centered on postnatal mammary gland development and breast cancer. Little is known about prenatal mammary gland development despite it being a fascinating process. The development of the mouse mammary gland starts in the embryo at E10.5 (Figure 1A). At birth, they are present as mammary rudiments. Due to hormonal regulation, there is extensive morphogenesis after puberty, and during pregnancy to prepare for lactation, milk let down, and breastfeeding. Their complexity lies in the fact that mammary glands are continuously transforming with various stages of life. The complex process of mammary gland development is regulated by various genetic and environmental factors, including hormones, growth factors, and interactions between epithelial and stromal cells.

Stages of fetal mammary gland development in mice.



Mice are an excellent research model to study mammary gland development because of their genetic similarities in human mammary networks and developmental staging, cost, and multiple pups per litter. The developmental phases of mice mammary gland have been summarized in Figure 1. Mice develop five pairs of rudiments, and each pair is specified in a unique manner that may resemble mammary development from other mammals. For example, the third pair most closely resembles human mammary development because of positioning and conservation of key regulatory genes (*Tbx3*, *Lef1*, *Trp63*, *Gata3*)⁴.

Table1: List of known mammary genes that regulate embryonic mouse mammary development.

Genes	Roles
Lef1	Mammary placode marker, mammary epithelium-mesenchyme interaction
Sox11	Embryonic mammary cells stemness and mammary progenitor plasticity
Tbx3	Mammary placode marker, mammary gland position determinant
Sox9	Early embryonic mammary epithelial specifier and luminal marker
Notch1	Luminal marker
Gata3	Luminal marker
Lmo4	Lobuloalveolar marker
Elf5	Luminal marker, prevents mammary epithelium-to-mesenchymal transition
Sox10	Early embryonic mammary epithelium stemness, postnatal luminal marker, mammary sprout branching, mammary epithelium-mesenchymal interaction
Snail2	Basal marker, mammary epithelium-to-mesenchymal transition
Pthlh	Mammary epithelium-mesenchyme mediator, early mammary tissue regulator, androgen-androgen receptor interaction
Trp63	Basal marker

Mammary gland development in mice is also divided into multiple stages: prenatal, prepubertal, post puberty, pregnancy, and lactation. During embryonic development at embryonic day 10.5 (E10.5), mammary lines start to appear ventrolaterally and extend antero-posteriorly from forelimbs to hind limbs (Figure 1A)⁵. They are ectodermal ridges with characteristic expression of *Wnt10b*¹⁰. At E11.5, the cuboidal cells along the mammary lines start to transform into columnar cells and migrate to form multilayering structures called mammary placodes (Figure 1C).

By E12, all five pairs of mammary placodes develop in asynchronous order (Figure 1B) ⁵. Placodes 3 are the first pair that develop followed by placodes 4. After that, placodes 1 and 5 appear around same time and placodes 2 arise last¹¹. The exact mechanism behind this asynchronous order of mammary placode formation is unknown. It has been reported that *Fgf10*, *Wnt* and *Shh* are involved in the development of mammary placodes¹². Studies have found that *Shh* signal in the mammary line is suppressed and *Wnt* signaling is induced for the formation of

the mammary placodes¹². Various clues might be responsible for variation in timing of Shh suppression and Wnt signal initiation in the different mammary line leading to this asymmetrical formation of the mammary placodes¹². Initially, placodes are raised structures of ectoderm. They eventually invaginate to form bulb-like structure known as mammary buds by E13.5 (Figure 1C). Meanwhile, the dermal mesenchyme adjacent to the mammary buds condenses and differentiates into primary or dense mammary mesenchyme. By E14.5, the subdermal mesenchyme underneath the primary mammary mesenchyme differentiates into fat pad precursors (also called secondary mammary mesenchyme). During E15.5, the mammary epithelial cells within the mammary buds proliferate and give rise to mammary sprouts that elongate distally into the primary mammary mesenchyme (Figure 1C). By E16.5, the mammary sprouts invade the secondary mammary mesenchyme and start to bifurcate. Branching morphogenesis, including secondary and tertiary branching, occurs within the rudimentary mammary ductal system by E18.5 (Figure 1C). The mammary buds from the second and third pairs have the most branches while the fifth pairs have the least. Alongside branching morphogenesis, the canalization of mammary epithelium and differentiation of skin around the stalk of mammary sprouts into nipple occur⁵. By birth, nearly all fetal mammary stem cells (fMaSCs) differentiate and give rise to mammary luminal and basal lineage progenitors¹³.

Mammary epithelium-mammary mesenchyme interaction.

Prior to lineage segregation, mammary glands are comprised of two types of tissues: mammary epithelium and mammary mesenchyme. Mammary epithelium will first segregate into two lineages: luminal and basal. The luminal lineage forms mammary ductal and luminal epithelial cells that differentiate into alveolar cells (milk secreting cells) just prior to lactation whereas the

basal lineage forms the myoepithelium that is responsible for milk let down from the alveoli to the gland cistern or mammary opening (nipple or teat). Mammary mesenchyme is also very crucial for the development of mammary gland. Reciprocal signaling between mammary mesenchyme and mammary epithelium determines the fate of mammary gland. It has been reported that the mouse mammary mesenchyme has a capacity to convert their salivary gland into mammary epithelium which highlights the importance of mammary mesenchyme¹⁴. On the other hand, mammary epithelium induces mesenchymal maturation and condensation⁵. A study done by combining skin epithelium from E13 rat or mouse with mammary mesenchyme from E13 mouse and grafting them together resulted in the transformation of skin to mammary duct-like structures¹⁵. However, grafting of mammary epithelium and salivary mesenchyme together in a host produced only salivary gland-like structures. Studies have also found that mammary epithelium depends on mammary mesenchyme for its differentiation and specification until E13-E14.5¹⁶. After that stage, the mammary epithelium gives rise to entire mammary gland irrespective of the presence or absence of mammary mesenchyme³.

Sexual dimorphism on fetal mammary gland development.

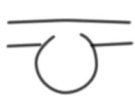
In vivo development of mammary rudiments						
Days of development	11	12	13	14	15	16
Female Embryos						
Male Embryos						

Figure 2: Sexual dimorphism of mouse mammary tissue becomes apparent by E14.5⁵⁹.

In humans, mammary gland development between males and females are similar until puberty. At puberty, there is surge in estrogen in females which leads to extensive development of female mammary gland with more branching of mammary ducts and lobuloalveolar initiation. Interestingly, the sexual dimorphism in the mouse mammary gland starts prenatally. The mammary gland development in both male and female mice is similar until E14. To prove the role of testosterone in male for the dimorphism in the mammary gland development between male and female mice, *in vitro* co-cultures of testes were explanted together with mammary rudiments from E14⁸. Within 18 hours of culture, condensation of mammary mesenchyme surrounding mammary epithelium started and was followed by detachment of mammary epithelium from epidermis. By 45 to 60 hours, there was complete regression of mammary rudiment¹⁷. Hence, testosterone production after E14 in male mice is responsible for the regression of the fetal male mouse mammary gland. Since fetal female mice do not produce testosterone, the mammary buds continue to develop. It has been demonstrated that in male mice, parathyroid hormone-like hormone (*Pthlh*) expressed from mammary epithelium binds to its receptor in mammary mesenchyme disrupting *Wnt* signaling pathway⁸. As a result, the programmed upregulation of *Lef1* in mammary mesenchyme and downregulation in mammary epithelium and epidermis is disrupted. Because of this imbalance, the development of mammary gland in male mice discontinues while there is undisturbed continuous crosstalk between the mammary mesenchyme and mammary epithelium in female fetal mice⁸. Around E11.5-E12.5, the mesenchyme surrounding mammary placode differentiates into mammary mesenchyme which contains activated androgen receptors (AR). The Leydig cells of fetal male mouse start to make testosterone around E12.5-E13¹⁸. As the testosterone level surges at E14.5, the mammary mesenchyme condenses around mammary

epithelium severing it from epidermis which degenerates. In CD-1 male mouse, the remnant of mammary epithelium remains throughout its life⁷.

Differentiation of fetal mammary stem cells.

Gene set enrichment analysis done on fMaSCs, adult basal cells, and adult luminal cells found that fMaSCs have more enriched basal cells markers compared to luminal cells markers (Figure 3). At E14, fMaSCs expressing both luminal and basal markers are present¹³. However, scRNA-Seq analysis found that *Sox10* is expressed in mammary cells at E18.5 but not in fMaSCs at E14¹³. This suggests that a homogenous population of multipotent mammary cells at E14 exists prior to lineage segregation. Immunohistochemistry was performed on mammary rudiments at E14 and E17 using basal markers cytokeratin 14 (Krt14) and Trp63 and luminal markers cytokeratin 8 (Krt8). At E14, all cells in the mammary bud expressed basal and luminal marker proteins with lower expression of Krt8 on the outer layer of the gland. At E17, cells on the inner side have higher Krt8 and no Trp63 expression while cells on the outer side have lower Krt8 but have Trp63 expression. Sox 9 is expressed in both groups of cells. Trp63 has been found to still be expressed in multipotent cells switching to unipotent basal cells. Overexpression of Trp63 in adult luminal cells switch the fate of cells to basal. Regulation of Trp63 expression and its targets are unknown¹³.

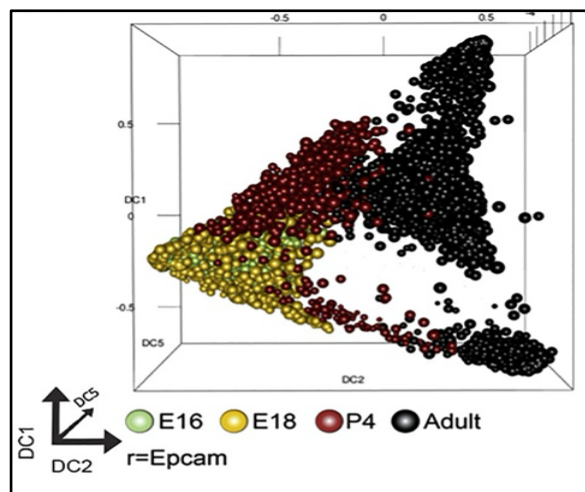
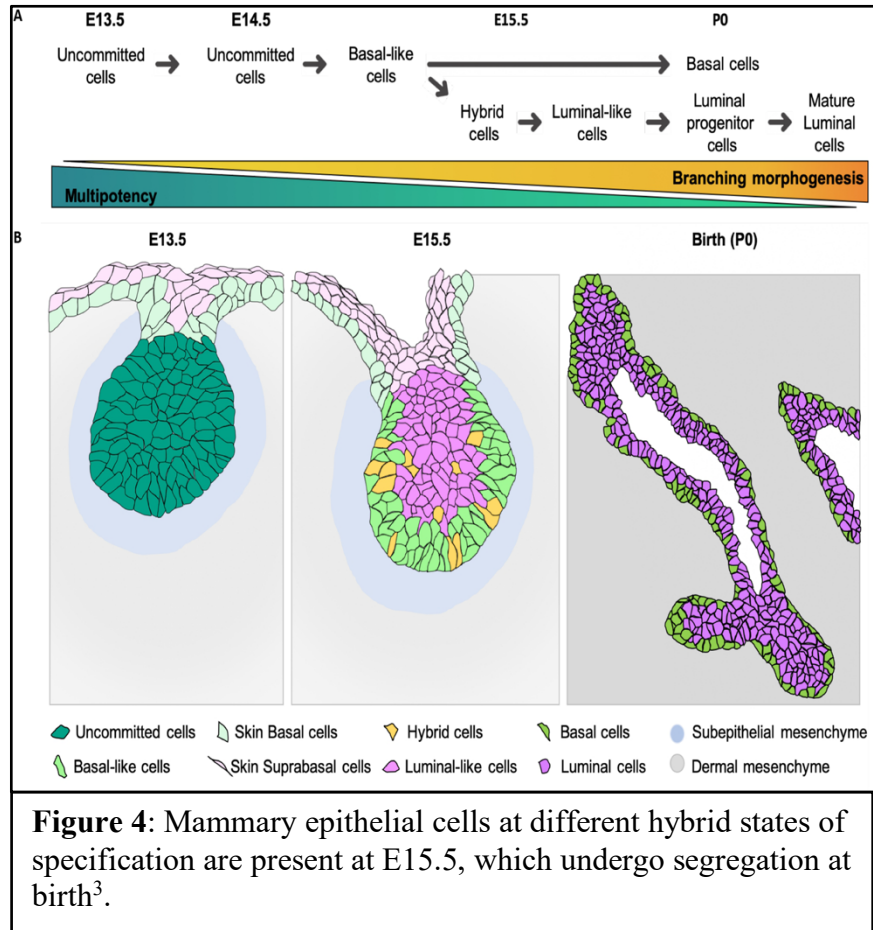


Figure 3: Diffusion graph of mouse mammary epithelial cell differentiation⁵³.

The fMaSC population numbers are few at early embryonic stages but increase as the embryo develops¹⁹. Numbers increase 9-fold from before E15.5 to E16.5 and 200-fold by E18.5. Stromal signaling has been suggested to be required for this increase in cell division. As embryos grow beyond E18 and pups are born, there is a reduction

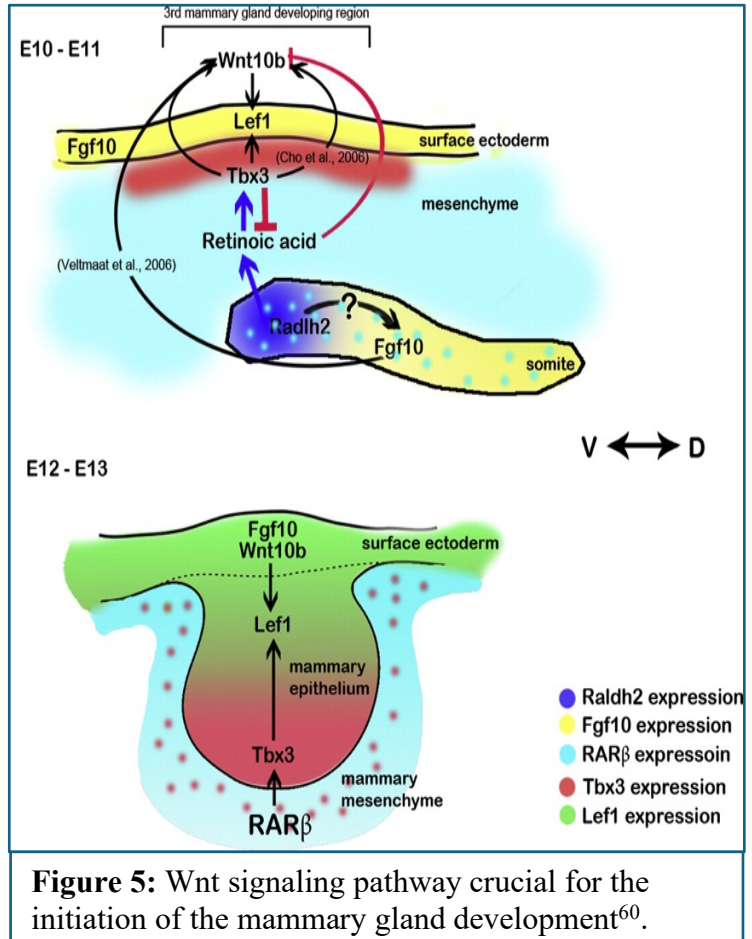


in fMaSC. To determine the relative degree of stem cell potential, fMaSCs were cultured in 2% Matrigel and evaluated for mammosphere formation, which indicates multipotency. It was found that only ~10% of fMaSC at E18.5 exhibit stem cell properties under these *in vitro* conditions. By E12.5, mammary rudiments are committed to become mammary gland. fMaSCs co-express luminal (*Krt8*) and basal markers (*Krt14*). Surprisingly, only 30% of fMaSCs at E13.5 are K14⁺/K8⁺¹⁹. A study suggested that lineage specification begins early on, but lineages are not fully segregated until birth (P4)². Their study was limited to their observations at E16.5, E18.5, P4 and adult mammary gland which is demonstrated in Figure 2. Carabana and colleagues studied earlier embryonic stages and found that the mouse mammary epithelium is uncommitted until E14.5 and convert into luminal-like and basal-like cells together with some hybrid cells at E15.5

as demonstrated in Figure 4³. They also identified three different cell populations in the mammary mesenchyme.

Specification of mammary epithelium and maintenance of multipotency.

Wnt signaling has been found to be one of the initial markers and drivers of mammary gland development¹⁰. It is expressed in the surface epithelium. *Wnt10b* was found to be locally expressed in the mammary epithelium at E11.5 with upregulated expression in the mammary buds¹⁰. *Wnt* signaling is important for placode formation and mammary gland specification²⁰. *Fgf10*, which is expressed at E10.5 in thoracic somites underneath the mammary line, is an upstream



regulator of *Wnt10b* as well as lymphoid enhancer binding factor 1 (*Lef1*)²¹. Heterozygous *Fgf10*^{+/-} mice show inhibition of *Wnt10b* and *Lef1* expression, abnormal mammary line formation, and absence of mammary buds 3 and 5²¹. The roles of *Wnt10b* and *Fgf10* in the mammary gland development are summarized in Figure 5.

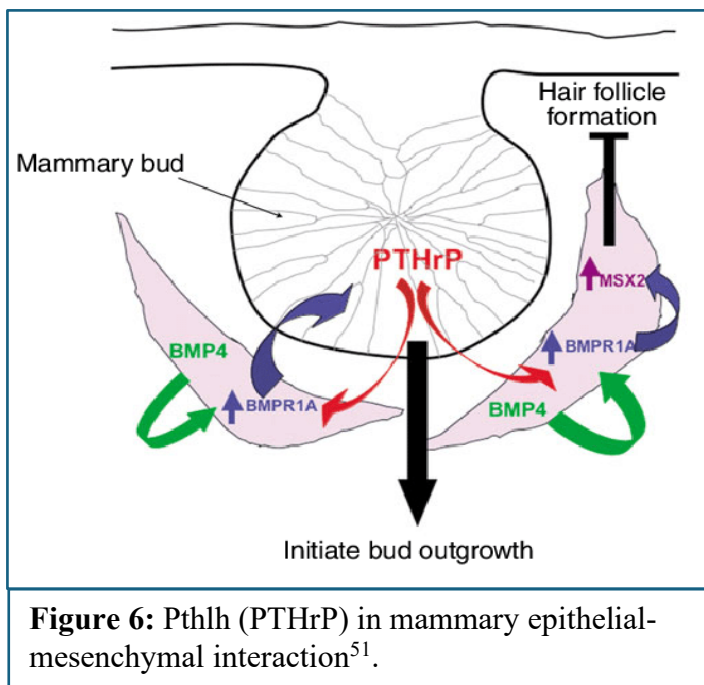
Tbx3 is a member of the T-box transcription factor family. Haploinsufficiency of *Tbx3* in humans causes ulnar-mammary syndrome, which results in defective formation of ulnar bones and mammary gland development²². *Tbx3* also regulates the development of other tissues such as the

heart, lungs, and limbs. In the mammary gland, *Tbx3* is expressed around E10.5 in the mesenchyme underlying the mammary line and is one of the very few known earliest markers of the mammary placode. It is expressed in mammary buds at E13.5 and in mammary mesenchyme underneath the nipple sheath and the rudimentary mammary ductal branches by E18.5. In *Tbx3* knockout mice, there is a complete absence of placode formation. In transgenic *Tbx3*^{+/+} mice, the branching of mammary ducts and nipple sheath formation are disrupted with more intense effects on mammary glands 1 and 2. *Wnt*, *Fgf* and *Tbx3* genes work together in a gene network to maintain the mammary gland development. Bone morphogenetic protein 4 (BMP4) is found to negatively control *Tbx3* which is important for determining the position of the mammary glands. As BMP4 represses the expression of *Tbx3*, it leads to aberrant *Wnt10b* expression, indicating that *Wnt10b* is regulated by *Tbx3*. Heterozygous *Fgf10*^(+/-) mice show inhibition of *Wnt10b* and *Lef1* expression, abnormal mammary line formation, and the absence of mammary buds 3 and 5. Neuregulin-3 (*Nrg3*), on the other hand, mediates signaling from *GLI3* for *Fgf10* expression.²¹ *Nrg3* mediates *Tbx3* and *Fgf* signals from somites to the ectoderm in the mammary line which results into mammary placode formation²² Mammary epithelial cells overexpressing *Tbx3* act as stem cells since *Tbx3* inhibits *NKκBIB*, a repressor of the *NFκB* pathway that maintains the proliferation of cells²³. In addition, *Tbx3* suppresses cyclin dependent kinase inhibitors, *Cdkn2a* (*p19^{ARF}* in mouse; *p14^{ARF}* in humans), *p16^{INK4a}*, and *p21^{CIP}*, which control cell proliferation. Hence, overexpression of *Tbx3* has been found to be one of the causes of breast cancer. *Wnt* signaling between epithelium and mesenchyme is important for the specification of epithelial tissues in various specified structures like hair follicles, sweat glands and mammary glands. Canonical *Wnt* signaling initiates in the underlying mesenchyme of the mammary line which induces mammary placode formation. On the other hand, the signal from mammary placode epithelium to the surrounding mesenchyme causes

condensation of the fibroblast in the mesenchyme and its specification to the mammary mesenchyme.

