Exploring the Wnt enhancer landscape in the mammary gland

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Chapter 1

General Introduction: WNT signaling in the mouse mammary gland
The mammary gland is a defining characteristic of mammals. Its primary function is to produce and secrete milk for the neonatal offspring\(^1\). In addition to satisfying these nutritional needs, the mammary gland also secretes immunoglobins to provide the newborn with immune protection\(^2\). Breast cancer finds its origin in the epithelial cells of this tissue, affecting 1 out of every 8 women in their lifetime\(^3\). Given the complex heterogeneous landscape of breast cancer subtypes, both in terms of histopathological features, molecular lesions and response to treatment, an important step towards understanding the origin of breast malignancies is learning how development and homeostasis is controlled in the healthy breast\(^4\). An excellent model system to study these properties, is the mouse mammary gland\(^5\).

The mouse has five pairs of mammary glands on contralateral sides of the body (Figure 1A). Each gland consists of a fat pad into which a network of epithelial ducts has grown out, starting at the nipple (Figure 1B). These two-layered ducts are composed of an inner luminal layer and an outer basal layer of epithelial cells and are surrounded by a collagen-rich extra-cellular matrix. Adipocytes are the main component of the mammary gland stroma, which also contains collagen-producing fibroblasts. Other components of the stroma are vascular and immune cells (Figure 1C).
Mammary gland development

Development of the mouse mammary gland starts at embryonic day 10 (E10). At this stage, two milk lines are formed on the ventral side, between the fore- and hindlimbs. Five pairs of multilayered ectodermal structures, called placodes, are detectable on the milk lines at day E11.5. At E12.5, these placodes develop into mammary buds, which are morphologically distinct bulbs of epithelial cells. These buds sink into the underlying dermal mesenchyme at E13.5, and by E14.5 they can no longer be detected externally. At E15.5, the mammary buds start forming a ductular structure, called the mammary sprout. This sprout invades the underlying fat pad precursor and
by E16.5 starts forming a lumen. The lumen starts to open on the skin side, where the nipple is formed. At E18.5, just before birth, the mammary sprouts have formed a small rudimentary ductal tree, which occupies only a small portion of the fat pad close to the nipple.

From birth to puberty, the mammary gland epithelium stays relatively quiet and grows at a rate that is isometric with body weight gain. Before puberty, the ductal network continues to occupy only a small part of the mammary fat pad (Figure 2A). At the onset of puberty, the mammary gland epithelium starts to undergo extensive branching under the influence of estrogen, in a process called branching morphogenesis. This is characterized by the appearance of terminal end buds (TEBs) that are located at the tips of invading ducts. The expansion of the epithelial ducts is caused by proliferation in the TEBs, which happens most rapidly between 4 to 7 weeks of age. When the distal end of the fat pad is reached, the TEBs regress. By the time the mouse reaches the end of puberty, the tree of epithelial ducts has fully invaded the mammary fat pad.

**Maintenance of the adult mammary gland**

During the reproductive life of a female mouse, the morphology of the mammary gland changes regularly. Subtle morphological changes can occur during the estrous cycle, which is repeated every 4 to 5 days. The estrous cycle consists of four stages: proestrus, estrus, metestrus, and diestrus. It has been reported that during the estrous cycle outgrowth and regression of lobuloalveolar epithelium can occur due to fluctuations of steroid hormone levels (Figure 2B). However, it is not completely clear when the outgrowth takes place; some studies report that this happens during late proestrus/estrus, while other studies show that the proliferative phase occurs during diestrus. However, there is consensus about the timing of apoptosis; this happens mainly in the diestrus stage. It is also important to note that this heightened proliferative state of the mammary epithelium does not occur during every estrous cycle. In summary, the morphology of the mammary gland can change during the estrous cycle, but there is no clear consensus about the timing of the outgrowth and these proliferative changes are not well understood.