Lef1 is the important *Wnt* pathway regulator in the fetal mammary placode. It is one of the earliest known markers of mammary placode formation. It forms complexes with a range of factors and activates/suppresses the transcription of genes based on its co-factors. For instance, *Lef1-Groucho* obstructs the *Wnt*-signaling pathway while *Lef1-β-catenin* activates the downstream genes of *Wnt* pathway. Initially, *Lef1* is expressed in the mammary placode around E12.5. In absence of *Lef1*, the mammary mesenchyme-mammary epithelium interaction gets blocked and placodes 2 and 3 do not form while placodes 1, 4 and 5 are poorly formed which eventually disappear by E15.5. It is unknown why there is disparity in effects of *Lef1* knockout between mammary placodes. It has been reported that, around E14.5-E15.5, its expression on the mammary epithelium downregulates while the expression on the mammary mesenchyme upregulates²⁴.

Parathyroid hormone like hormone (*Pthlh*) signaling is one of the primary pathways responsible for mammary epithelium-mammary mesenchymal crosstalk. It is responsible for *Lef1* expression in mammary mesenchyme. *Pthlh* is secreted from the mammary epithelia and binds to its receptor, parathyroid hormone receptor 1 (*Pthr1*) expressed in mammary mesenchyme as depicted in Figure 6. *Pthlh* and parathyroid hormone (*Pth*) are peptides which are



homologous in their amino terminal and act through their G-protein coupled receptors. Pth is produced by parathyroid glands while Pthlh is produced by varieties of prenatal and postnatal tissues. Pthlh signaling is crucial for mammary bud formation, invagination into fat pad, and branching. A study was done examining the changes in the mammary gland development at E12-13, E15 and E18 in *Pthlh*^{-/-} mouse fetuses²⁵. No drastic changes in E12-13 and E15 embryos were observed. At E18, elongation of mammary buds to the fat pad, branching, and nipple sheath formation all failed to occur²⁵.

Sox10 is another key regulator of mammary cell stemness. It is expressed in the fetal mammary stem cells and postnatally in bipotent mammary progenitor cells and unipotent luminal progenitor cells. A study was done to determine the role of *Sox10* in fetal mammary gland development by replacing the open reading frame of *Sox10* with a *lacZ* reporter²⁶. Both homozygous and heterozygous *lacZ* fetal mice developed 5 pairs of mammary buds that invaginated into the fat pad like wild type fetuses. However, the homozygous *lacZ* mutant mice exhibited failure of branching of mammary sprouts indicating the importance of *Sox10* for branching morphogenesis of the mammary ducts. Heterozygous fetal mice had resumption of branching of mammary sprouts, but not until approximately six weeks after birth. A similar experiment was done by making transgenic mice in which *Sox10* was deleted in the mammary epithelium by combining floxed *Sox10* alleles with MMTV:Cre(A) transgene. Mammary ducts invaded the fat pads in 6-8 weeks old female fetal mice, but ductal trees were underdeveloped regarding size and the number of branches versus wildtype glands. The ductal system did not flourish even during pregnancy, the number of alveoli was fewer, mothers were not able to feed pups, and involution was impaired postweaning. Hence, *Sox10*'s role in mammary gland development extends beyond fetal development into adulthood. Data also suggests that *Sox10*

participates in mammary epithelial-mesenchymal cross talk via canonical *Wnt* signaling as deletion of *Sox10* was found to be inhibiting the branching of mammary sprouts ²⁶.

Specification of fMaSCs towards basal lineage.

Trp63 codes for two isoforms: transactivating transcription factor and dominant negative isoform. It is expressed in the fetal mammary cells and regulates epithelial-mesenchymal status. Later in mammary development, *Trp63* expression becomes restricted to basal mammary epithelial cells. *Trp63* in myoepithelial cells controls the expression of neuregulin (*Nrg*) 1 and 2. *Nrg1* activates *ErbB4* in the myoepithelial cells and *Stat5* in the luminal epithelial cells (Figure 7). Activation of

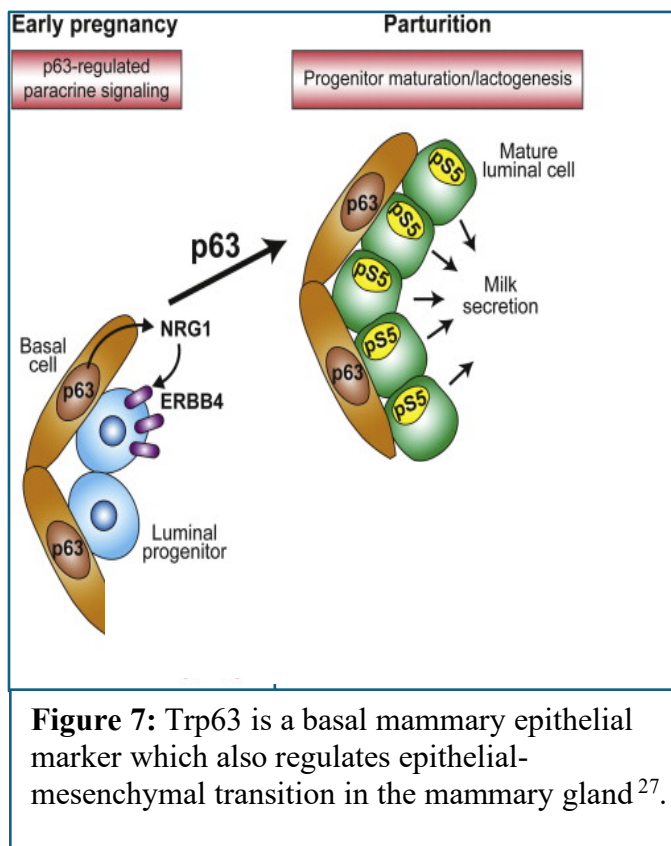


Figure 7: Trp63 is a basal mammary epithelial marker which also regulates epithelial-mesenchymal transition in the mammary gland ²⁷.

Stat5 is necessary for the luminal progenitor cell differentiation and maturation during pregnancy. This basal paracrine signaling mediated by Trp63/Nrg1 to the luminal mammary epithelium also activates luminal E74- like factor 5 (*Elf5*) expression ²⁷.

Spatiotemporal expression of *Sox11* is restricted to fetal mammary populations and is repressed before birth ²⁸. Its expression had been identified at around E14.5 when mammary epithelial cells are multipotent. In *Sox11*-overexpressing mammary epithelial cells, both basal and luminal cell markers have been observed which suggests their role in maintaining fetal mammary cells stemness and mammary progenitor plasticity. In the human mammary epithelial cell line

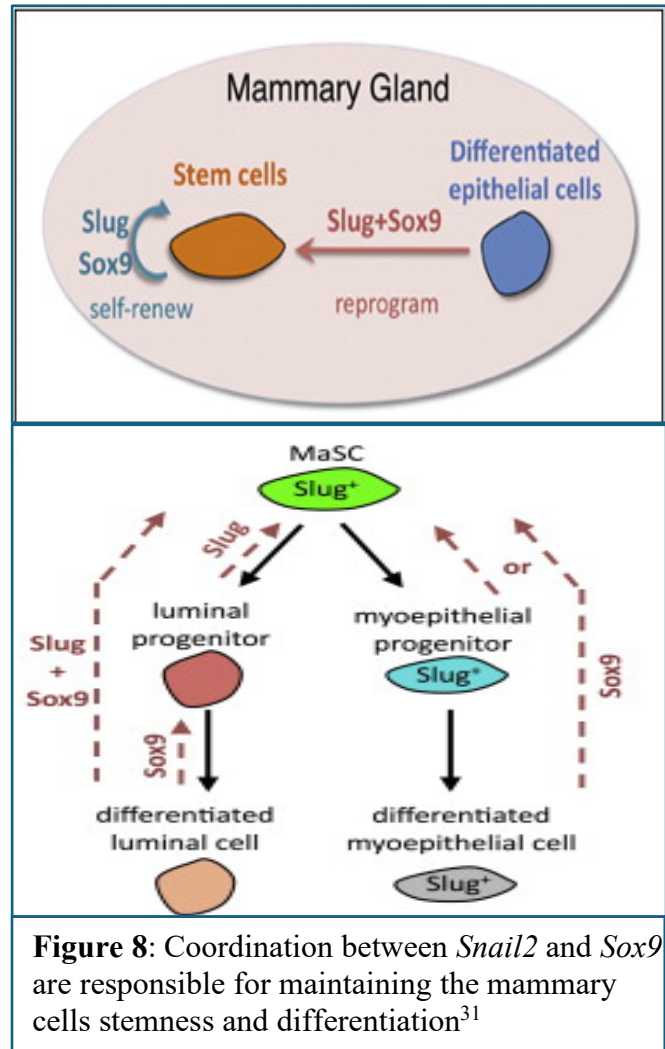
MCF10A, upregulation of *Sox11* increases myoepithelial colony and formation of mammospheres (clusters of luminal and basal progenitor cells). It promotes expression of mesenchymal cell markers such as N-cadherin and Fibronectin1 in various organs including mammary gland ²⁸.

Snail2, also known as *Slug*, is a zinc finger transcriptional repressor. It represses the promoter of *Cdh1* (*E-cadherin*) and regulates the epithelial-mesenchymal transition²⁹. In mammary gland tissue, *Snail2* expression becomes restricted to the basal lineage. Downregulation of *Snail2* results in increased expression of luminal lineage genes such as *Gata3*, *Era*, *Epcam*, *E-cad*, *CD24*, and *Muc1* and reduced basal lineage cell numbers ²⁹. It acts together with LSD1 (Lysine Specific Demethylase 1) to prevent the transcription of genes responsible for the differentiation of mammary epithelial cells to the luminal fate.²⁹ *Snail2* and *Sox9* function together in the network that convert the mature luminal mammary epithelial cells into mammary stem cells.

Markers of the luminal progenitor cells.

Transcriptome analysis of undifferentiated mammary epithelial cells and myoepithelial cells identified enhanced expression of *Sox9* as one of the key genes maintaining stemness in mammary epithelial cells ³⁰. In *Sox9^{flox/flox}* mutant mice, cell proliferation was adversely affected, and both mammary gland elongation and branching were impaired. These effects, however, were not observed in basal epithelial cells and maintain the cell population of luminal progenitor cells. Myoepithelial cells can be derived from *Sox9* knockout mammary epithelial stem cells, however luminal counterparts are not viable. Ectopic expression of *Sox9* together with *Snail2* reprogrammed mature luminal mammary epithelial cells into mammary stem cells, which upon transplantation into transient as they disappeared by 8 weeks of age. *Sox9*-enriched cells give rise to the luminal cleared mammary fat pads formed mammary gland-like structures. The human of differentiated mammary luminal epithelial cells. Following the overexpression of *Sox9* and MCF7

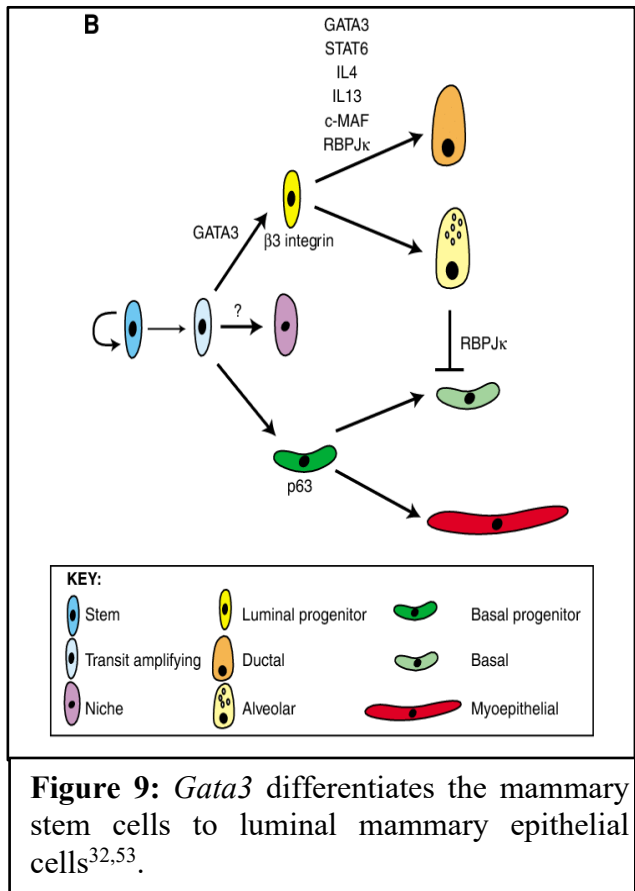
cell line, a breast cancer cells derived from 69-year-old white female, possesses properties decreasing Wnt-1-induced signaling protein-2 (*WISP2*) expression, differentiated MCF7 cells started showing stem cell-like properties. Breast cancer cells in general have higher expression of *Sox9* and lower levels of *miR-140*, which antagonizes *Sox9*. It has also been found that *Sox9* upregulates the *Wnt/β-catenin* pathway³⁰. Exogenous expression of both *Snail2* and *Sox9* in fate-determined luminal cells converts them into mammary stem cells. When fluorescence-activated cell sorting (FACS) on mammary epithelial



cells is used to sort out the epithelial cell population into CD49f^{high}/ CD61⁺ fMaSC-enriched basal cells, CD49f^{low}/ CD61⁺ luminal progenitors, and CD49f^{low}/ CD61[−] differentiated luminal cells³¹, *Snail2* was found to be richly expressed in in mammary stem cells and basal cells. Exogenous expression of *Snail2* in fMaSC-enriched basal cells did not show many changes while it induced stemness in luminal progenitor cells but not the differentiated luminal cells. Intriguingly, when *Sox9* was co-expressed with *Snail2* in the murine-differentiated luminal cells, the cells could form organoids upon culturing on the Matrigel. This indicates that coordination between *Sox9* and *Snail2* in fetal mammary cells maintains stemness (Figure 6). Differentiated luminal cells induced

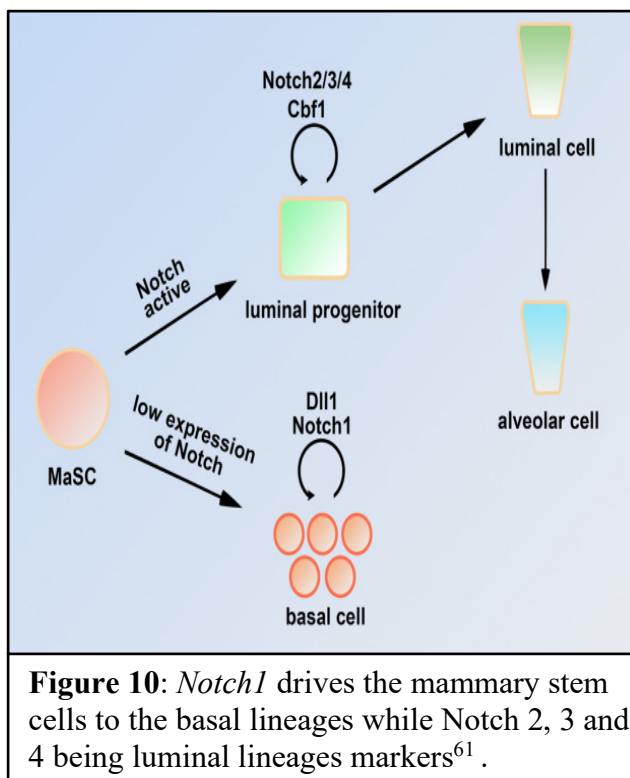
to express *Sox9* alone form mammary acini with lumens. Hence, it was concluded that the co-expression of *Snail2* and *Sox9* together transform differentiated luminal cells back into mammary stem cells as shown in Figure 8. Knockdown of *Snail2* and overexpression of *Sox9* in basal cells also failed to induce stemness in those cells ³¹.

Gata3 is a zinc finger transcription factor that is highly expressed in luminal mammary cells. Its loss-of-function leads to proliferation of the luminal progenitor cells and its gain-of-function in mammary stem cells causes differentiation and maturation of luminal progenitor cells (Figure 9). By E12.5, *Gata3* is expressed in the mammary buds. In adults, it is expressed in both ductal epithelium and lobuloalveolar cells. It has been reported that myoepithelial cells do not express *Gata3*³². *Gata-3^{lox/flox}* mice, when crossed with cytotokeratin 14 (*Krt14*) *cre* recombinase



transgenic strains, show deleted *Gata-3* expression in fetuses by E11.5 ³². *Lef1* is a marker of mammary placode formation. All observed six *Krt14-cre; Gata3^{lox/flox}* embryos showed absence of mammary placodes indicating the role of *Gata3* in mammary gland specification and regulation of *Lef1*³².

There are different paralogs of *Notch* gene. Among the different *Notch* genes, *Notch 1*, *2*, *3* and *4* are important for the mammary gland development which can be seen in Figure 10. *Notch1*, more specifically, is important for embryonic mammary gland development. Interestingly, *Notch1* overexpression can convert basal mammary cells into estrogen receptor-negative luminal mammary cells³³. Its downregulation makes luminal cells acquire the property of basal

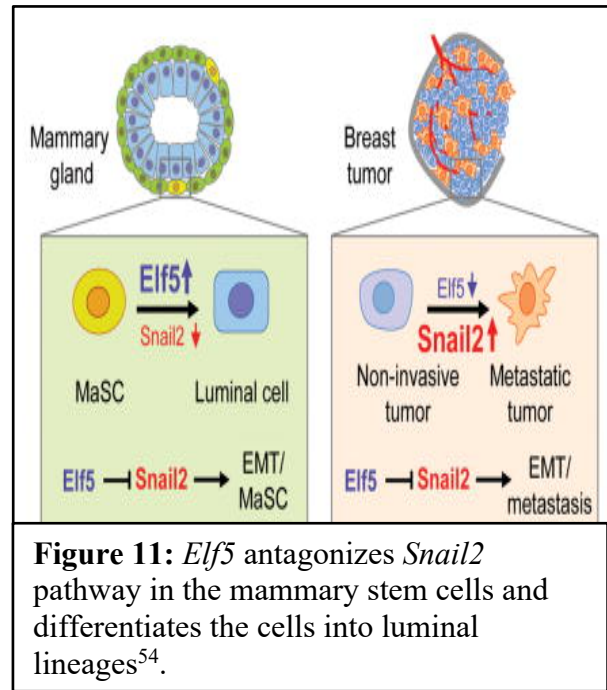


cells. *Notch3* and *Notch4* are richly expressed in the luminal progenitor cells and bipotent colony forming cells, respectively³³. Mice with overexpression of *Notch1* or *Notch3* have lower levels of milk protein β -casein expression within differentiated luminal secretory cells as compared to wildtype³³. *Notch2* is required for tertiary branching and alveolar proliferation. There exists a cross talk between *Notch* and *Wnt* pathways to regulate the normal mammary gland development³³. *Notch* ligand *Dll1* is one of the markers of fMaSCs³³. *Dll1* activates *Notch* signaling and upregulates *Wnt* ligand expression in the mammary macrophages. The mammary macrophage in turn induces *Wnt* signaling to the fMaSCs maintaining their stemness³³.

Lmo4 protein do not have DNA binding sites. They serve as adapter proteins and interact with transcription factors to regulate gene expression. Depending on their protein interactions, *Lmo4* can be localized to both the cytoplasm and nucleus. At E14.5, expression of *Lmo4* was found to be low in mammary bud while the underlying mesenchyme has relatively higher

expression³⁴. This expression downregulates during lactation and upregulates back during involution. Lmo4 forms a complex with Ldb1 and keeps cells in an undifferentiated state. Overexpression of Lmo4 and Ldb1 was found to prevent differentiation of mammary epithelial cells into β -casein-producing cells²⁸. Overexpression of Lmo4 has been found in ~50% of breast cancers as well³⁴.

Elf5 is a member of the ETS (E twenty-six) domain transcription factor family. *Elf5* activation has been found to inhibit *Snail2* expression by binding to the *Snail2* promoter³⁵. As *Snail2* regulates mammary cell stemness and epithelial-mesenchymal transitions, *Elf5* is involved in mammary epithelial cell fate determination. The knockout study of *Elf5* on the adult mammary gland demonstrated the promotion of epithelial-mesenchymal transitions



as indicated by downregulation of *E-cadherin*, enriched *Vimentin*, and *Snail2* expression in luminal epithelial cells. Its loss of function in females prevents differentiation of the luminal progenitor cells, blocks lobuloalveologeneis, and impairs lactation³⁵. *Elf5* has been extensively studied postnatally at the pubertal and pregnancy stages³⁶. *Elf5* is methylated in the mammary stem cells. Partial methylation of *Elf5* differentiates the mammary stem cells into luminal progenitor cells which respond to both the estrogen and progesterone hormones. With complete demethylation of *Elf5*, its expression increases, estrogen and progesterone receptors in the luminal

progenitor cells get completely abolished and the cells turn into the secretory alveolar cells³⁶. Hence, *Elf5* is a luminal marker and *Snail2* is a basal marker (summarized in Figure 11).

Discussion and conclusion:

The mammary gland is an extensively studied field in biology. However, those studies focus mostly the mammary gland development post pubertal, during pregnancy, and post parturition. Mammary gland development starts *in utero* and is vulnerable to the maternal environment. Very little research has been done on fetal mammary gland development, however, so that mammary gland issues with an origin during fetal development may go unnoticed until puberty or the start of lactation. If those problems could be identified early on, many mammary gland issues could be resolved. To identify abnormalities occurring during fetal mammary gland development, we first need to understand the normal developmental changes and gene expression patterns at different embryonic stages. Hence, we studied spatiotemporal gene expression patterns or mammary regulators from E13.5 to E15.5 to identify the unique gene signatures within the luminal- and basal-primed cells. This study can help in building the gene atlas of the fetal mouse mammary gland and used as a reference to study any anomalies associated with mammary gland development.

CHAPTER 3

DEFINE THE SPATIOTEMPORAL GENE EXPRESSION PROFILES OF FETAL MAMMARY STEM CELLS IN THE MOUSE EMBRYONIC MAMMARY RUDIMENT.

3.1 Abstract:

The adult mammary epithelium is comprised of luminal and basal lineages that are defined by the spatiotemporal expression of validated basal and luminal markers (Supplementary Figure1). There is a knowledge gap, however, as to when and where these markers are expressed during fetal development. Multiplex spatial transcriptomics is a powerful tool that can be used to determine the spatiotemporal distribution of various gene markers in the fetal mammary epithelium. Based on multiplexed RNA-FISH using two RNA probes (*Anxa1*, luminal marker and *Cxcl14*, basal marker) at certain fetal stages and postnatal stages, Carabana *et al.*, 2024 found that lineage specification begins at E15.5 with enriched *Anxa1* expression on cells located in the center of the mammary sprout and enriched *Cxcl14* expression in the peripheral cells of the mammary sprout. We hypothesize that multiplexed RNA-FISH with probes corresponding to twelve mammary lineage specification genes will identify additional hybrid gene signatures corresponding to various states of specification. Therefore, we performed multiplexed RNA-FISH using 12 probe sets (4 probes multiplexed per mammary tissue) on E13.5, E14.5 and E15.5 mammary rudiments to construct gene expression patterns that will indicate when and where multipotent mammary epithelial cells in hybrid states specify and segregate into luminal and basal lineages. We observed the variation in *Notch1*, *Lef1*, *Pthlh*, and *Snail2* expression levels between the peripheral and the center cells of the mammary rudiments at E14.5 which suggests priming for

lineage specification by E14.5. *Sox9* and *Gata3* expression were expressed in the mammary rudiments only from E13.5 to E15.5. *Lmo4* and *Sox10* were observed to be evenly expressed in the mammary epithelium and the mammary mesenchyme. *Sox11* expression was qualitatively observed to be upregulated in E15.5 as compared to E13.5 and E14.5 stages. Together, our data provides spatiotemporal profiles for 9 mammary regulator genes within a crucial window of fetal mammary development. Furthermore, our qualitative RNA-FISH data is the first step for performing quantitative multiplex spatial transcriptomics to better understand the genetic and epigenetic changes that occur throughout embryonic mammary gland development.

3.2 Introduction:

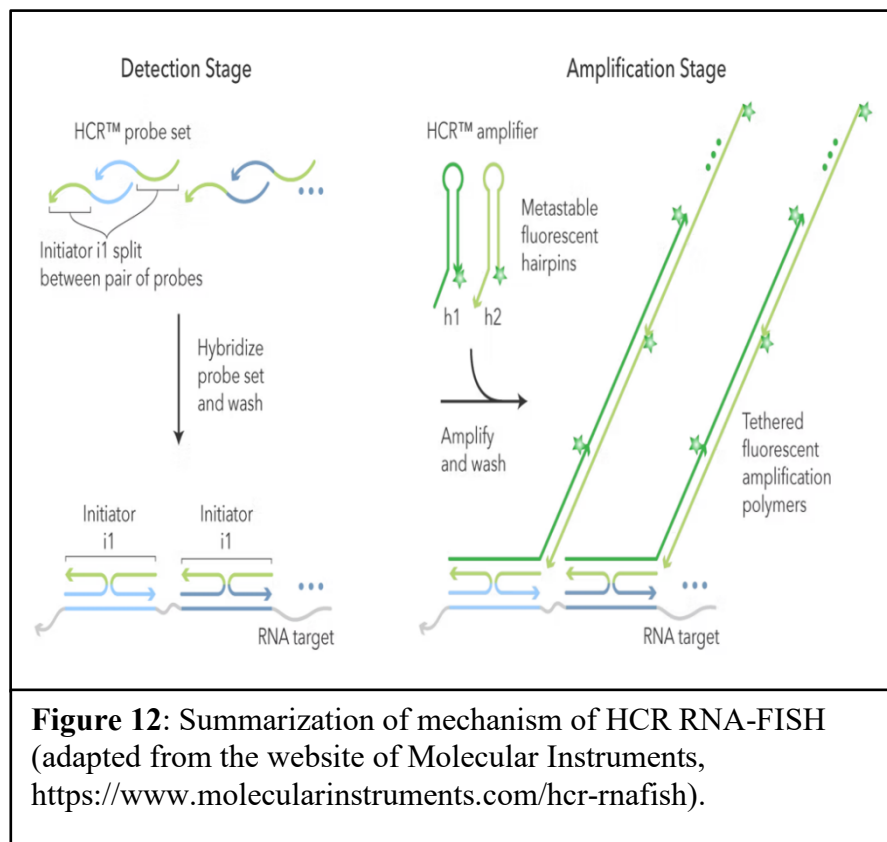
The mouse is an extensively used animal model to study human mammary gland development. Genes that are required for human mammary gland development are known to be expressed in mouse mammary tissue. Mouse knockout studies of genes whose mutations are known to cause mammary congenital defects in humans exhibit abnormality in either all mammary rudiments or specific pairs specifically affected upon knockout of those genes³⁷. This knockout study demonstrated that the third pair of mouse mammary glands are more closely related to the human mammary glands in terms of genetic regulation³⁷.

Mammary glands possess plasticity, and mammary tissue undergoes significant transformations during pregnancy, lactation, and involution. Surprisingly, only two progenitor cell lineages are known for replenishing cell numbers and types during these constant changes. Within the luminal and basal lineages, there could be distinct progenitor cell types within each lineage and there may be even more than two lineages. Many defined regulators of mammary gland involved in early mammary specification and maintenance of multipotency are also expressed later in the networks of the luminal lineage (*Sox9*, *Gata3*, *Notch1*, *Elf5*, *Sox10*, *Lmo4*), or basal lineage

(*Pthlh*, *Trp63*, *Snail2*). Alternatively, many regulators are primarily expressed to maintain epithelial tissue as uncommitted mammary epithelium (*Lef1*, *Pthlh*, *Sox11*, *Tbx3*)³. Many of these genes have been extensively studied in the adult mammary tissue and breast cancers. They have been reported to be expressed in the mouse embryonic mammary gland development², however, their spatiotemporal information is unknown. Multiplex *in situ* hybridization using RNA probes of these gene sets corresponding to uncommitted, luminal-specified, and basal-specified identities could identify additional cell states of developmental potential in the fetal mammary epithelium. Understanding their multiplexed spatial distribution in the mammary gland at different fetal stages is important to better understand the genetic basis of congenital mammary gland defects.

Hybridization Chain Reaction (HCR) RNA-FISH is an important tool which is widely used to locate the mRNA expression of each individual cell which is summarized in the Figure 12.

This technique has two major steps. The first step is the hybridization step where probe sets designed to be complementary to mRNAs hybridize to the mRNA. Each probe set is composed of multiple probe pairs that hybridize to the respective mRNA such that the maximum



number of these probe pairs should successfully hybridize with the mRNA to visualize the mRNA signal. Along with this, the specificity of this technique is increased by the split initiators which increase the specificity of the hybridization chain reactions . During the second step, hairpins are used to tag the properly hybridized probes. Each hairpin set contains two hairpins, h1 and h2. The hairpins are designed for a particular probe set in such a way that hairpin h1 is complementary in nucleotide sequence to the split initiators and another hairpin h2 is complementary to the hairpin h1. This design of hairpin leads to the amplification of hairpin so that each mRNA signal could be detected.

3.3 Methods:

Mice

All experiments were performed on outbred CD-1 timed-pregnant mice purchased from the Charles River Laboratories (Wilmington, MA, USA). Female fetal mammary tissues from E13.5, E14.5, and E15.5, and E18.5 were used for all experiments. Only the third mammary rudiments from the left side were analyzed. All mice were handled according to IACUC-approved guidelines of the Animal Study Protocol [R-APR-23-21]. Animals were appropriately housed at the Animal Facility within the Department of Animal Sciences at the University of Maryland, College Park, and were provided with daily food, water, and health checks. Euthanasia was performed by CO₂ administration followed by secondary cervical dislocation.

Mammary tissue processing

Mouse embryos at E13.5, E14.5, E15.5 and E18.5 were processed for multiplexed fluorescent RNA *in situ* hybridization (RNA-FISH). Upon fetus extraction, tail pieces were collected for genotyping. Head and limbs were then removed, and anterior-posterior incisions were made along

the lateral left and right flanks from the cervical region to the dorsal limbs. The dorsum of the embryo was removed, and any attached organs were separated from the ventral flank. Flanks were fixed in 4% paraformaldehyde (PFA) for 2 hours on ice and then washed 4 x 30 minutes with RNase-free 0.2% PBST on ice. Flanks were dehydrated through a gradient of RNase-free ethanol (EtOH) washes: a) 30% EtOH + 70% 0.2% PBST; b) 50% EtOH + 50% 0.2% PBST; c) 70% EtOH + 30% 0.2% PBST; and d) 100% EtOH. Steps a-d were done on ice for 15 minutes each. The tissues were then changed to fresh 100% EtOH and kept at -80°C for at least 48 hours before RNA-FISH.

Primer design

Primers were designed for reverse transcriptase PCR (RT-PCR) using NCBI Primer BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The parameters for designing primers were amplicon size of 300-500 nucleotides, GC content of 60%, primer length of 18-22 nucleotides, and melting temperature of 55-60°C.

Fetal mouse sex determination

Embryo sex was determined by genotyping genomic DNA with ZFY and HPRT primer sets. Fetuses were genotyped using ZFY (a gene turned on in Y chromosome) primers and HPRT (a gene turned on in X chromosome) primers to identify their sexes. ZFY primer sequences are (sense) 5'-GAC TAG ACA TGT CTT AAC ATC TGT-3' and (anti-sense) 5'-CCT ATT GCA TGG ACA GCA GCT TAT G-3' ³⁸. Primer sequences for HPRT are (sense) 5'-GAC TGA AAG ACT TGC TCG-3' and (anti-sense) 5'-CTA GGA TGA ATG AAG CTC GG-3'. Tail pieces (~3mm long) were minced using a 2 ml tube and a pestle. Next, genomic DNA was extracted according to kit instructions (QuantaBio, Beverly, MA, USA). Briefly, 100 ul of extraction reagent was added to each sample. The samples were then incubated at 95°C for 30 minutes. Samples were then cooled for ~10 minutes at RT. Next, 100 ul of stabilization buffer was added

to each sample, which were subsequently vortexed and micro centrifuged. Extracted genomic DNA (1ul) was then used for both ZFY and HPRT PCR reactions. The setup for PCR (35 cycles total) for both primer sets was a) initial denaturation at 94°C for 3 minutes, b) denaturation at 94°C for 30 seconds, c) annealing at 60°C for 30 seconds, d) extension at 72°C for 30 seconds, e) final elongation at 72°C for 10 minutes, and f) hold at 4°C until samples could be analyzed by gel electrophoresis. PCR products were then run on 2% agarose gels at 110 V for 50 minutes and images were obtained on a Chemi-doc from the Department of Animal and Avian Sciences at University of Maryland College Park. Amplicons for both ZFY and HPRT indicated male while an HPRT-only amplicon indicated female.

RNA extraction

RNA was extracted from bipotent mouse mammary HC11 cells for RT-PCR (HC11 information below). Cell pellets were ground using pestle. Lysis/binding solution was then added in the volume of 100-500 ul depending on cell counts and lysates were mixed by pipetting up and down. Lysates were transferred to a new 2 ml tube and briefly vortexed. Next, 64% EtOH was added in the same volume as the lysis buffer and samples were mixed by gentle pipetting. Lysates were then transferred into a filter cartridge assembled with a collection tube (max volume of 700 ul). The filter cartridge was centrifuged at 12,000 RCF for 5 minutes. The flow through was discarded. Wash solution #1 (700 ul) was loaded, and filter cartridges were centrifuged at 12,000 RCF for 5 minutes. After the flow through was discarded, wash solution #2/3 (500 ul) was added to the filter cartridges which were then centrifuged for 5 minutes. This step was repeated twice. The cartridges were centrifuged for 1 minute to remove any leftover wash solution. Cartridges were then transferred to fresh collection tubes and 60 ul of elution buffer preheated at 75°C was loaded into each cartridge. Cartridges were centrifuged at 12,000

RCF for 30 seconds at RT. Another 20 ul of preheated elution buffer was added into each cartridge and centrifuged at 12,000 RCF for 30 seconds at RT. To eliminate genomic DNA contamination, eluted RNAs were treated with 10 ul of 10x DNase buffer 1, 1 ul of DNase 1 and 9 ul of ultrapure PCR H₂O was added to eluted RNA and incubated at 37°C for 30 minutes. Next, 0.8 ul of DNase inactivation reagent was added, and samples were incubated at RT for 2 minutes. Samples were centrifuged at 10,000 RCF for 1 minute before being transferred into new 1.5 ml tubes and stored at -80° C.

Reverse transcriptase PCR (RT-PCR)

Reverse transcriptase (RT) master-mixes were prepared using 2 ul of 10x RT buffer, 4 ul of 25 mM of MgCl₂, 2 ul of 0.1 M DDT and 1 ul of 40 U/ul RNase OUT according to manufacturer's instructions (Superscript III, Thermofisher, Carlsbad, CA, USA). Primer mixes were made by adding 1 ul of 50 ng/ul random hexamers and 1 ul of 10 mM dNTP to each reaction. Reactions were then incubated at 65°C for 5 minutes. Tubes were placed on ice for at least 1 minute. For the experimental sample, 9 ul of RT mix and 1 ul of 200 U/ul of Superscript III reverse transcriptase were added while for the negative control, 9 ul of RT mix and 1 ul of distilled pure H₂O were added. The tubes were briefly centrifuged and incubated at 25°C for 10 minutes, 50°C for 50 minutes, and 85°C for 5 minutes. Tubes were then chilled on ice for at least 1 minute followed by brief centrifugation. To remove the RNA template strand from new cDNA, 1 ul of RNase H was added to each tube. The tubes were incubated at 37° C for 20 minutes. cDNAs were then stored at -20° C until PCR was done.

Fluorescent *in situ* hybridization

Hybridizations were performed according to published protocols and RNase-free commercial buffers provided by Molecular Instruments (Los Angeles, CA, USA). All probe sets and

fluorophore-conjugated hairpins were also supplied by Molecular Instruments. The dehydrated tissues at -80° C were rehydrated through a reverse gradient of RNase-free EtOH: a) 70% EtOH + 30% 0.2% PBST; b) 50% EtOH + 50% 0.2% PBST; c) 30% EtOH + 70% 0.2% PBST; and d) 100% 0.2% PBST. Rehydration steps were done on ice for 15 minutes each per step. The 3rd mammary buds from left side of female embryos from three separate mothers were collected for each stage (E13.5 to E15.5 and E18.5, with experimental units of at least 3 for each stage). Excised buds were incubated in new 0.2% PBST at RT for 15 minutes. Tissues were then post-fixed in 4% PFA at RT for 20 minutes, washed with 0.2% PBST for 3 x 10 minutes at RT, and then incubated in 100 ul of probe hybridization buffer at RT for 10 minutes. Old probe hybridization buffer was replaced with new 100 ul of probe hybridization buffer and prehybridized for 1 hour at 37°C. Tissues were then incubated in 100 ul probe solution made by mixing 0.4 pmol of 4 probes each. Incubations were done for 48 hours at 37°C. The tissues were washed afterward with probe washing buffer for 4 x 30 minutes at 37°C followed by washing with 5x SSCT for 2 x 10 minutes at RT. Tissues were then pre-amplified with 100 ul of amplification buffer for 10 minutes at RT. Hairpin cocktails (1.5 ul of 3 uM stock of each hairpin, h1 and h2) were mixed in separate tubes for each tissue. Hairpins were heated at 95°C for 90 seconds and then cooled at RT for 30 minutes in the dark. Hairpin solutions were made by mixing 1.5 ul of both h1 and h2 of all 4 hairpin sets in 100 ul. The amplification buffer was replaced with the hairpin solution and samples were incubated for 48 hours at RT in dark. Next, tissues were washed with 5x SSCT at RT for 2 x 10 minutes followed by 2 x 1-hour washes and a final 10-minute wash.

Tissue Embedding

Following fluorescent *in situ* hybridization, tissues were incubated with 5% sucrose/ PBS for 2 hours at 4°C. The 5% sucrose was then replaced with 15% sucrose and incubated for 4 hours at

4°C. Tissues were placed in embedding molds containing OCT media and then frozen in liquid N₂. Frozen OCT/tissue chunks were kept in 2 ml tubes and stored at -80°C until dissection. All tissues were cryosectioned at minus 24-25°C. Cryosection thickness was set at 20 microns.

Post dissection degelatinization, mounting, and imaging

OCT media was removed from cryosections by incubating slides in 1x PBS at 42°C for 20 minutes. Slides were then washed for 2 x 5 minutes in 0.2% PBST and then washed with 1x PBS for 5 minutes at RT. Slides were then dried and mounted with Fluoromount mounting media containing DAPI. Slides were cured in a slide holder overnight at RT in the dark and then stored at 4°C. A TI-2 Nikon widefield microscope (Schiffmacher lab) was used to locate and mark sections with buds at 20x using eyepiece with FITC below 5% intensity. The slides were then imaged using Leica Stellaris 8 confocal microscope (University of Maryland Imaging Core, Cellular and Molecular Biology). Marked sections were imaged with an 63x oil immersion lens. Sections were quickly scanned to adjust the gain and intensity of lasers for all 5 channels (383, 488, 563, 647 and 730) in order to prevent oversaturation of pixels. Laser intensities were kept below 10% for all the channels except 730 channel (around 15%). Negative control tissue sections (only hairpins treated) were utilized to prevent signal from background and autofluorescence.

Segmentation of cells in mammary tissue

Cover glasses were removed by submerging in PBS for up to 48 hours at RT. The duration depended on how long the slides had been mounted with mounting media. The slides were then washed 2 x 10 minutes with 0.2% PBS and then 2 x 10 minutes with 2x SSC. Mammary sections were covered with hybridization chambers and 10 ul of DNase I (1U/ul) was added to destroy all DNA probes attached to mRNA in the tissue. The tissues were incubated with DNase I for 1 hour at RT. The DNase I was inactivated by 2 x 15 minutes washes with probe wash buffer/ 2x SSC at

RT followed by 2 x 15 minutes washes with 2x SSC at RT. Sections were then blocked with 5% goat serum for 1 hour at RT. The tissue sections were incubated with 1:50 dilution of 250 ug/ml E-cadherin antibody (610181, BD Transduction Laboratories) in 5% goat serum overnight at 4°C. Slides were washed for 4 x 30 minutes with 0.2% PBST, and Alexa Fluro 647-conjugated secondary antibody (850 ug/ml) was used at 4 ug/ml to label the E-cadherin primary antibody. Covered slides were then incubated overnight at 4°C and then washed for 4 x 30 minutes with 0.2% PBST. Slides were then dried, mounted with mounting media without DAPI and left to cure in the dark overnight. The slides were imaged for DAPI and 647 (63x oil) using the Leica Stellaris 8 confocal microscope.

Image Analysis

Images from RNA-FISH and segmentation were overlaid using ADOBE Photoshop software. Each gray dot observed in the RNA-FISH image of a particular gene indicates a single mRNA molecule of the specific gene.

3.4 Results:

3.4.1 Validation of RNA-FISH probes-hairpins on HC11 cells.

HC11 cells (ATCC CRL-3062) are heterogenous bipotent mammary epithelial cells derived from BALB/c mice. HC11 cells which have been reported to express all twelve genes of interest listed in Tables 2 and 3^{39,40}. HC11 cells were cultured according to ATCC protocols and media formulations. We performed RT-PCR using RNA extracted from HC11 cells to confirm this gene expression. RT-PCR results are listed in table 3. Our RT-PCR data verified the expression of all the genes of our interest except *Lefl*. We then performed RNA-FISH on HC11 cells and observed the expression of all the genes of our interest and validated all of our probe sets (Figure 13).