More dramatic morphological changes occur during pregnancy, when the mammary gland is preparing for the lactation stage. Throughout pregnancy, progesterone causes extensive side-branching of epithelial ducts and initial growth of alveolar buds (Figure 2C). Together with prolactin, these hormones induce growth of alveolar buds into large lobuloalveolar structures. These structures produce milk for the offspring during lactation, which is squeezed from the alveolar cells by surrounding myoepithelial cells. At the end of lactation, when weaning has caused a lack in milk demand, the process of involution is started. There are two phases of involution. The first, reversible, phase is induced by accumulation of milk in the lumen of the ducts and is characterized by initial apoptosis of epithelial cells. After approximately 72 hours, the irreversible phase starts, accompanied by widespread apoptosis and active tissue remodeling. At the end of involution, after 10 to 15 days, the mammary gland has been restored to its pre-pregnancy state. The cycle of alveolar differentiation, lactation, and involution is repeated with every pregnancy.
General Introduction

Figure 2: The mouse mammary gland is a dynamic tissue with high regenerative capacity. A) Illustration of mammary gland morphology during puberty. At the start of puberty, the epithelial ducts form a rudimentary tree. Under the influence of hormones, this rudimentary tree grows out into the fat pad during puberty. This phase is characterized by terminal end buds (TEBs). At the end of puberty, the epithelial network has reached the distal end of the mammary fat pad. B) Representation of the morphological changes that can occur during the estrous cycle. Outgrowth and regression of lobuloalveolar epithelium during the estrous cycle has been reported, although there is no consensus about the timing of the proliferative phase. Regression occurs mainly in diestrus. C) Representation of the morphological changes in the mammary gland during pregnancy. Lobuloalveolar structures, which contain milk producing cells, grow out during pregnancy. See text for details. This cycle of lobuloalveolar outgrowth, lactation, and involution, is repeated with every pregnancy.

Mammary stem cells

The high regenerative potential of the mammary gland, shown by the many cycles of growth and regression during the estrous cycle and pregnancy, suggests that stem cells are present in the epithelium. Stem cells are defined by their capacity to self-renew and generate daughter cells that can differentiate into more specialized cell types.

Stem cell like behavior of mammary epithelial cells was first demonstrated by transplantation assays in the late 1950's and early 1960's. In these assays, fragments of normal donor epithelium were transplanted into a cleared (i.e. de-epithelialized) fat pad of the recipient mouse. The transplanted fragments were capable of reconstituting an epithelial ductal tree that filled the entire

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fat pad. In the 1990’s it was suggested that one single cell had the capacity to grow an entire functional ductal network after transplantation into a fat pad\textsuperscript{29}. Evidence for this was presented in 2006, when cell populations enriched for mammary stem cells (MaSCs) could be isolated using Fluorescence-Activated Cell Sorting (FACS)\textsuperscript{30,31}. Both luminal and basal cells were derived from one single transplanted, multipotent cell. Serial transplantations of the epithelial outgrowth demonstrated the self-renewing capacity of these MaSCs. Although transplantation assays have been the gold standard assay for evaluating MaSCs\textsuperscript{32,33}, they do not show evidence that these cells also act as stem cells during physiological processes, such as tissue development and maintenance. Therefore, cells that are able to reconstitute an epithelial ductal network after transplantation, are currently referred to as mammary repopulating units (MRUs), rather than MaSCs. In addition to the question whether MRUs are important for the physiological growth and maintenance of the epithelial ducts, there are other concerns regarding the transplantation assay. The conditions in which these assays are carried out can dramatically influence the efficiency of the outgrowth. For example, epithelial cells from a donor in the diestrus stage, showed a 14-fold increase in absolute number of MRUs compared to cells from a donor in estrus, showing that the estrus cycle can change the reconstitution efficiency\textsuperscript{14}. Furthermore, the use of Matrigel has shown to greatly increase the transplantation efficiency\textsuperscript{34}. It is also possible that environmental cues influence the behavior of the injected donor cells, as for transplantation assays surgical resection is needed, which might induce a “wound healing” response\textsuperscript{35}. Moreover, transplantation assays can stimulate a higher level of plasticity, which is not observed under physiological conditions\textsuperscript{36,37}. Overall, even though it is important to keep in mind these disadvantages, the transplantation assay has been instrumental in demonstrating the regenerative capacity of the mammary gland.