Although our effort to amplify any *Lef1* cDNA was unsuccessful, we later validated our *Lef1* RNA probes by confirming its expression profiles from previous studies^{39,40}.

Table 2: RT-PCR results of HC11 cells using primers designed for genes studied under RNA-FISH.

Genes	Accession number	Primer sets	RT-PCR Result
<i>Lef1</i>	NM_010703.5	Sense: 5'-TCACTGTCAGGCGACACTTC-3' AS: 5'-GCTGTCATTCTGGGACCTGT-3'	x
<i>Sox11</i>	NM_009234.6	Sense: 5'-AGCTAGTGTCCACAGGGCTA-3' AS: 5'-TCTGTGGGTCTAGAGTCGGG-3'	+
<i>Trp63</i>	NM_001127259.1	Sense: 5'- CTTCTGTGATTAACTCTGATGCCA-3' AS: 5'-CACGAGAAATGAGCTGGGGT-3'	+
<i>Tbx3</i>	NM_011535.3	Sense: 5'-GGATCAGTTAGTGGGGGCTG-3' AS: 5'-GGGAAGGCCAAAGTAAATCCG-3'	+
<i>Pthlh</i>	NM_008970.4	Sense: 5'-TGAAGAGGGGTTTGACCTGC-3' AS: 5'-TGGATGGACTTGCCCTTGTC-3'	+
<i>Notch 1</i>	NM_008714.3	Sense: 5'-CTTGCCAGGTTTTGCTGGAC-3' AS: 5'-CTTTGCCGTTGACAGGGTTG-3'	+
<i>Gata3</i>	NM_008091.4	Sense: 5'-GAAGGCAGGGAGTGTGTGAA-3' AS: 5'-CGGGTCTGGATGCCTTCTTT-3'	+
<i>Sox9</i>	NM_011448.4	Sense: 5'-CACAAGAAAGACCACCCCGA-3' AS: 5'-TGTTGGAGATGACGTCGCTG-3'	+
<i>Snail2</i>	NM_011415.3	Sense: 5'-GCCTCCAAGAAGCCCAACTA-3' AS: 5'-GCCGACGATGTCCATACAGT-3'	+
<i>Lmo4</i>	NM_010723.3	Sense: 5'-GCAGTGTTGCAATATAGGGGAG-3' AS: 5'-GCTATGCCAGTAGCTGTCCA-3'	+
<i>Sox10</i>	NM_011437.1	Sense: 5'-GACGATGACAAGTTCCCCGT-3' AS: 5'-CATGGGGGAGCCTTCTTCTG-3'	+
<i>Elf5</i>	NM_010125.3	Sense: 5'-GGACCTAGCCACCACTTGTC-3' AS: 5'-CTTGCGAGCGAATGTTCTGG-3'	+

Note: + indicates that gene expression was validated performing RT-PCR on HC11 cells and x indicates the failure to validate. AS is the abbreviation for anti-sense primer.

Table 3: Probe sets designed for RNA-FISH on mouse mammary tissue.

Gene	Gene Accession number	Amplifier	Fluorophore	Probe validation
<i>Lef1</i>	NM_010703.5	B5	488	+
<i>Sox11</i>	NM_009234.6	B3	563	+
<i>Trp63</i>	NM_001127259.1	B1	750	+
<i>Tbx3</i>	NM_011535.3	B1	750	+
<i>Pthlh</i>	NM_008970.4	B2	647	+
<i>Notch1</i>	NM_008714.3	B2	647	+
<i>Gata3</i>	NM_008091.4	B3	563	+
<i>Sox9</i>	NM_011448.4	B2	647	+
<i>Snail2</i>	NM_011415.3	B3	563	+
<i>Lmo4</i>	NM_010723.3	B5	488	+
<i>Sox10</i>	NM_011437.1	B5	488	+
<i>Elf5</i>	NM_010125.3	B1	750	+

3.4.2 Gene expression in mammary epithelium varies through E14.5.

Mammary gland development starts at E10. Studies have shown that the early embryonic mammary epithelial cells are multipotent and uncommitted but then begin to simultaneously express regulators for both luminal and basal lineages. Giraddi and colleagues provided evidence that multipotent mammary epithelial cells become lineage-specified by E18 and eventually commit and segregate to either lineage by birth, with a few hybrid cells remaining that still express both

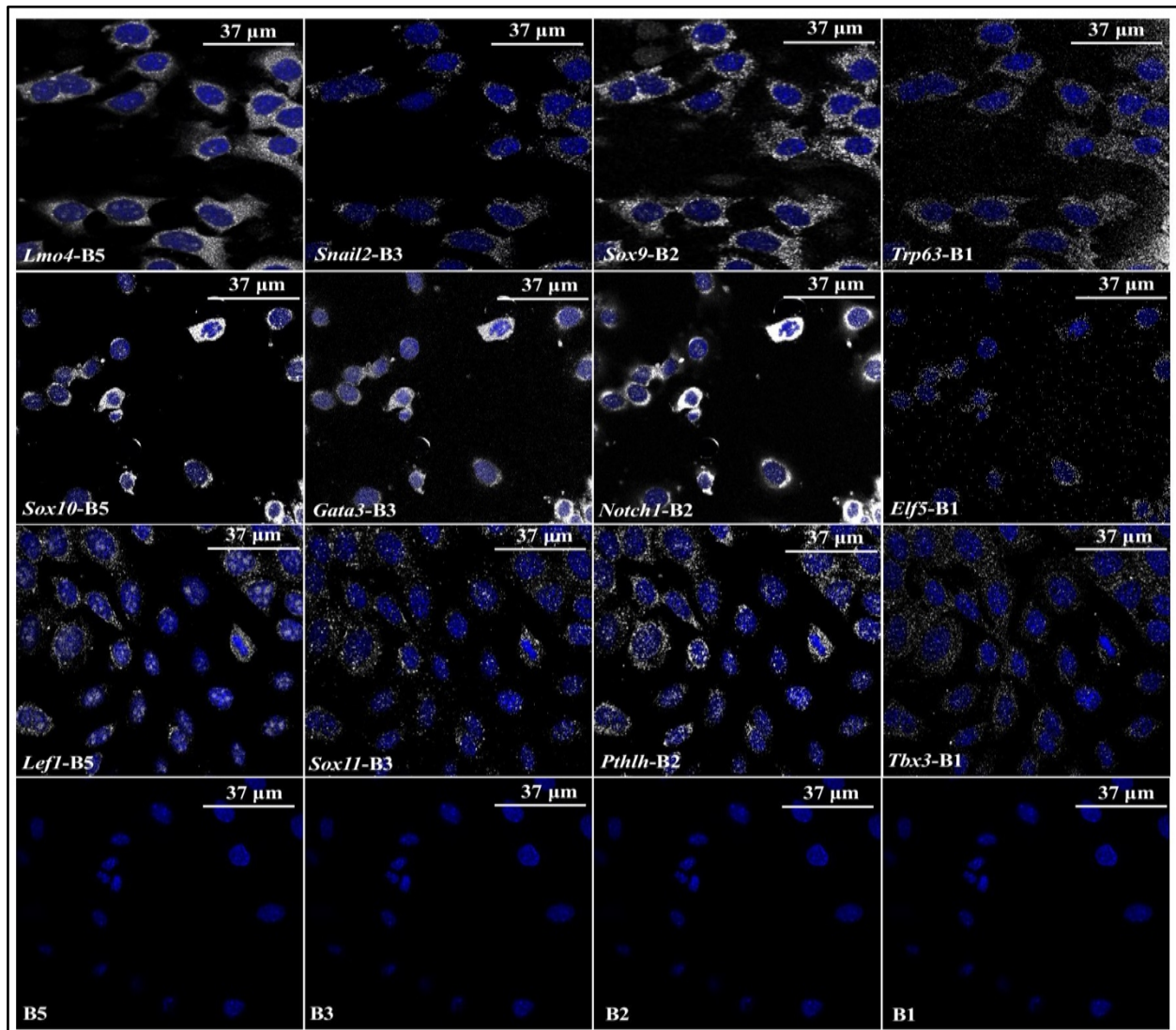


Figure 13: Validation of mouse RNA-FISH probe sets on HC11 cells. HC11 cells were cultured on coverslips and four probe sets designed for four different amplifiers were tested per coverslips. Images were taken in Leica Stellaris 8 confocal microscope under 60x oil immersion. Negative controls (bottom row: No probes, amplifiers only) were imaged using the same parameters that were set across all individual probe sets.

basal and luminal markers ². A recent study, however, reported that the mammary epithelial cells are multipotent until E14.5 and specify into luminal-primed and basal-primed cells by E15.5. We seek to understand how mammary epithelial cells transition from being multipotent to lineage progenitor cells. Therefore, we investigated the spatiotemporal expression patterns of key mammary genes during this critical window (E13.5-E15.5), including mammary epithelial cell specifiers (*Tbx3*, *Sox11*, *Pthlh*), luminal specifiers (*Notch1*, *Lmo4*, *Sox9*, *Elf5*, *Gata3*) and basal specifiers (*Trp63*, *Pthlh*, *Snail2*, *Lef1*, *Sox10*). Multiplexed Fluorescent *in situ* hybridization (RNA-FISH) was performed on the left third mammary buds at three distinct stages: E13.5, E14.5 and E15.5 (n=3).

3.4.2.1 Higher levels of *Sox11* expression in E15.5 compared to the earlier embryonic stages.

Sox11 is a marker of mammary specification that starts to express at E12.5 in mouse mammary epithelium. It has been suggested to maintain mammary epithelial cells stemness ⁴¹. It has been reported that *Sox11* expression is downregulated by E16.5. We also found that *Sox11* is only expressed within the mammary bud and not the surrounding mesenchyme ^{28,41}. We observed that expression is upregulated in E15.5 as compared to E13.5 and E14.5 (Figure 15 and Supplemental Figure 2). This expression pattern might be associated with multipotency as the number of mammary stem cells has been reported to increase with the maximum number of stem cells reported to present at E18.5 ².

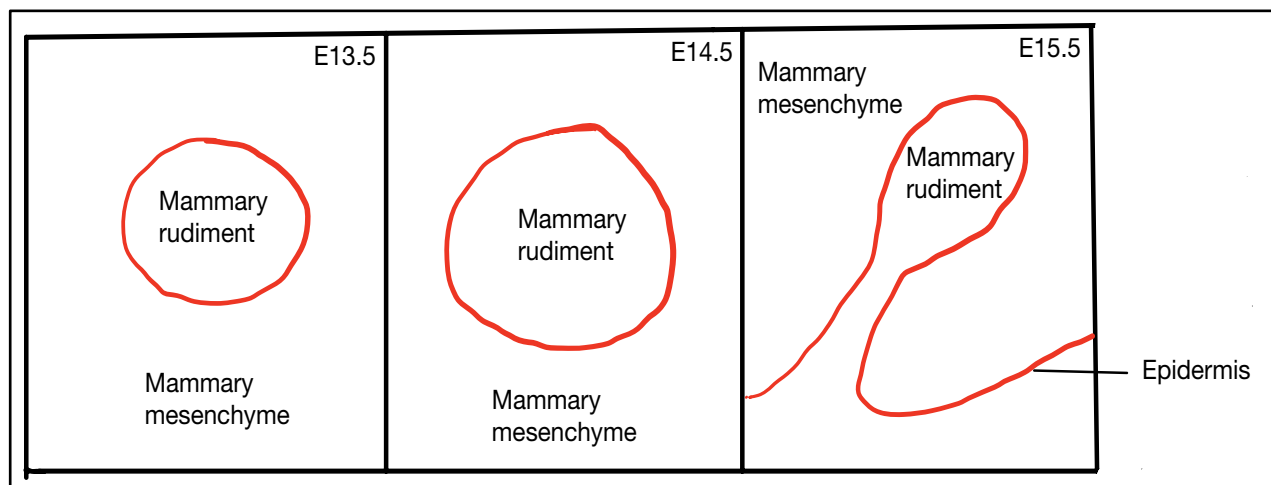


Figure 14: Diagram of mammary tissue from E13.5-E15.5

The first, second and third box from left to the right indicate mouse mammary tissue at E13.5, E14.5 and E15.5. The circular red outlined structure at E13.5 and E14.5 are mammary rudiments and part of the mammary tissue outside the red circles is the mammary mesenchyme. For E15.5, invaginated structure from the epidermis which is outlined by the red highlight is the mammary rudiment while rest of the area outside the mammary rudiment is mammary mesenchyme. This diagram can be used as reference to identify mammary rudiments, mammary mesenchyme and epidermis in our RNA-FISH data where the mammary rudiments are highlighted red as in Figure 15 to Figure 22.

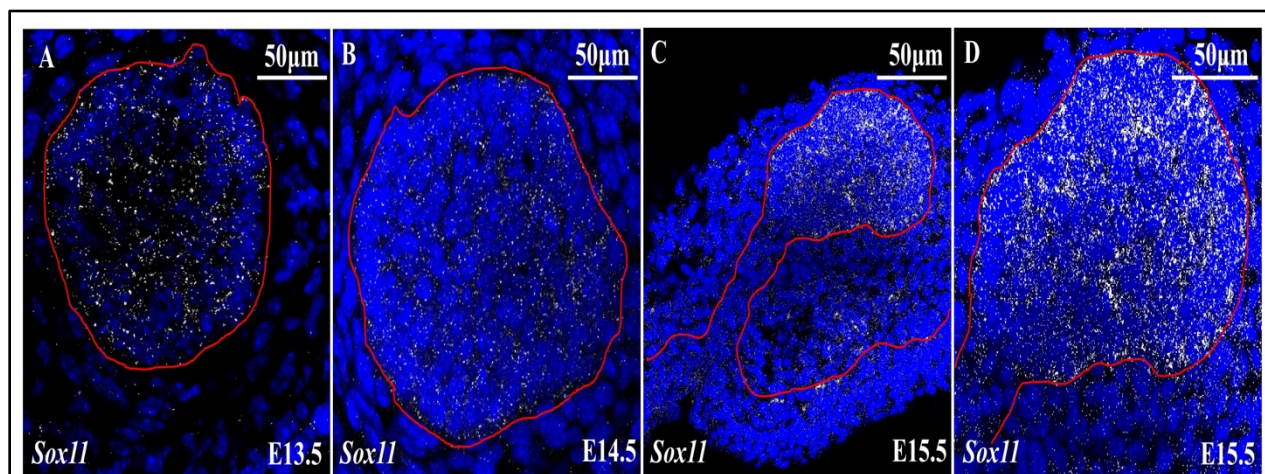


Figure 15: *Sox 11* expression profile in fetal mammary rudiments from E13.5 to E15.5.

Confocal microscopy shows lower level of *Sox 11* expression at E13.5 (A) and E14.5 (B) as compared to E15.5 (C and D). D is the magnified figure of E15.5 (C). All the images were taken at 63x oil immersion (n=3). The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Sox11*.

3.4.2.2 Mammary epithelium is primed for lineage restriction by E14.5.

A recent study found that the mammary epithelium at E15.5 is comprised of 3 clusters of gene expression signatures (luminal-like, basal-like and hybrid cells)³. We hypothesized that changes in the expression levels of luminal and basal genes are observed earlier before E15.5 and the mammary epithelium is primed for lineage specification by E14.5. Notch1 is a mammary lineage

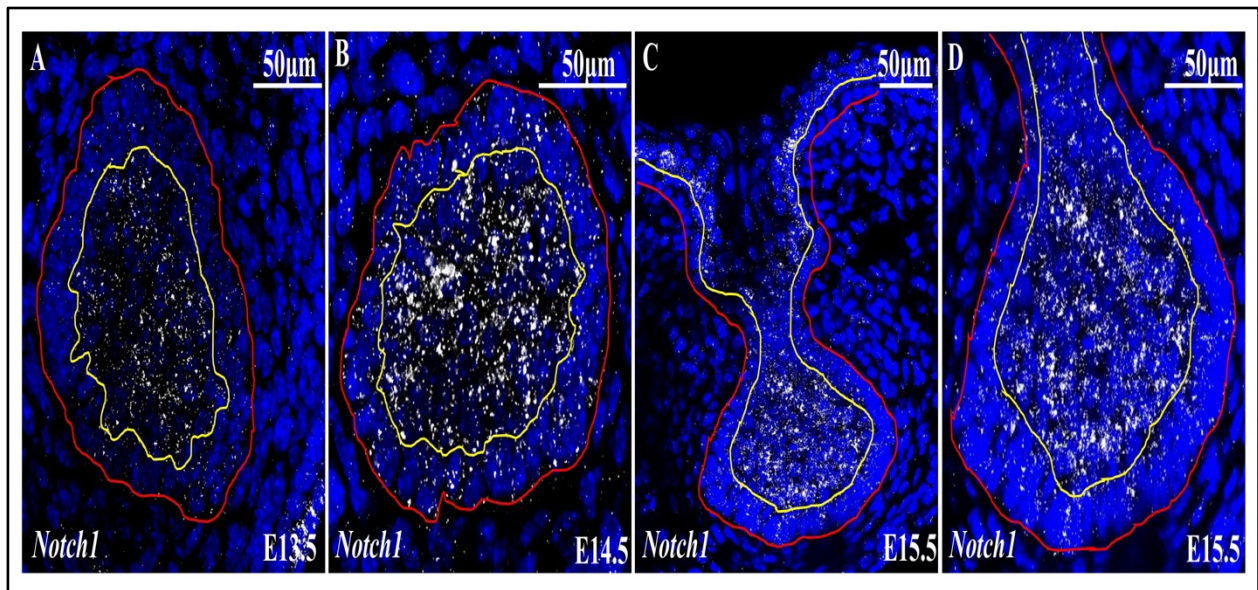


Figure 16: Spatiotemporal changes in *Notch1* expression between E13.5 to E15.5.

RNA-FISH data of *Notch1* showing two different cell population in fetal mouse mammary epithelium starting by E13.5. *Notch1* expression is higher in the center cells of the mammary rudiments in comparison to the peripheral cells in E13.5 (A), E14.5 (B) and E15.5 (C and D). D is the magnified version of E15.5 (n=3). The yellow highlight is used to separate two regions in the mammary rudiments having different level of *Notch1* expression. The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Notch1*.

specifier and later, a luminal mammary epithelial regulator that directs the fetal mammary epithelial stem cells towards luminal fate⁶. It is abundantly expressed in luminal progenitor cells. Our study found out that its expression is more in the center of mammary rudiment and less in the periphery of the mammary rudiments as early as E14.5 (Figure 16 and Supplemental Figure 7). Since Carabana and colleagues reported that the mammary epithelium initiates lineage

specification by E15.5³, our observation of spatiotemporal expression patterns of *Notch1* indicate that priming of multipotent mammary epithelial cells towards the luminal fate occurs as early as E14.5. RNA-FISH data for three mammary epithelial stem cell and later basal lineage regulators (*Pthlh*, *Lef1* and *Snail2*) align with the priming we observed with *Notch1* from E13.5 to E15.5.