Another method to investigate stem cell populations in the mammary gland, is \textit{in situ} lineage tracing. Using lineage tracing, stem cells can be tracked and their contribution to the development of a tissue can be investigated\textsuperscript{38}. Like the transplantation assay, lineage tracing of MaSCs has also not been free of confusion, especially regarding the potency of MaSCs. Single color lineage tracing showed that during puberty, unipotent stem cells give rise to either the basal lineage or the luminal lineage\textsuperscript{36}. In contrast, another study that used multicolor lineage tracing, suggested that during puberty stem cells are present that contribute to both lineages\textsuperscript{39}. Bipotent stem cells that were identified in this study were labeled for either Krt5 and Krt14 (both basal cell markers), and Lgr5 (putative stem cell marker). In addition, also a unipotent luminal stem cell population was identified in the pubertal mammary gland, marked by Elf5 expression\textsuperscript{39}. During pregnancy, the lobuloalveolar structures that are formed are thought to arise from luminal progenitors\textsuperscript{36,39}. Bipotent stem cells that are labeled \textit{Axin2+} cells, showed that both basal and luminal alveolar cells are labeled during pregnancy, while \textit{Axin2} is only expressed in basal cells in the adult virgin mammary gland\textsuperscript{40}. This suggests that alveolar cells do not (only) arise from unipotent luminal progenitors, but from bipotent stem cells.

The discrepancies between the different lineage tracing studies can in part be explained by the different gene labeling techniques and mouse models that were used. In addition, different
concentrations of the labeling-inducing agent can result in different labeling efficiency. Furthermore, in the inducible CreER(T2)-loxP system, tamoxifen is used as labeling-inducing agent, which may influence MaSC behavior. Moreover, when tracing a lineage, it can be difficult to distinguish patches that are located close to each other. This is less difficult when using multicolor lineage tracing, but it is still possible that cells in one patch were not derived from the same labeled cell.

To conclude, the mammary gland is a dynamic tissue with high regenerative potential, as demonstrated by transplantation assays. Lineage tracing assays have given us unprecedented insights into MaSC biology, but further research in this area is needed to unravel the potency of different stem cell populations and their contribution to mammary gland development and maintenance.

The WNT/CTNNB1 pathway

The behavior of stem cells is controlled by signals that come from their microenvironment, which is called the stem cell niche. WNT ligands have been identified as niche factors in several tissues. The highly conserved Wnt gene family comprises 19 members in mammalian genomes. For the WNT/CTNNB1 pathway to be active, WNT ligands need to be correctly modified and secreted from the stem cell niche. In the endoplasmic reticulum (ER), Porcupine (PORCN) modifies the newly translated WNT proteins by attaching a palmitoleic acid group. Generally, this is considered to be essential for the secretion and functionality of WNT proteins, although PORCN independent WNT secretion has also been described. After their palmitoylation, WNT proteins bind to Wntless (WLS), which is required for trafficking to the plasma membrane and the secretion of WNT ligands into extracellular space. In the WNT/CTNNB1 pathway, WNT proteins bind to a heterodimer complex between Frizzled (FZD) and co-receptor Low density lipoprotein receptor-related protein 5 or 6 (LRP5/6) on the surface of neighboring stem cells. This leads to the recruitment of the CTNNB1 destruction complex (DC) to FZD and LRP5/6 in the cytoplasm. The DC consists of several components, including Dishevelled (DVL), AXIN, Casein Kinase-1 (CK1), Glycogen synthase kinase 3b (GSK3b), and Adenomatous polyposis coli (APC). CTNNB1 is targeted for destruction by the DC. Phosphorylation of CTNNB1 by GSK3b and CK1 is followed by ubiquitination and degradation by the proteasome. When the DC is recruited to the FZD-LRP5/6 complex however, the DC is inactivated. This results in an accumulation of CTNNB1 in the cytoplasm. CTNNB1 then enters the nucleus, where it binds to T-cell factor/lymphoid enhancer factor (TCF/LEF), which leads to the transcription of target genes. Through the years, multiple target genes of the WNT/CTNNB1 pathway have been identified. In the mammary gland they include, amongst others, Axin2, Ccn1d, c-Myc, and Tgif.
Figure 3: The WNT/CTNNB1 pathway. WNT proteins are palmitoylated by PORCN in the ER, after which they bind to WLS. WLS controls trafficking to the plasma membrane and secretion into the extracellular space. WNT proteins bind to the receptor complex FZD-LRP5/6, which leads to the recruitment and subsequent inactivation of the CTNNB1 destruction complex. This results in accumulation of CTNNB1 in the cytoplasm and translocation to the nucleus. In the nucleus, CTNNB1 binds to TCF/LEF, which causes transcription of WNT target genes.

**Wnt expression in the mammary gland**

Several members of the Wnt gene family are expressed during various phases of mammary gland development. Already during embryonic development, Wnt genes are expressed in structures that will later develop into the mammary gland (Table 1). In the milk line Wnt5a, Wnt6, and Wnt10b expression has been detected. These Wnt genes are also expressed during placode formation, together with Wnt1, Wnt2, Wnt3, Wnt7b, Wnt10a, and Wnt11. During mammary bud formation, Wnt1, Wnt2, Wnt3, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b, Wnt10a, Wnt10b, and Wnt11 are expressed.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Expressed Wnt genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk link formation</td>
<td>Wnt5a, Wnt6, Wnt10b</td>
</tr>
<tr>
<td>Placode formation</td>
<td>Wnt1, Wnt2, Wnt3, Wnt5a, Wnt6, Wnt7b, Wnt10a, Wnt10b</td>
</tr>
<tr>
<td>Mammary bud formation</td>
<td>Wnt1, Wnt2, Wnt3, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b, Wnt10b, Wnt11</td>
</tr>
</tbody>
</table>

Table 1: Wnt gene expression during embryonic mammary gland development. Adapted from Yu et al.

In the pubertal mammary gland, Wnt2 is expressed in the TEB epithelium and the stroma around the TEBs (Table 2). Wnt4, Wnt5b, and Wnt6 are detected in both TEB and ductal epithelium. Wnt5a and Wnt7b are only expressed in the epithelium of the TEBs. Wnt5a has also been detected in the stromal compartment during puberty. In the adult mammary gland, Wnt4
and Wnt7b are exclusively expressed in luminal cells. Wnt5a is expressed in luminal, basal, and stromal cells, while Wnt5b is only expressed in the epithelial cell compartment. Wnt10a expression is detected in basal cells, and Wnt11 expression in both basal and stromal cells. Wnt5b is only expressed in the epithelial cell compartment. Wnt10a expression is detected in basal cells, and Wnt11 expression in both basal and stromal cells. Expression of Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b, Wnt10a, Wnt10b, and Wnt11 was also detected in the adult mammary gland in studies where different (epithelial) cell types were not separated. In the mammary glands of pregnant mice, expression of Wnt2, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b and Wnt10b has been detected. Wnt5b and Wnt6 are expressed at low levels during the lactation phase. During involution, Wnt2, Wnt5a, Wnt5b, and Wnt7b are expressed.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Cell type</th>
<th>Expressed Wnt genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puberty</td>
<td>TEB epithelium</td>
<td>Wnt2, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b</td>
</tr>
<tr>
<td></td>
<td>Ductal epithelium</td>
<td>Wnt4, Wnt5b, Wnt6</td>
</tr>
<tr>
<td></td>
<td>Stroma</td>
<td>Wnt2, Wnt5a</td>
</tr>
<tr>
<td></td>
<td>Whole mammary gland</td>
<td>Wnt2, Wnt5a, Wnt7b</td>
</tr>
<tr>
<td>Adult</td>
<td>Luminal cells</td>
<td>Wnt4, Wnt5a, Wnt5b, Wnt10a, Wnt11</td>
</tr>
<tr>
<td></td>
<td>Basal cells</td>
<td>Wnt5a, Wnt5b, Wnt10a, Wnt11</td>
</tr>
<tr>
<td></td>
<td>Stroma</td>
<td>Wnt2, Wnt5a, Wnt11</td>
</tr>
<tr>
<td></td>
<td>Epithelial cells</td>
<td>Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b</td>
</tr>
<tr>
<td></td>
<td>Whole mammary gland</td>
<td>Wnt5a, Wnt5b, Wnt10b</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>Luminal cells</td>
<td>Wnt4</td>
</tr>
<tr>
<td></td>
<td>Epithelial cells</td>
<td>Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b</td>
</tr>
<tr>
<td></td>
<td>Whole mammary gland</td>
<td>Wnt2, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b</td>
</tr>
<tr>
<td>Lactation</td>
<td>Epithelial cells</td>
<td>Wnt5b, Wnt6</td>
</tr>
<tr>
<td></td>
<td>Whole mammary gland</td>
<td>Wnt5b, Wnt6</td>
</tr>
<tr>
<td>Involution</td>
<td>Whole mammary gland</td>
<td>Wnt2, Wnt5a, Wnt5b, Wnt7b</td>
</tr>
</tbody>
</table>

**Table 2: Wnt gene expression during postnatal mammary gland development.** This table shows the expression of individual Wnt genes in different cell types of the mammary gland. The rows of “whole mammary gland” and “epithelial cells” in the “cell type” column indicate that in these studies the mammary gland (epithelial) cell types were not separated (It does not mean that these Wnt genes are expressed in all epithelial cell types). Techniques that were used for the detection of Wnt gene expression that are mentioned in this table include qRT-PCR, RNA in situ hybridization, northern blot, and microarray-based gene expression profiling. Adapted from Yu et al.56.