Pthlh is an important regulator of mammary gland. Its expression has been reported in

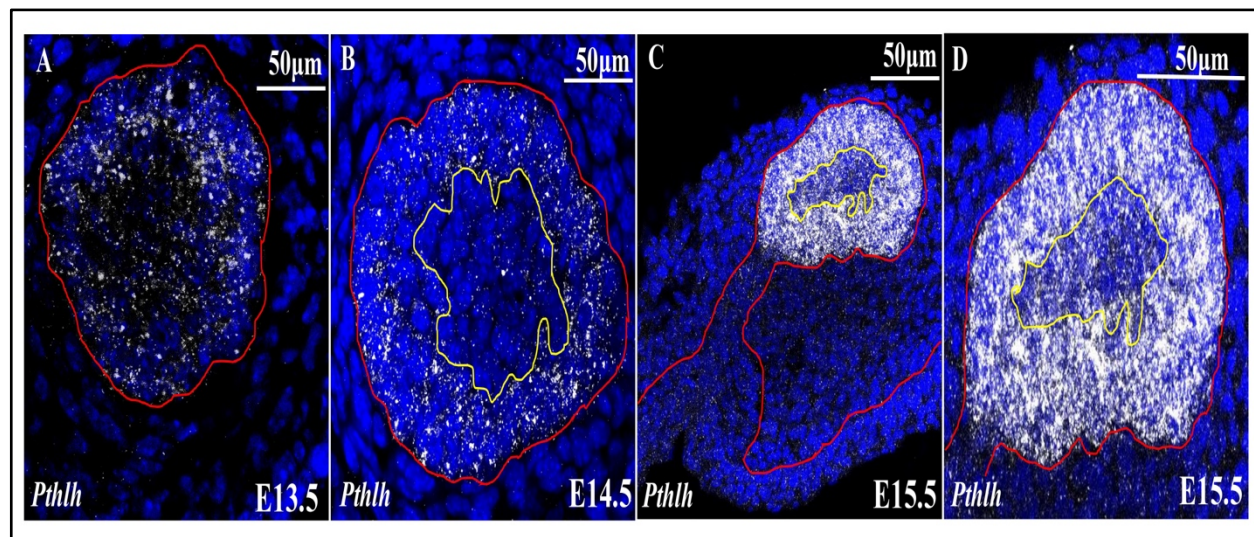


Figure 17: Possible priming of mouse mammary rudiments by E14.5 towards lineage specification. A. The expression level of *Pthlh* (basal marker) is almost same in all cells of rudiments at E13.5. B, C, and D. *Pthlh* was observed more expressed in the peripheral cells of the mammary rudiment as compared to the cells in the center at E14.5 (B), E15.5 (C and D). D. The magnified mammary terminal bud at E15.5 (n=3). The yellow highlight separates two regions in the mammary rudiments having different level of *Pthlh* expression. The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Pthlh*.

the mammary gland at E10.5 when the mammary lines are formed. Its expression is also restricted to the mammary epithelium similar to the other mammary epithelium specifiers including *Gata3* and *Sox11*⁴². Our RNA-FISH data from E13.5-E15.5 show that *Pthlh* expression is exclusive to the mammary epithelium, and at E13.5, its expression appears downregulated in the center of the mammary rudiment (Figure 17 and Supplemental Figure 3). By E14.5, two populations of mammary epithelium were evident where cells in the center of the mammary rudiment expressed less *Pthlh* transcripts versus the peripheral cells. *Snail2* is important for epithelial-to-mesenchymal

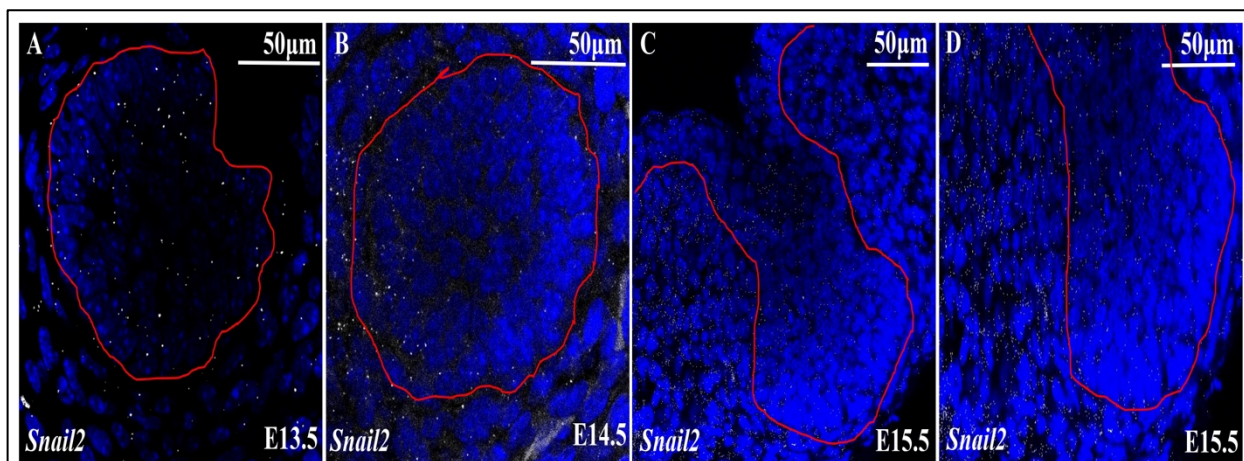


Figure 18: The expression level of *Snail2* in mouse embryos from E13.5-E15.5 is low in both the mammary rudiments and the mammary mesenchyme. Confocal microscopy at 60x oil immersion showing low expression level of *Snail2* in the mammary rudiments and the mammary mesenchyme at E13.5(A), E14.5(B) and E15.5(C and D). The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Snail2*.

transition and branching of mammary rudiment⁴³. The branching of mammary rudiments starts around E16.5⁴⁴ when *Snail2* expression is upregulated. Our RNA-FISH data from E13.5-E15.5 observed that there is low expression of *Snail2* in both the mammary rudiments and the mammary mesenchyme in all three studied embryonic stages (Figure 18 and Supplemental Figure 5). *Lef1* is a part of Wnt signaling between the mammary epithelium and the mammary mesenchyme. Its expression in the mammary epithelium starts at E12.5 and is also expressed in the surrounding mesenchyme by *Pthlh* signaling from the mammary rudiment at E15.5⁴⁵. Studies have found that the expression of *Lef1* downregulates in the mammary rudiment at E13.5 but was then enriched in the outer layer of the mammary rudiment and the surrounding mesenchyme at E14.5. *Lef1* expression was found more abundantly expressed in the mesenchyme as compared to the mammary rudiments at E15.5⁴⁶. Our study also found a similar expression pattern (Figure 19 and Supplemental Figure 1).

Based on our RNA-FISH done on the mouse mammary tissue from E13.5-E14.5, there is clear demarcation in the expression of luminal marker (*Notch1*) and basal markers (*Pthlh*) at

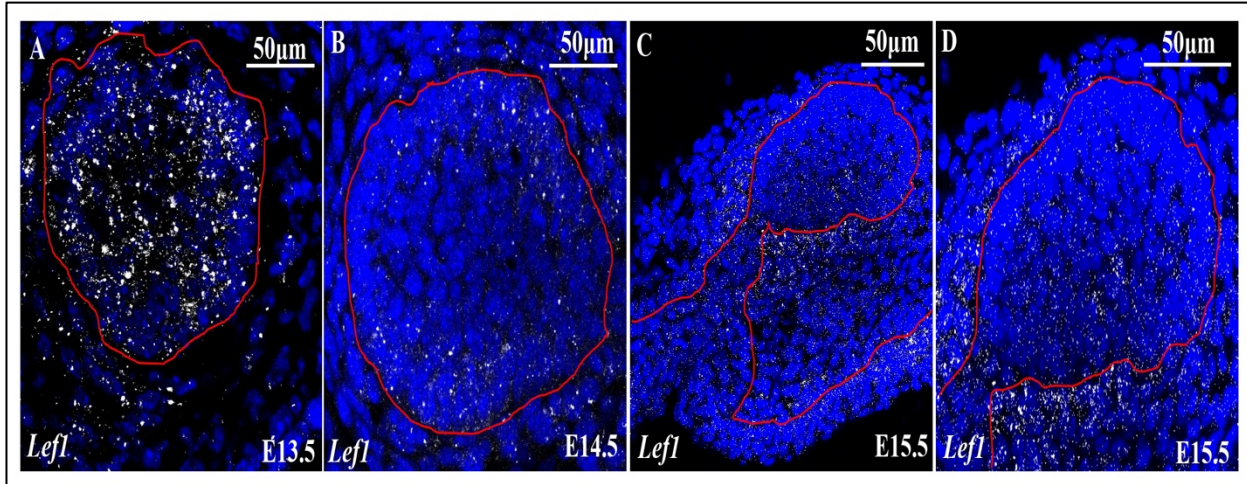


Figure 19: Changes in *Lefl* expression from 13.5 to E15.5. A. Ubiquitous expression of *Lefl* in the mammary rudiments and the neighboring mesenchyme at E13.5. B. Down regulation of *Lefl* expression in the center cells of mammary rudiments at E14.5. C and D. There is increased *Lefl* expression level in the mammary mesenchyme compared to the cells in the mammary rudiments at E15.5 (n=3). The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Lefl*.

E14.5, when *Notch1* expression was observed to be downregulated in the peripheral cells of the mammary rudiments while *Pthlh* expression was observed to be downregulated in the center cells of the mammary rudiments. Genes that eventually direct basal lineage formation are more expressed in the outer layers as compared to the inner layer of mammary rudiments. It is possible that the mammary epithelium gets primed for lineage specification by E14.5 and then gets specified into basal-like, luminal like cells and hybrid cells at E15.5 as stated by Carabana and colleagues³.

3.4.2.3 *Sox10* is expressed in mammary epithelium by E13.5.

Sox10 is one of the regulators of stemness of multipotent mammary epithelial cells. It is expressed in both luminal and basal adult mammary epithelial cells. Its overexpression in mammary epithelial cells was found to induce mammary epithelial-to-mesenchymal transition⁴⁷. A previous study reported that *Sox10* is not expressed at E14.5 but induced at late embryonic stage E18.5 with

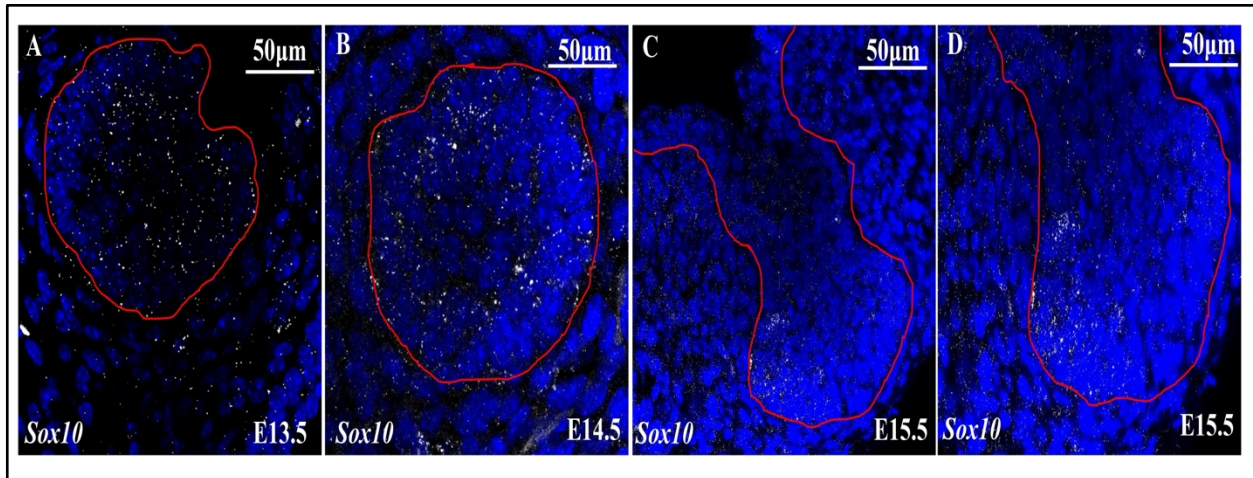


Figure 20: *Sox10* expression from E13.5 to E15.5 in female mouse fetuses.

Sox10 expression starts by E13.5(A) in the mammary rudiments and the surrounding mesenchyme. Qualitatively, the expression level is similar in the mammary rudiments and the mesenchyme at E14.5 (B) and E15.5 (C and D) as well (n=3). The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Sox10*.

maintained expression in the adult mammary gland¹³. Interestingly, we observed that *Sox10* is ubiquitously expressed in the mammary bud and the surrounding mesenchyme from E13.5 to E15.5 (Figure 20 and Supplemental Figure 4). The expression of *Sox10* was low at all three embryonic stages which could be the reason the previous study using scRNA-seq could not detect its expression in the early embryonic mammary gland¹³.

3.4.2.4 *Lmo4* is expressed in the mammary rudiment and the mesenchyme.

Immunohistochemistry (IHC) analysis of *Lmo4* done by Sum *et al.*, 2005 found that *Lmo4* levels are higher in the surrounding mesenchyme as compared to the mammary bud at E14.5⁴⁸. Qualitatively, our study found almost similar level of its mRNA expression between the mammary rudiment and the mesenchyme with slightly more expression in the mammary rudiment (Figure 21 and Supplemental Figure 9). It may be that translational or post-translational regulation of

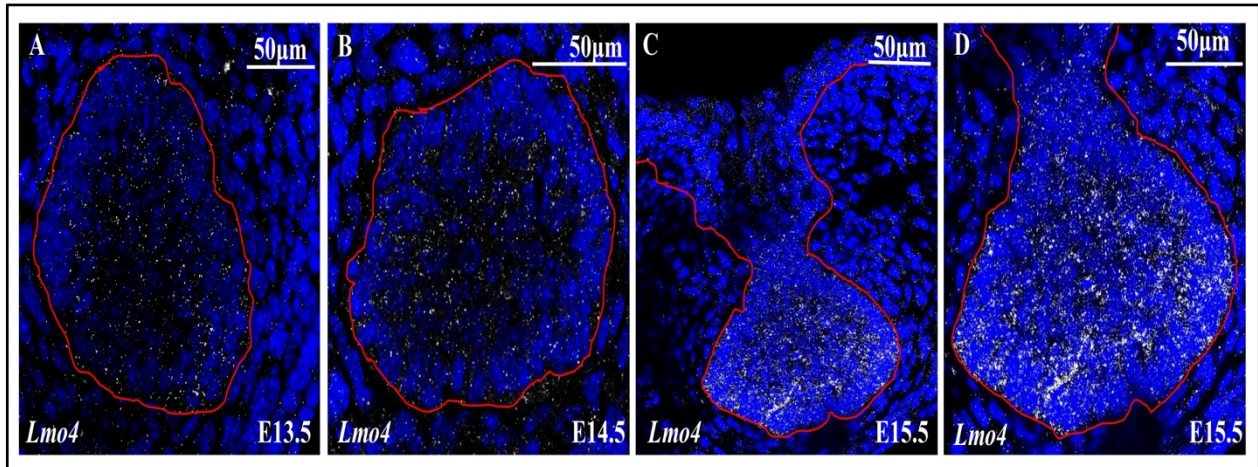
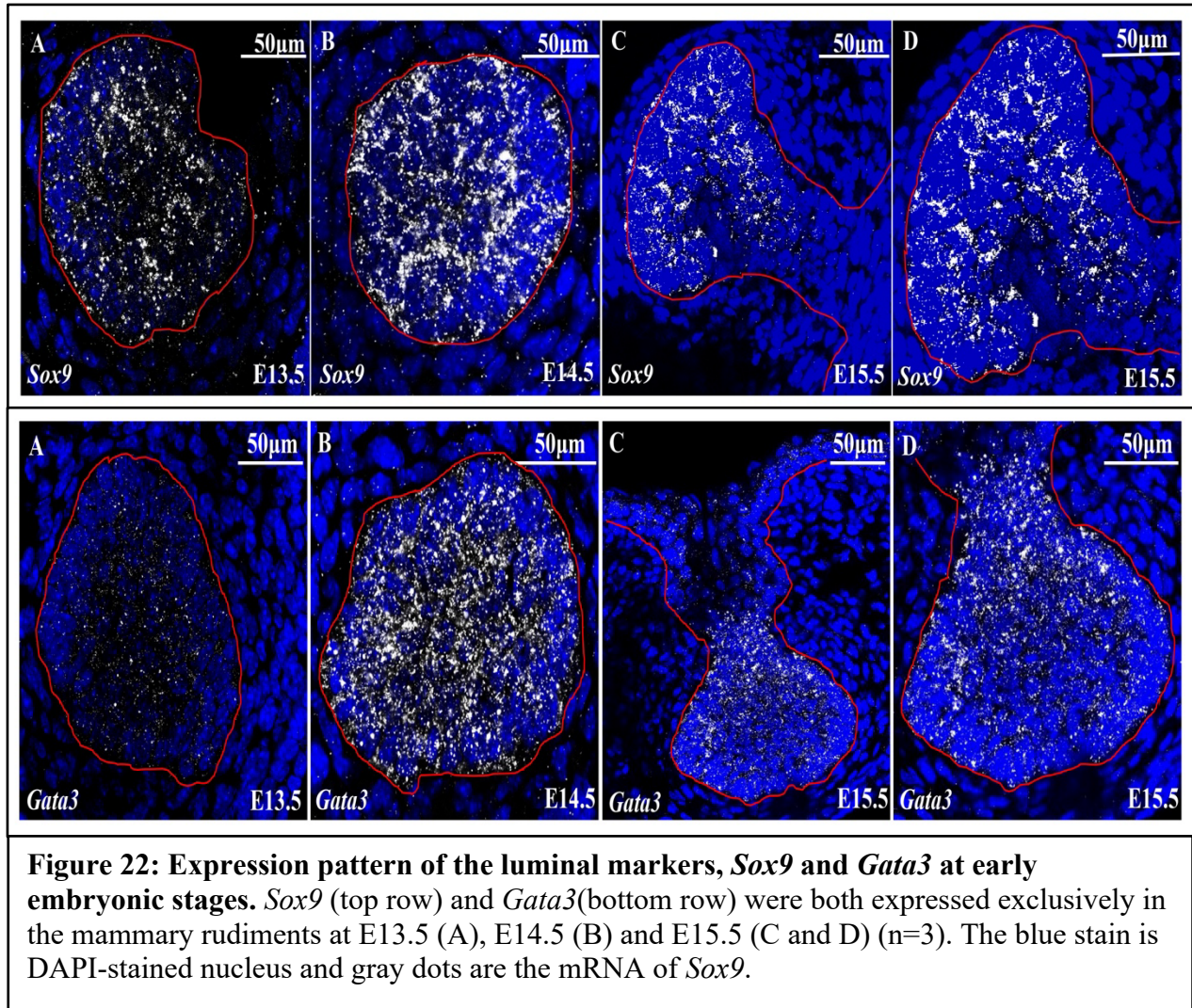


Figure 21: *Lmo4* expression profile from 13.5 to E15.5. *Lmo4* is best known as a luminal marker expressed more in mammary lobular-alveolar cells during lactation. Its expression is ubiquitous in the mammary rudiment and the mammary mesenchyme at E13.5 (A), E14.5 (B) and E15.5 (C and D) (n=3). The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Lmo4*.

Lmo4 is discordant with its transcription. There may also be mouse strain-specific variation as their study was performed on C57BL/6 and BALB/c mice while we studied CD-1 mice.

3.4.2.5 *Sox 9* and *Gata3* expression are restricted to the mammary epithelium.

Sox9 and *Gata3* are mammary epithelial luminal marker genes. *Sox9* is one of the earliest expressed transcription factors in mouse mammary epithelium. It has been reported to be richly expressed in mammary rudiment at E14.5⁴⁹. Our study also found its expression strictly in the mammary rudiment in all 3 stages (Figure 22 and Supplemental Figure 6). However, we observed a merging of high intensity pixels rather than single dots, indicating over-saturation or over-amplification of



signal. *Gata3* has been reported to be expressed by E12.5 where it is spatially exclusive to the mammary epithelium⁵⁰. As previously reported, we also found the expression of *Gata3* exclusively in the mammary epithelium (Figure 22 and supplemental Figure 8). Its expression in the distal ends was more prominent compared to the rest of the mammary rudiment at E15.5.

3.5 Discussion

Mammary gland development starts *in utero* around E10.5⁵¹. Embryonic mammary gland development can be affected by various changes within the maternal environment, for

example, maternal heat stress during late gestation have been reported to decrease fat pad mass, mammary parenchymal mass and mammary epithelial cells proliferation⁵². Any possible issues causing irreversible fetal reprogramming of the embryonic mammary gland may remain unrealized until women reach the stage of lactation. We performed gene expression profiling of twelve genes using RNA-FISH technology to understand their spatial distribution pattern and investigate the time frame of the mammary epithelial priming towards luminal or basal lineages. We studied mammary epithelial specifiers (*Tbx3*, *Sox11*), luminal lineage specifiers (*Sox9*, *Gata3*, *Elf5*, *Notch1*, *Lmo4*), and basal lineage specifiers (*Snail2*, *Trp63*, *Lef1*, *Pthlh*), and *Sox10* which are expressed in both the luminal and basal lineages.

Table 4: Summarization of our RNA-FISH study from E13.5-E15.5

Genes	Previous finding	Our RNA-FISH data
<i>Lef1</i>	Expression in the mammary epithelium begins by E12.5. E14.5-E15.5: the expression in the mammary mesenchyme upregulates and the expression in the mammary rudiments downregulates. ⁴⁵	E13.5: similar level of expression in mammary rudiments and the mammary mesenchyme E14.5: Expression downregulates in the center cells of mammary rudiments, the expression level in the peripheral cells of the mammary rudiments and the mammary mesenchyme similar E15.5: Similar level of expression in the center cells and peripheral cells of the mammary rudiments, expression in the mammary mesenchyme upregulated
<i>Sox11</i>	Expression begins by E12.5 and stops by E16.5 ^{28,41}	E13.5-E15.5: Expressed in the mammary rudiment; negligible expression in the mammary mesenchyme
<i>Trp63</i>	Expression begins by E12.5 ²⁷	Spatiotemporal information undetermined
<i>Tbx3</i>	Expression begins by E12.5 ²³	Spatiotemporal information undetermined
<i>Pthlh</i>	Expression begins from E10.5, restricted expression in the mammary epithelium ⁴²	E13.5: similar level of expression in all cells of the mammary rudiments. E14.5-E15.5: Higher expression level in the peripheral cells than the center cells of the mammary rudiments Negligible expression in the mammary mesenchyme from E13.5-E15.5.
<i>Notch1</i>	Expression begins by E12.5 ⁶	E13.5-E15.5: Expression level in the center cells more than the peripheral cells of the mammary rudiments, negligible expression in the mammary mesenchyme
<i>Gata3</i>	Starts expressing by E12.5, restricted expression in the mammary epithelium ⁵⁰	E13.5-E15.5: expressed in the mammary epithelium and epidermis, negligible expression in the mammary mesenchyme
<i>Sox9</i>	Expression in the early embryonic mammary epithelium ⁴⁹	E13.5-E15.5: Restricted expression in the mammary rudiments
<i>Snail2</i>	Expressed in the early embryonic mammary epithelium ⁴³	E13.5-E15.5: Low level of expression both in the mammary epithelium and the mammary mesenchyme
<i>Lmo4</i>	E14.5: Higher expression level in the mammary mesenchyme as compared to the mammary rudiments ⁴⁸	E13.5-E14.5: Similar level of expression in the mammary rudiments and the mammary mesenchyme E15.5: Higher level of expression in the mammary rudiments than the mammary mesenchyme
<i>Sox10</i>	Not expressed until E18.5 ¹³	E13.5-E15.5: Expressed both in the mammary rudiments and the mammary mesenchyme
<i>Elf5</i>	Expression begins by E12.5 ³⁶	Spatiotemporal information undetermined

Our RNA-FISH data is summarized and compared with previous findings in the table 4. Our study on *Notch1*, *Snail2*, *Lef1* and *Pthlh* showed two distinct populations of cells within mammary epithelium. One group of cells are in the center of the mammary rudiments and another group of cells are in the periphery of the mammary rudiments. The cells in the center were found to be expressing more *Notch1*(luminal marker) compared to the peripheral cells, while basal markers like *Snail2*, *Pthlh*, and *Lef1* were more expressed in the peripheral cells. Carabana and colleagues stated that the mammary epithelial lineage is specified at E15.5³. Our data demonstrate an uneven distribution of basal and luminal markers at E14.5 between center and the periphery of the mammary rudiment. This suggests that the mammary epithelium could be primed to get specified into two lineages by E14.5.

One of the interesting findings of our study is that *Sox10* is expressed in CD-1 mouse mammary tissue by E13.5. It was previously reported that *Sox10* is not expressed in the embryonic mammary tissue until E18.5¹³. There is a possibility of mouse strain variation, as we used the CD-1 mouse strain while they used the C57BL/6 mouse strain¹³.

Lmo4 is a gene which is a lobuloalveolar cell specifier. Its expression had been reported to be upregulated during lactation. IHC data of *Lmo4* done at E14.5 mammary tissues reported that the expression level of *Lmo4* is higher in the mesenchyme compared to the mammary rudiment⁴⁸. Interestingly, our study found that *Lmo4* is expressed at similar levels in the mammary epithelium and the mammary mesenchyme between E13.5 to E15.5. This difference in mRNA and protein expression could be due to post-transcriptional regulation, or strain variation. It would be interesting to perform IHC on CD-1 mouse mammary tissue in this time frame to compare the protein expression profile with the mRNA expression data.

*Sox9*⁵³ and *Gata3*⁵³ are luminal markers during later development. They have been

reported to be specifically expressed in the mammary epithelium from the beginning of their expression in the mammary gland, which is by E14.5 for *Sox9* and by E12.5 for *Gata3*^{49,50}. Our data aligns with the previous findings. These two genes could be used as markers of mammary epithelium. *Sox11* is a mammary epithelium specifier. It has been known to maintain the stemness of mammary epithelium^{28,41}. Multipotent mammary stem cells have been reported to increase in number until E18.5, and then decrease and disappear by P4². Considering this fact, we anticipated seeing the upregulation in its expression in E15.5 compared to E13.5. Our study also observed that the expression level of *Sox11* was higher in the mammary epithelium at E15.5 as compared to E13.5 and E14.5. It has also been reported that *Sox11* expression downregulates by E16.5^{28,41}. It is possible that the *Sox11* expression is maintained until E15.5 and then downregulates from E16.5 to be eventually turned off by birth.

We also evaluated *Trp63*, *Tbx3* and *Elf5* probes on all three embryonic stages (supplemental Figure 12). *Trp63*²⁷ is a basal lineage specifier, *Elf5*⁵⁴ is a luminal lineage specifier, and *Tbx3*^{22,23} is a mammary epithelium specifier. All these genes have been reported to be expressed in mouse mammary tissue by E12.5^{23,27,36}. While optimizing the whole-mount RNA-FISH protocol for the embryonic mammary tissue, third mammary tissue at E14.5 was used. We had observed the expression of *Trp63* (restricted to mammary rudiment) and *Tbx3* (low expression but expressed both in the mammary rudiment and the mammary mesenchyme) during optimization of the protocol. However, we did not see the expression of *Elf5* at E14.5 even though all of them were tested at the same time. While repeating the same RNA-FISH protocol on our experimental units, due to photobleaching of 730 conjugated hairpins, we could not determine their expression pattern from E13.5 to E15.5. We are reperforming the RNA-FISH experiment to identify the spatial distribution of these three genes.

Of note, our study is a qualitative analysis of RNA-FISH. Upon quantification, we would be able to observe various cells at different hybrid states of specification.

CHAPTER 4

OPTIMIZATION OF A SINGLE-CELL RNA SEQUENCING PROTOCOL TO COMPARE MALE VS FEMALE MAMMARY RUDIMENTS AT E14.5.

4.1 Abstract:

The mammary gland is highly regulated by the neuroendocrine system. At each stage of female life, including puberty, pregnancy, lactation, involution, menstrual/estrus cycle and menopause, mammary development and homeostasis are influenced by hormones including progesterone, estrogen, testosterone, prolactin, and/or oxytocin. Each stage is influenced by a distinct set of hormones. For example, the mammary gland proliferates due to estrogen and branches due to progesterone during pregnancy and lactation while downregulation of those hormones post-weaning leads to involution mammary gland returning it to pre-pregnancy or pubertal phase mammary gland⁵⁵. This indicates the plastic nature of the mammary gland. Giraddi *et al.*, 2018 reported that mammary epithelium begins to segregate after E18.5 and are segregated by birth.² Carabana *et al.*, 2024 noted the presence of uncommitted cell types expressing both the luminal and basal markers at E13.5 and at E14.5, and clustered all epithelial cells identified at E15.5 into 3 cell types: luminal-primed, basal-primed and hybrid cells³. CD-1 mice are an outbred strain in which the male mammary remnant remains after degeneration due to androgen signaling. In inbred strains of mice, the male mammary tissue completely degenerates⁵⁶. There could also be mouse strain-specific variation in the timing of mammary epithelial lineage specification, types of cells detected during lineage specification, and strain-specific variation in gene profiles of the mammary tissue at E14.5. There is a knowledge gap in what kind of transcriptomic changes are

made in the mammary epithelium and mammary mesenchyme of male mouse fetuses once their reproductive system starts making testosterone. scRNA-seq is an important tool which can be used to determine the difference in gene expression and hybrid cell types of the mammary epithelium and the mammary mesenchyme between male and female embryos at E14.5 by when the change in the mammary tissue begins to become apparent under the influence of testosterone in male embryos. The power of scRNA-seq data depends on how many cells are read and how many transcript reads are done per cell. It is important to collect enough cells in order to perform scRNA-seq effectively. We hypothesized that the published single-cell mammary tissue dissociation protocol ^{2,3} needs to be optimized for efficient dissociation of male and female embryonic mammary rudiments and mammary mesenchyme at E14.5 in order to read sufficient number of cells during scRNA-seq. We have optimized the single-cell dissociation protocol and collected two-third of the required quantity of cells. At E14.5, we could observe the histological variation between the male and female mammary rudiments where male rudiments were relatively smaller and difficult to isolate.

4.2 Introduction:

Breast feeding is important for newborn babies for balanced nutrition, strong immunity, and proper development. The mammary gland is intricately regulated by the endocrine system associated with the reproductive axis. In the female body, there are constant changes in progesterone and estrogen levels. During pregnancy and lactation, the mammary gland undergoes proliferation and elongation, while during involution, the mammary gland remodels itself back to the pre-pregnancy state. We hypothesized that these dynamic organ changes are facilitated by multiple resident cell types. However, there are very few cell types known in the mammary gland. Giraddi *et al.*, 2018 identified a pool of multipotent mammary epithelial cells that segregate into luminal and basal

lineages by birth ². Recently, Carabana and colleagues investigated multipotent mammary epithelial cells at E14.5 and identified three distinct transcriptomic signatures in the mammary mesenchyme. Carabana's data, together with the Giraddi data, strongly indicate that multipotent fetal mammary epithelial cells begin lineage specification by E15.5³. The male fetuses' reproductive system secretes testosterone by E14.5 causing degeneration of mammary gland. Understanding the differences in gene expression profiles of both male and female mammary epithelial and mesenchymal tissues at E14.5 is an important initial step towards understanding how mammary cell development potential is controlled by sex-specific cues. This information will ultimately shed light on the effects of high maternal testosterone present in some women during pregnancy, for example, women with polycystic ovarian syndrome.

Since we are using a different strain of mouse, CD-1, which is an outbred mouse, and we are not introducing biased cell selection due to FACS sorting of mammary epithelium and mammary mesenchyme with single marker antibodies, we anticipate getting different results than previous studies. This chapter includes the optimized protocol for single-cell dissociation of mammary epithelium and mammary mesenchyme at E14.5 which was used to collect cells for performing scRNA-seq.

4.3 Methods:

Mice

CD-1 fetuses were obtained from E14.5 timed pregnant dams received from Charles River. All mice were handled in accordance with the Animal Study Protocol [R-APR-23-21] approved by Institutional Animal Care and Use Committee (IACUC). Dams were properly housed in the Animal Facility within the Department of Animal Sciences in the University of Maryland College

Park with routine daily care. On the day of experimentation, pregnant dams and fetuses were euthanized using CO₂ followed by cervical dislocation.

Mammary tissue dissection

Both male and female mammary tissues at E14.5 were analyzed to determine the differences in gene expression within mammary epithelial and mesenchymal cell types in response to androgen signaling in males vs females. Cells to be submitted for scRNA-seq (Azenta, Burlington, MA, USA) will consist of pooled, dissociated mammary rudiment cells from all five pairs of all female or male embryos harvested from three dams. The sex of each fetus was determined by genotyping as described in Chapter 3. Cells were harvested from fetuses from one dam per day. E14.5 embryos were pinned down on their flank in cold DPBS, tail pieces were excised for genotyping, and the heads and limbs were removed. Deep incisions were made dorsal to the limbs from anterior end to the posterior end on each side. Dorsal tissue and any attached viscera were removed from the ventral flank containing all ten rudiments. Excised tissues (ventral flanks contain pairs #1-3 or #4-5) were kept in RPMI without phenol containing 1x Anti-Anti and 10% FBS until digestion.

Mammary tissue dissociation into single cells

Pooled mammary rudiments from each fetus were temporarily stored in RPMI without phenol/1x Anti-Anti/ 10% FBS and then transferred to 1ml of freshly made 2.5 U/ml Dispase II solution (Roche, 04942078001). Pooled rudiments were incubated on ice until the epidermis started to detach from the underlying tissue (~45 minutes). Dispase II was replaced with pancreatin-trypsin solution and then incubated at room temperature for 5 minutes. The pancreatin-trypsin solution was made by first mixing 0.375 g of 1000-2000 unit/mg trypsin with 13.5 ml Tyrode's solution,

1.5ml of 10x pancreatin solution, and 30 ul of 10,000 U/ml penicillin-streptomycin. The detachment of epidermis and digestion of mesenchyme were observed in 5 minutes of incubation. Rudiments were then quickly transferred to the RPMI solution and kept on ice for 30 minutes to inactivate the pancreatin-trypsin. Mammary epithelial buds were isolated from the tissue with a few layers of mammary mesenchyme attached and kept in the RPMI media on ice. Once all the buds were isolated and fetuses genotyped, the male and female buds were pooled together by sex in separate 2 ml tubes. Next, 1.5 ml collagenase (4mg/ml) -hyaluronidase (300 U/ml; Sigma, H3884) solution in DMEM/F12 were added to the tubes and samples were incubated in a thermomixer at 37°C for 90 minutes at 500 rpm. Pooled dissociated rudiments were then transferred to 15 ml tubes and cells were pelleted by centrifuging the tubes at 22°C for 3 minutes at 300 g. The supernatant was aspirated, and cells were reconstituted with 1ml of DME/F12 media supplemented with 10% FBS and centrifuged again at 300 g. The supernatant was removed, and the fresh 0.5 ml of cell recovery freezing media was added to the tube. Cell numbers per sample were counted using a Corning Cell Counter. The reconstituted cells were transferred to the cell freezing tube and then kept in -80°C in Mr. Frosty. Samples were then transferred to liquid N₂ storage the next day.

4.4 Preliminary Results:

4.4.1 Trouble shooting of single-cell dissociation protocol. We adapted the protocol published in Carabana *et al.*, 2024 to do single-cell dissociation of mammary epithelium and mammary mesenchyme³. The protocol is mentioned in the methods. Initially, we started digestion of dissected tissue in 1.25U/ml Dispase II for 15 minutes on ice according to the previously published protocol. However, we could not see the separation of epidermis as suggested. Hence, we doubled the concentration of dispase II to 2.5U/ml and incubated the tissue on ice for ~45 minutes as

performed by Giraddi *et al.*, 2018 ². While isolating mammary bud from mesenchyme, we observed the morphological differences between male and female mammary buds. The male mammary buds were smaller and more difficult to identify and isolate following trypsin-pancreatin digestion as compared to female mammary buds.

4.4.2 Quantification of number of cells collected.

We dissociated mammary buds from E14.5 of both male and female mouse embryos. We dissociated mammary buds with a few layers of surrounding mesenchyme attached as we want to explore the different cell types present in mammary mesenchyme. The number of cells collected is listed in Tables 1, 2, 3 and 4.

Table 5: Number of mammary buds dissociated, and number of cells collected on day 1.

Sample Collection:	Mouse #1	
Fetus	Male	Female
1	4	
2		5
3		4
4	10	
5		5
6	9	
7		5
8		5
9		2
10		5
11	7	
Total Rudiments	30	31
% Retrieval	75%	44%
Cell/ml	244000	337000
Total Cells Frozen	122000	168500
Cells per rudiment	4067	5435

Table 6: Number of mammary buds dissociated, and number of cells collected on Day 2.

Sample Collection:	Mouse #2	
Fetus	Male	Female
1		1
2		7
3		4
4	7	
5	2	
6	5	
7		1
8		6
9		6
10		9
11		1
12	3	
Total Rudiments	17	35
% Retrieval	43%	50%
Total Cells Frozen	0*	0*

Note: For mouse #2, both pooled single-cell samples were lost due to supernatant aspiration error.

Table 7: Number of mammary buds dissociated, and number of cells collected on Day 3.

Sample Collection:	Mouse #3	
Fetus	Male	Female
1		5
2		8
3		7
4	6	
5	5	
6		7
7	9	
8		5
9	7	
10		6
11	6	
12	7	
Total Rudiments	40	38
% Retrieval	67%	63%
Cells/ml	103000	103000
Total Cells Frozen	51500	51500
Cells per rudiment	1288	1355

Table 8: Number of mammary buds dissociated, and number of cells collected on Day 4.

Sample Collection:	Mouse #4	
Fetus	Male	Female
1		3
2	4	
3		6
4	7	
5		6
6	4	
7	2	
8	5	
9	2	
10		4
11		3
Total Rudiments	24	22
% Retrieval	40%	44%
Cells/ml	205000	352500
Total Cells Frozen	103000	176250
Cells per rudiment	4292	8012

4.4.3 Encountered pitfalls.

There are mechanical and enzymatic ways of cellular dissociation of tissue. The application of mechanical dissociation technique in the embryonic mammary rudiments is not feasible as the mammary rudiment is deeply embedded in mesenchyme underneath a highly elastic and attached epidermis. The mechanical dissociation can also cause damage to the cells. Hence, we chose to do the enzymatic digestion. We faced some difficulties during the single-cell dissociation of mammary rudiments with attached mammary mesenchyme at E14.5. We optimized the previously published protocol of enzymatic dissociation of mammary tissue^{2,3} so that we could collect as much cells as possible. The duration of dispase treatment and its concentration can affect the experiment. Initially, when we treated the mammary tissue from E14.5 with 1.25U/ml dispase II for 15 minutes, we did not obtain separation of epidermis. This led to greater difficulties in

microdissections and significantly longer dissection times. The incubation time of tissue with pancreatin-trypsin should not be more than 5-8 minutes. Longer incubations can cause over digestion of the bud and the mesenchyme. Because of the risk of losing buds during dissociation, in order to collect sufficient cells, embryos from at least 3-4 pregnant dams with the average litter size of 12 should be used. When pelleting the collected cells, it is important to control the rate of aspiration and use conical 15 ml tubes than shallow 50 ml tubes. It should be anticipated that freeze-thawing of cells, cell death will also decrease yield and viability.

4.5 Discussion:

Single-cell RNA sequencing (scRNA-seq) is a widely used technology to identify unique cell types in a tissue⁵⁷. Giraddi² and Carabana³ used this technique to investigate lineage specification and segregation of fetal mouse mammary epithelium. Collectively, their data showed that the mouse mammary epithelium is uncommitted until E14.5, undergoes lineage specification at E15.5, and begins lineage segregation into luminal and basal lineages by P4. There could be strain-specific variation in these embryonic stages when the lineage specification begins in CD-1 mouse (the strain of mouse we studied) versus C57BL/6 (the mouse strain Giraddi and Carabana studied)³. We observed two different population of cells in terms of *Pthlh*, *Lef1*, *Snail2* and *Notch1* at E14.5 in our RNA-FISH data described in Chapter 3. The mouse mammary tissue undergoes sexual dimorphism by E14.5 as the male fetus starts producing testosterone by E14.5. There should be variation in gene profile of the mouse mammary epithelium and the mammary mesenchyme at E14.5. It is possible that more variants of cell types than reported by Carabana and colleagues³ exist at E14.5. Hence, we are performing scRNA-seq of the mammary epithelium and the mammary mesenchyme of male and female mouse embryos at E14.5.

Chapter 4 describes our optimization efforts of a scRNA-seq protocol based on Giraddi² and Carabana³ single-cell dissociation methods. We initially applied their exact protocols, however, isolation of mammary rudiments from the overlying epidermis and difficulty in locating the mammary rudiments, especially male rudiments, led us to troubleshoot. The optimized protocol is described in the methods of Chapter 4. We have collected 1.73×10^5 cells (mammary epithelial + mammary mesenchymal) from the E14.5 male embryos and 2.2×10^5 cells (mammary epithelial + mammary mesenchymal) from the E14.5 female embryos. Because cells losses are inevitable during freeze-thawing, we will collect cells from one more mouse litter and send our samples to the Azenta Biotechnology Company for scRNA-seq. We anticipate seeing different gene profiles and different cell types between male and female mammary tissue at E14.5. Our scRNA-seq data of male mammary tissue will provide insight into the effects of testosterone on the genetic signatures of embryonic female mammary rudiments from mothers exhibiting elevated levels of testosterone circulation in their body during pregnancy, such as in pregnant women with polycystic ovarian syndrome.

CHAPTER 5:

CONCLUSIONS AND FUTURE DIRECTIONS

We performed RNA-FISH on the mammary tissues from E13.5, E14.5 and E15.5 to understand spatiotemporal gene expression patterns of early mammary regulators during embryonic mouse mammary gland development. The data were analyzed qualitatively. The mammary epithelium was observed to be primed for lineage segregation by E14.5 where *Notch1* was expressed more in the center cells and *Pthlh*, *Lef1* and *Snail2* were more expressed in the peripheral cells of the mammary rudiments. *Sox11* expression levels were upregulated in E15.5 versus levels at E13.5 and E14.5. *Sox10*, and *Lmo4* were expressed in both the mammary epithelium and the mammary mesenchyme in all three stages studied. *Sox9* and *Gata3* were exclusively expressed in the mammary epithelium. We optimized the single-cell dissociation protocol for collecting the mammary epithelial and mammary mesenchymal cells from male and female embryos at E14.5 which will be used for performing scRNA-seq.

Out of 12 genes studied for the first aim, the spatiotemporal expression pattern of *Tbx3*, *Trp63*, and *Elf5* could not be determined. We encountered photobleaching issues while imaging 750 fluorophore conjugated hairpins. The probes for these three gene are designed to be tagged by 750 fluorophore conjugated hairpins. We are redoing RNA-FISH for these three genes and image using confocal microscope set at the lowest possible gains and intensity. All our RNA-FISH data are from whole-mount RNA-FISH which limited us to study four probes at a time. One of our future directions is to optimize RNA-FISH protocol for tissue sections so that all our 12 genes of interest can be tested on the same section with DNase treatment after each RNA-FISH with four probe sets and imaging. It will increase the probability of finding more cells at different hybrid states of specification. All of our present data would be quantified by doing IHC of sections with

E-cadherin /647 conjugated fluorophore. E-cadherin is a membrane bound protein that is expressed in mammary epithelial cells. Wheat Germ agglutinin (WGA) staining has been used to stain and outline the cell membrane of the mammary tissue⁵⁸. It has been used mostly in breast cancer cells so its reactivity to the embryonic mammary epithelium would be interesting to observe. Since it stains both the epithelial and mesenchymal cell membrane, overlaying the WGA-stained image and RNA-FISH image might be difficult without DAPI staining of the nucleus.

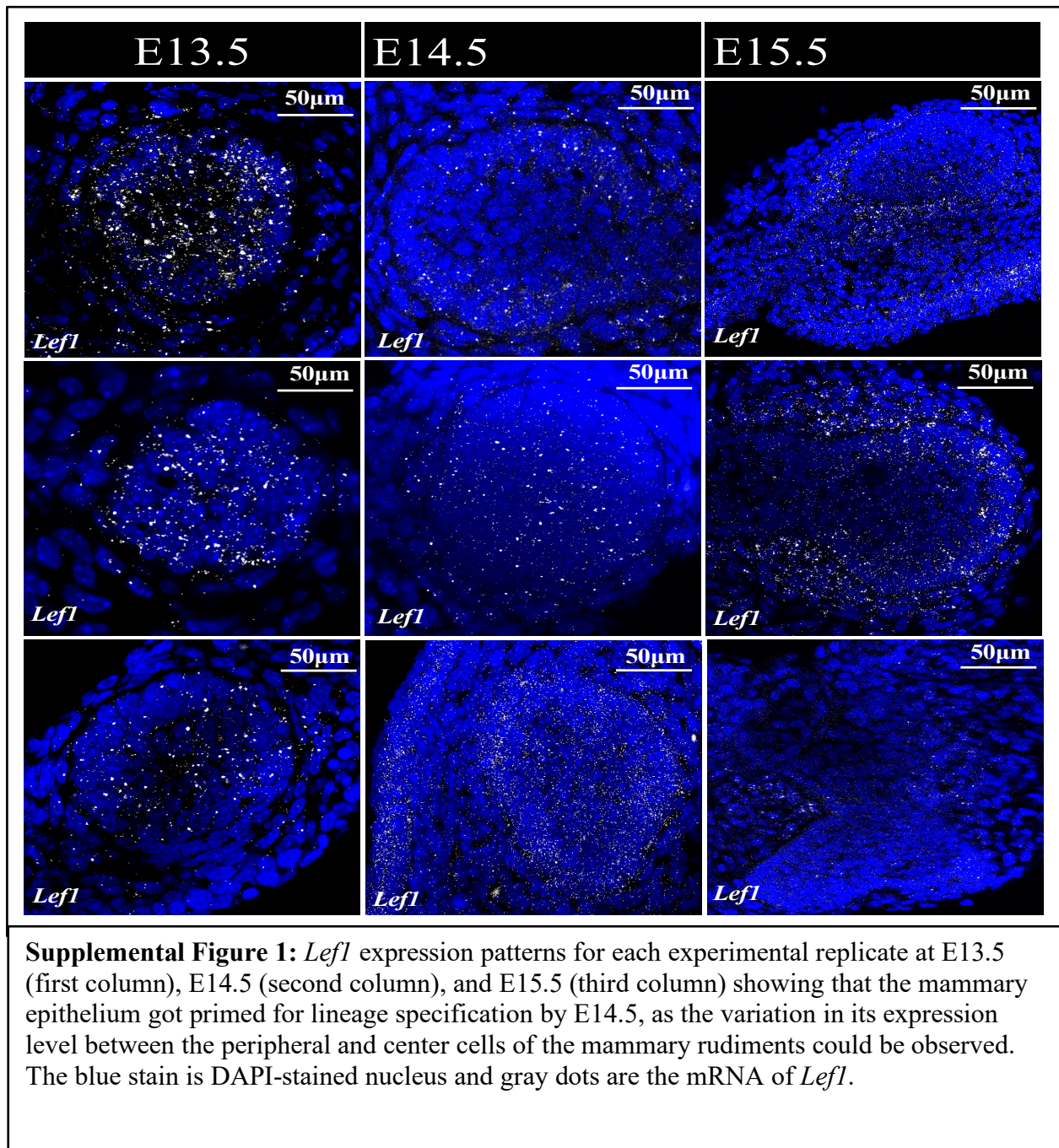
After collecting the required number of cells for scRNA-seq, they will be sent for scRNA-seq. The scRNA-seq data will identify the cells of different hybrid specifications present in the male and female embryonic mammary epithelium and mammary mesenchyme at E14.5. This data will be used to understand the genetic differences in the male mouse mammary tissue due to testosterone exposure. We might be able to detect proteases, if any, that are involved to sever the mammary rudiment from the epidermis in male embryos. We will be able to observe the downregulation of mammary epithelial receptors for various ligands as the mammary epithelium undergoes degeneration in male embryos. The scRNA-seq data will be used to determine more gene markers of the early embryonic mammary gland which can be used for doing more RNA-FISH in the future. One of our major future directions is to understand the effects of high testosterone levels present in circulation of pregnant female in the mammary gland of her female embryos. For instance, women with polycystic ovarian syndrome have been reported to have higher levels of testosterone, which could affect the mammary rudiment as ARs are present in mammary mesenchyme at this stage.

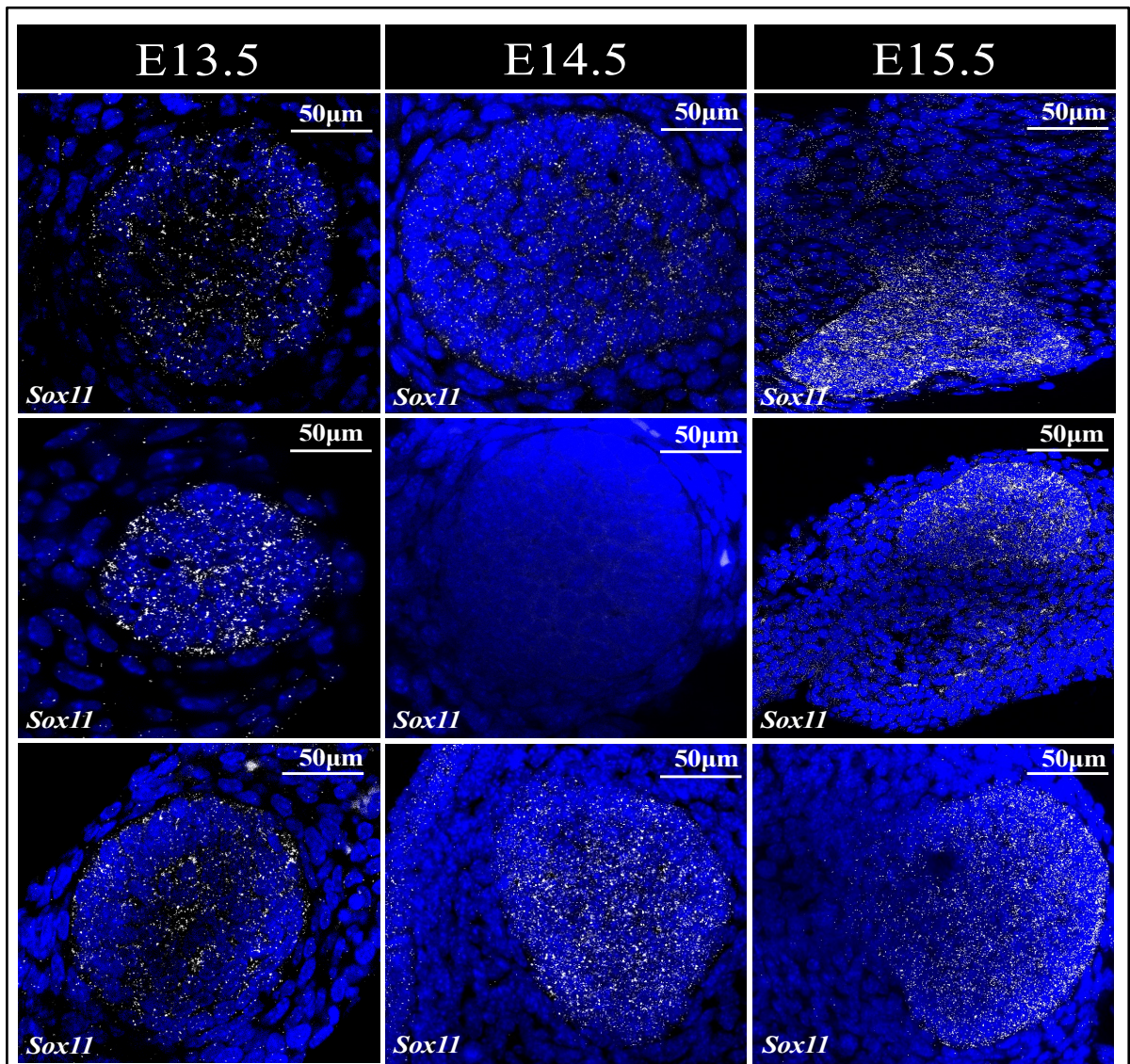
The overall goal of this research project is to understand the normal gene regulatory network of the embryonic mammary gland so that it can be used as a reference to determine genetic and epigenetic changes in the embryonic mammary gland due to exposure to various adverse

maternal environment. Identification of these changes protect fetuses from adverse maternal environment.

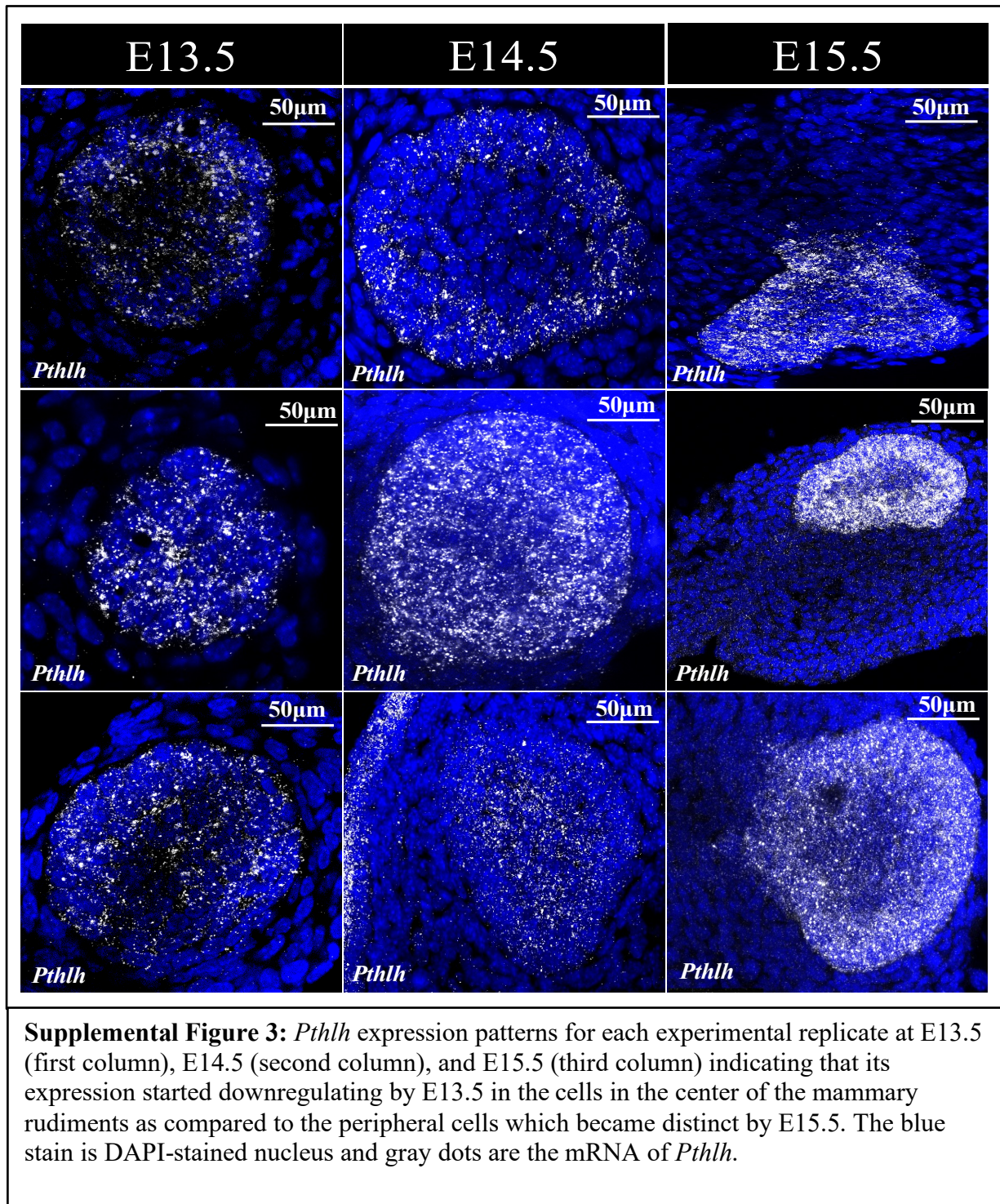
APPENDIX: SUPPLEMENTARY FIGURES

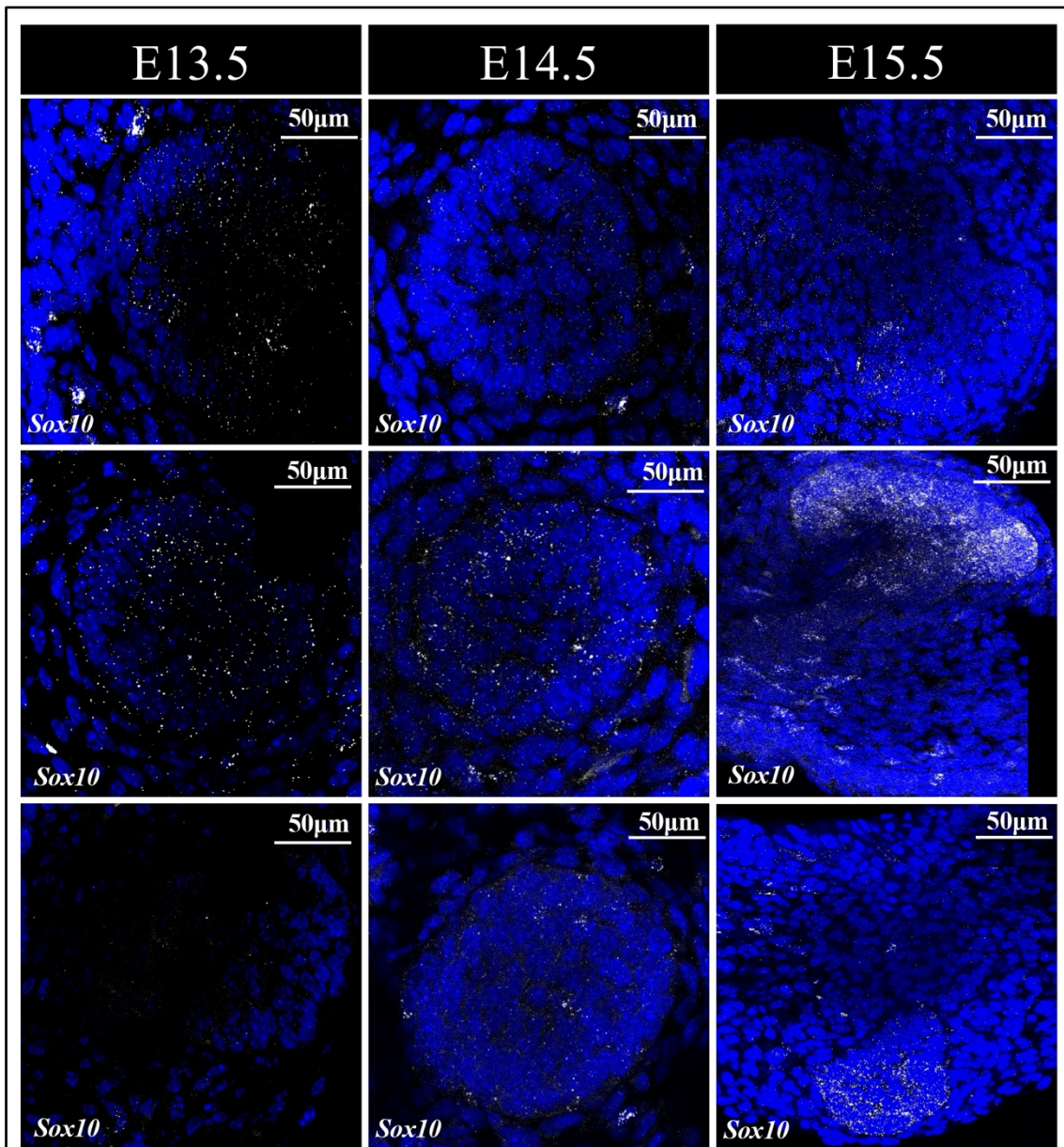
Our supplementary figures include the compiled images of n=3 RNA-FISH images taken under the confocal microscope (63x oil immersion) of all three embryonic stages (E13.5, E14.5, and E15.5) for *Lef1* (supplementary figure 1), *Sox11* (supplementary figure 2), *Pthlh* (supplementary figure 3), *Sox10* (supplementary figure 4), *Snail2* (supplementary figure 5), *Sox9* (supplementary figure 6), *Notch1* (supplementary figure 7), *Gata3* (supplementary figure 8), and *Lmo4* (supplementary figure 9). Supplementary figures 4, 5 and 6 include n=3 RNA-FISH data for E13.5 and E14.5, and n= for E15.5 for *Sox10*, *Snail2*, and *Sox9* respectively. The first column of supplementary figures 1 to 9 includes all RNA-FISH data for E13.5, the second column for E14.5, and the third column for E15.5 (tiled images). The scale bar for each image is 50µm. The supplementary figure 10 is the aggregate of RNA-FISH data for *Elf5* (first row), *Trp63* (second row), and *Tbx3* (third row). The first column (A) indicates their images from E13.5, the second column (B) for E14.5, third column (C) and fourth column (D) for E15.5 where D is the magnified version of image C focusing on the terminal bud, and the last column € is the negative control where RNA-FISH was done with just hairpins only.



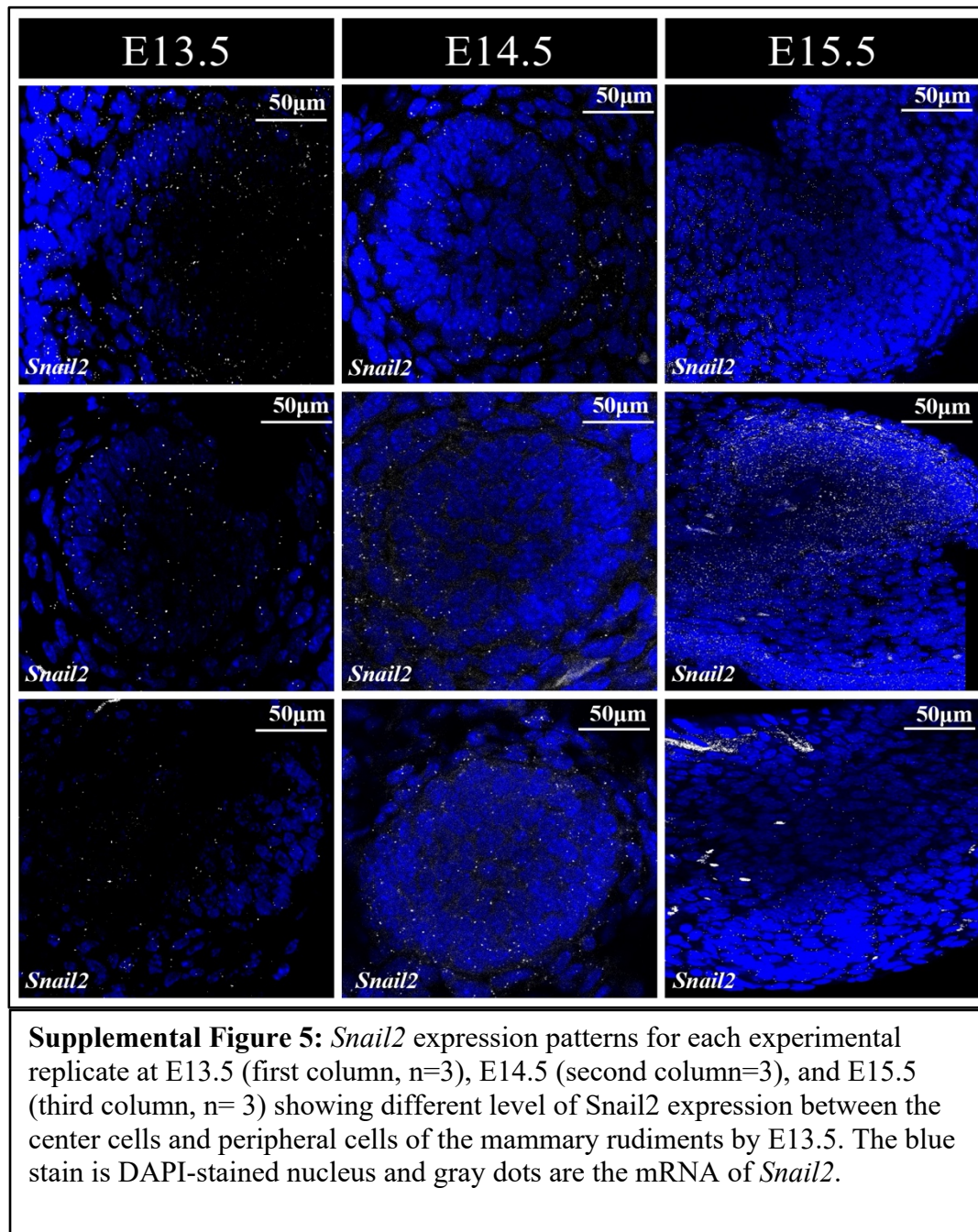


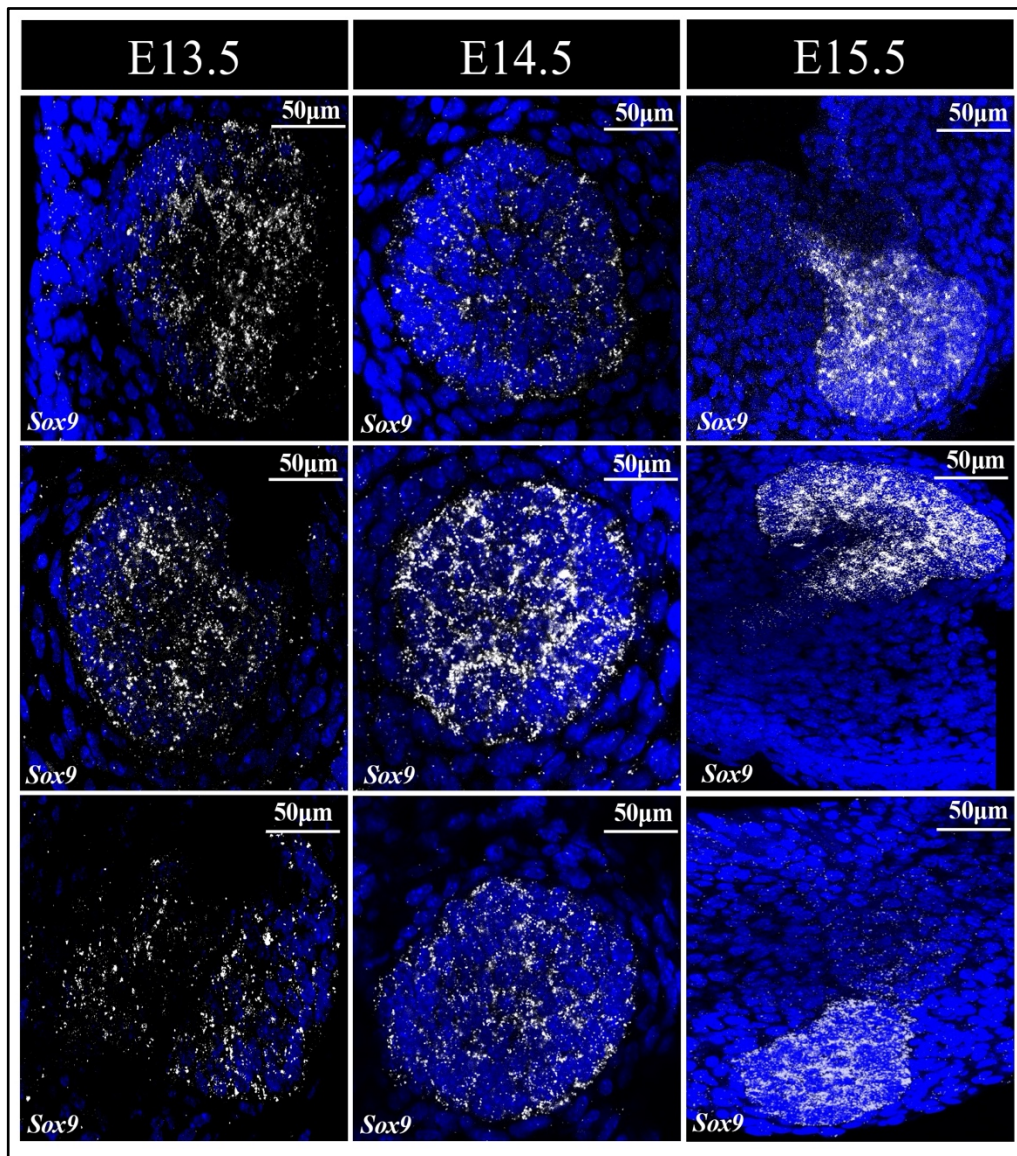
Supplemental Figure 2: *Sox11* expression patterns for each experimental replicate at E13.5 (first column), E14.5 (second column), and E15.5 (third column) indicating that Sox 11 was observed to be expressed by the mammary epithelium and overlying epidermis. Its expression was found to be higher at E15.5 as compared to E13.5 and E14.5. The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Sox11*.



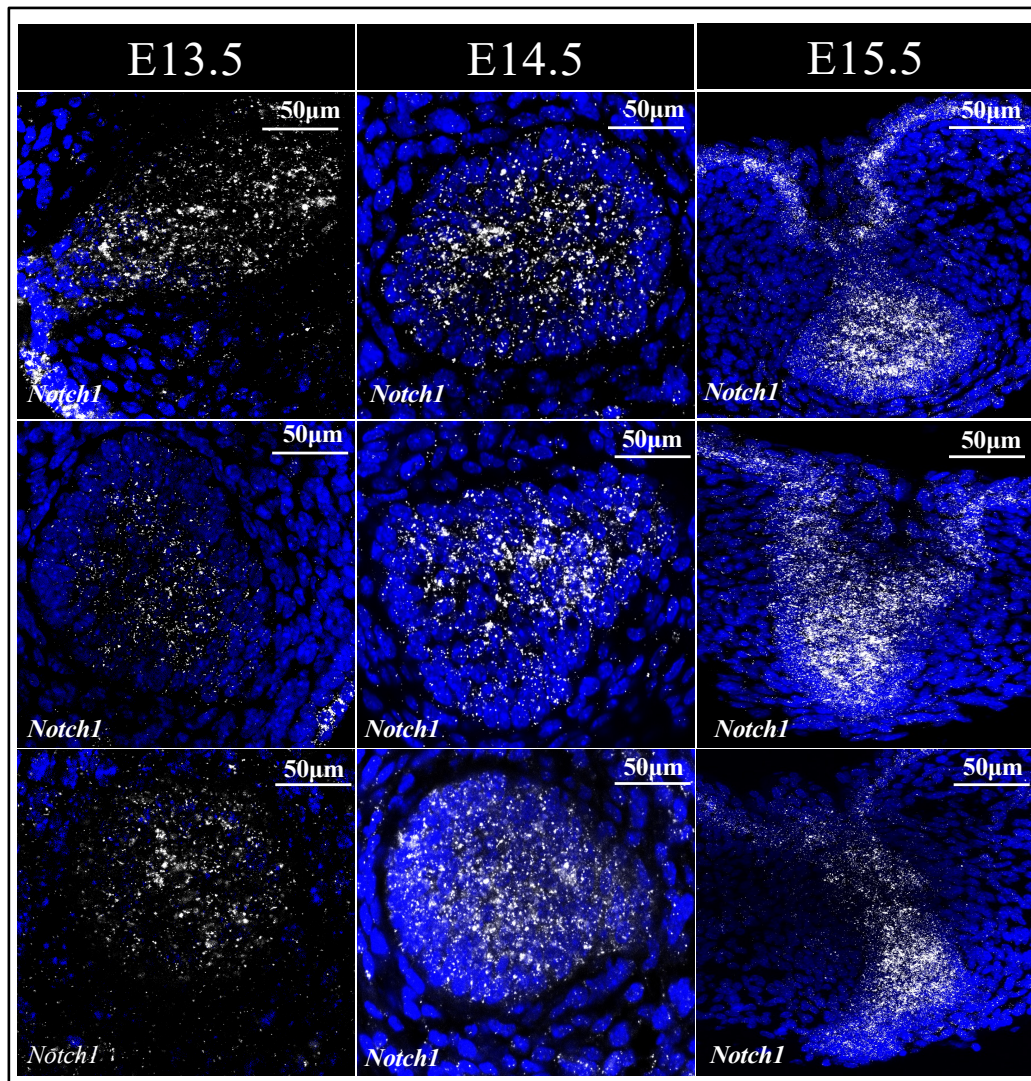


Supplemental Figure 4: *Sox10* expression patterns for each experimental replicate at E13.5 (first column), E14.5 (second column), and E15.5 (third column, n= 3) showing that *Sox10* was observed to be expressed in the mouse mammary rudiments by E13.5. The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Sox10*.

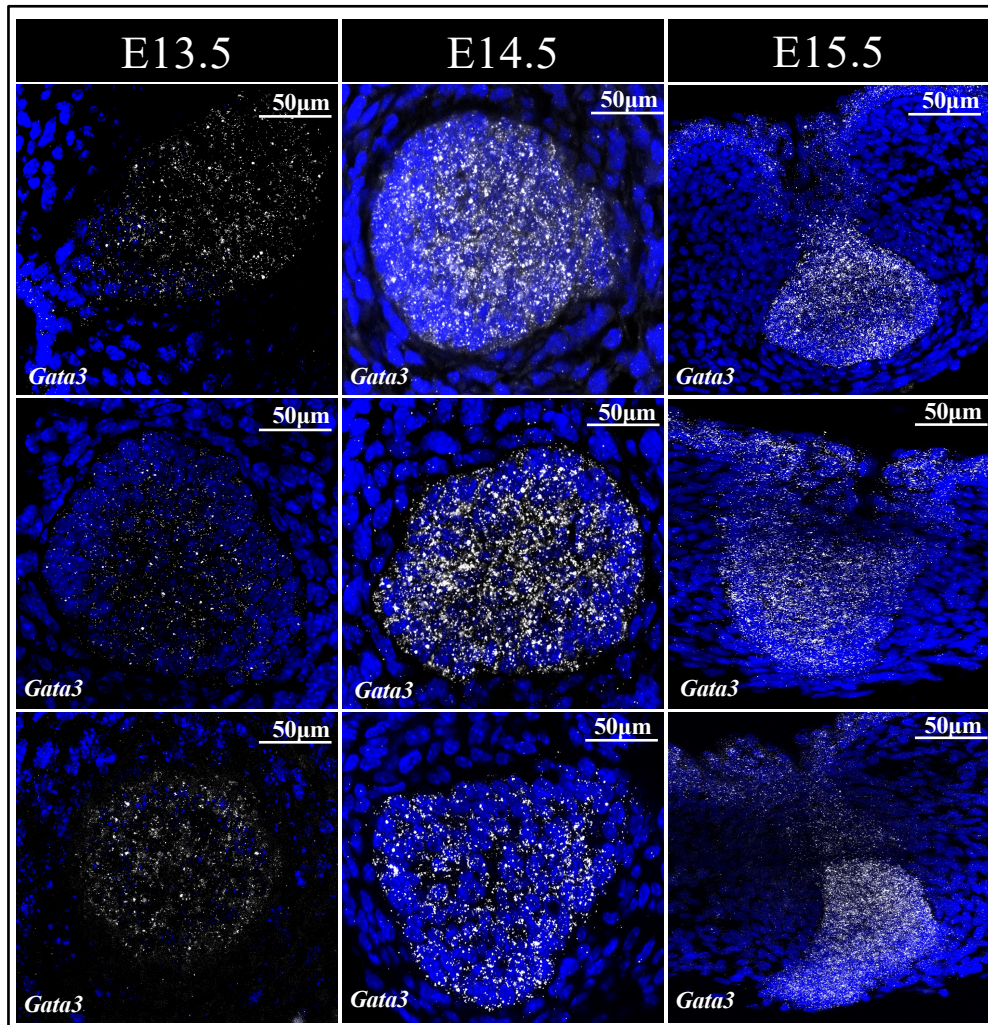




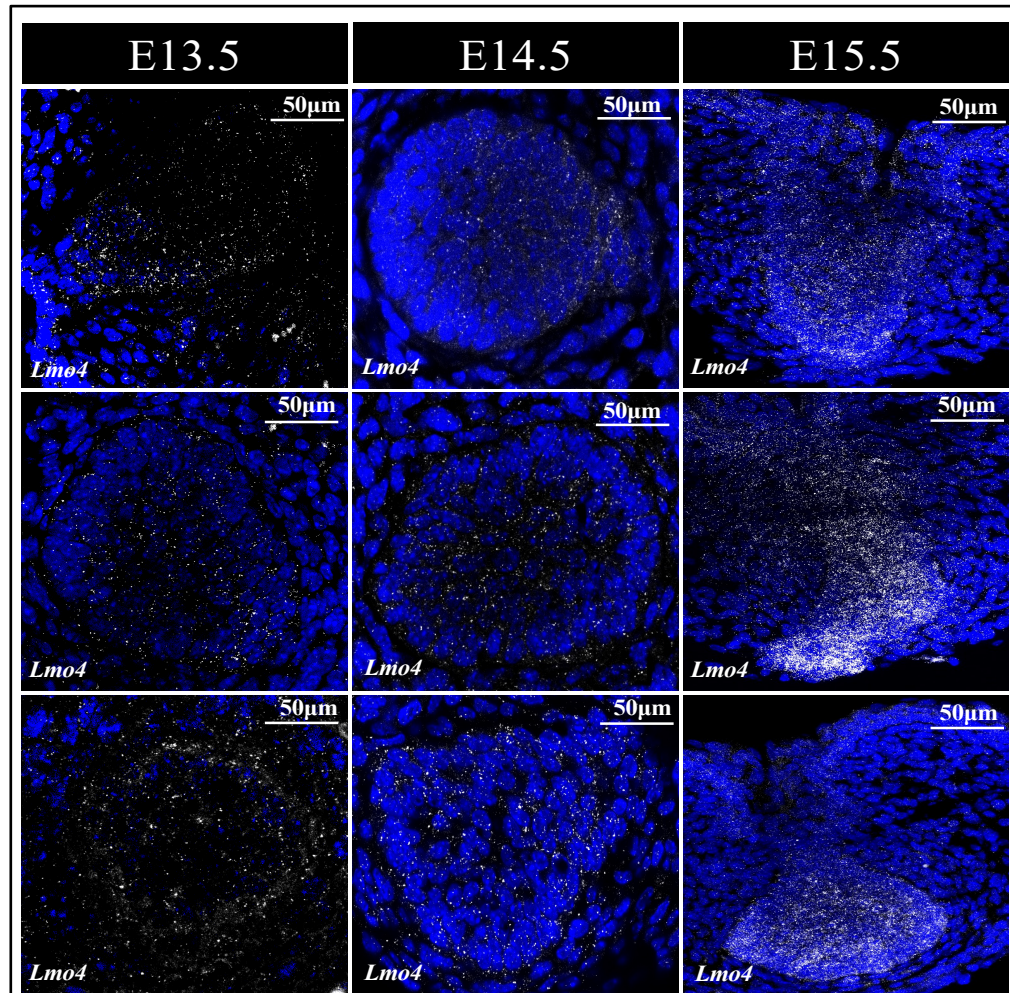
Supplemental Figure 6: *Sox9* expression profile representing its abundant expression level in the mammary rudiment in all three stages, E13.5 (first column, n=3), E14.5 (second column, n=3), and E15.5 (third column, n=3). The expression of *Sox9* was exclusively by the mammary rudiment where its expression in the mammary mesenchyme was negligible. The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Sox9*.



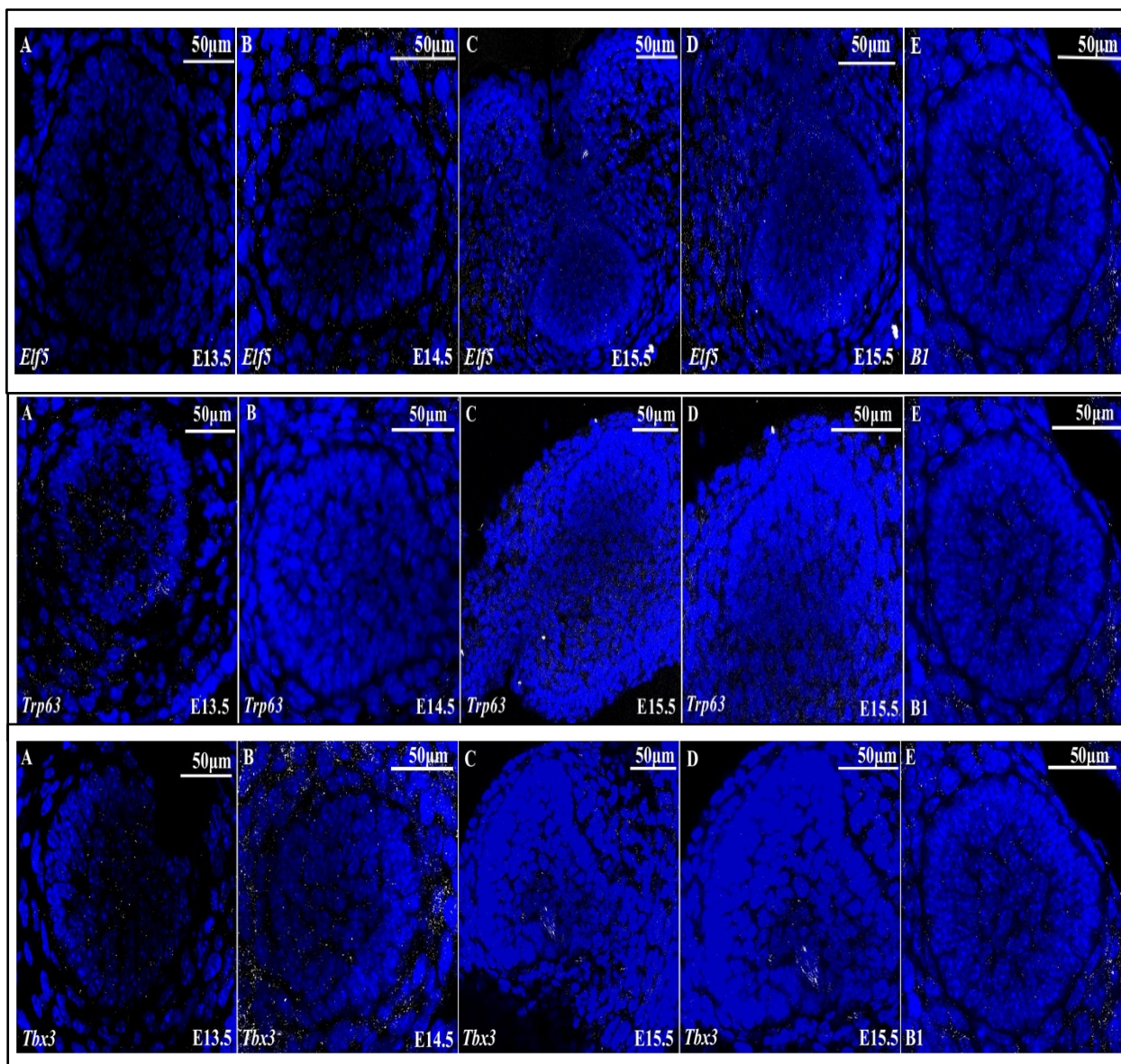
Supplemental figure 7: *Notch1* expression at E13.5 (first column), E14.5 (second column), and E15.5 (third column) indicating the change in *Notch1* expression pattern between peripheral and center cells of the mammary rudiments by E13.5. The peripheral cells were observed to have downregulated *Notch1* expression while the center cells have upregulation indicating that mammary cells are primed for lineage specification by E13.5-E14.5. The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Notch1*.



Supplemental Figure 8: Qualitative analysis of RNA-FISH data showed that *Gata3* was expressed exclusively by mammary epithelium like *Sox9* at E13.5 (first column, n=3), E14.5 (second column, n=3), and E15.5 (third column, n=3). The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Gata3*.



Supplemental Figure 9: *Lmo4* was observed ubiquitously expressed in the mammary rudiment and the mesenchyme at E13.5 (first column), E14.5 (second column), and E15.5 (third column). At E15.5, its expression was more in the mammary rudiment in comparison to the mammary mesenchyme. The blue stain is DAPI-stained nucleus and gray dots are the mRNA of *Lmo4*.



Supplemental Figure 10: *Elf5*, *Trp63* and *Tbx3* have been reported to expressed in the mouse mammary tissue by E12.5. We could not observe their expression patterns from E13.5 to E15.5 in the mouse mammary tissue potentially due to accidental photobleaching. The blue stain is DAPI-stained nucleus.

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