**WNT signaling in the mammary gland**

The WNT/CTNNB1 pathway is involved in proper embryonic development of the mammary gland, as is shown by studies that use genetically modified mouse models in which the WNT/CTNNB1 pathway is perturbed. For example, embryos that lacked WNT co-receptors LRP5 or LRP6, or transcription factor LEF1, had smaller or fewer mammary placodes at E12.5 and underdeveloped mammary glands at birth. Also ectopic overexpression of WNT/CTNNB1 pathway antagonist Dickkopf1 (DKK1) resulted in impaired mammary gland development before the mammary bud stage.
Evidence is emerging that in the postnatal mammary gland, WNT ligands have a role as niche factors that control MaSC behavior. For example, the mammary cell population that expresses Axin2 (i.e. cells that are WNT reporter positive), is enriched for MaSCs, which were capable of mammary outgrowth after transplantation in cleared fat pads. Furthermore, in the presence of WNT3A, MaSCs could be expanded in vitro for many generations and kept their regenerative capacity in transplantation assays. The importance of the WNT/CTNNB1 pathway for the regenerative capacity of MaSCs was also demonstrated by the loss of stem cell activity of LRP5-/- mammary gland cells in transplantation assays. Furthermore, the same study showed that mammary glands of pubertal LRP5-/- mice showed fewer TEBs. Lineage tracing with an Axin2CreERT2 reporter mouse has shown the presence of WNT-responsive stem cells in the mammary gland. It was also suggested that lineage tracing using Lgr5CreERT2 reporter mice demonstrated active WNT/CTNNB1 signaling in the mammary gland. It is not clear however, whether Lgr5, which is a bona-fide WNT target and stem cell marker in the intestine, marks WNT/CTNNB1 responsive stem cells in the mammary gland. Procr on the other hand, was identified as a WNT target gene that specifically marks MaSCs. Procr expression marks a unique MaSC population, that shows high regenerative capacity in transplantation assays. Furthermore, lineage tracing showed that Procr positive cells can differentiate into both mammary epithelial lineages. To conclude, several studies have shown evidence that WNT responsive stem cells exist in the mammary gland, which contribute to different lineages at different stages of mammary gland development.

Although evidence suggest that the WNT/CTNNB1 pathway plays a role in mammary gland homeostasis by controlling MaSC behavior, the specific function of most individual WNT ligands remains far from clear. One exception is Wnt4, which has shown to be required for proper outgrowth of the mammary epithelium. Wnt4 has been linked to stem cell maintenance in various tissues, including the mammary gland. The role of Wnt4 in mammary gland development has been investigated using Wnt4 deficient mammary epithelium. Due to kidney failure, Wnt4-/- mice die within 24 hours of birth, but the epithelium from mammary buds can be isolated. Transplantation of Wnt4-/- and wildtype (WT) epithelium in a WT host, revealed that during pregnancy, Wnt4-/- implants showed considerably less branching and outgrowth of lobuloalveolar epithelium compared to the WT implants. A different study tested the regenerative capacity of Wnt4-/-, Wnt4-/-, and Wnt4-/- epithelium in serial transplantations. They showed substantial impairment of reconstitution capacity in Wnt4-/- epithelium compared to Wnt4-/- and Wnt4-/- epithelium, which indicates that Wnt4 plays a role in maintaining stem cell function in the mammary gland. Another study has shown that WNT4 cooperates with RSPO1, which is a WNT/CTNNB1 pathway agonist, to promote MaSC self renewal. Taken together, different studies have shown that Wnt4 can regulate MaSC activity in the mammary gland.

In conclusion, the WNT/CTNNB1 pathway is important for mammary gland development and adult tissue homeostasis. Wnt genes are expressed throughout mammary gland development in various mammary cell types, and as such, responsible for all the WNT dependent processes.
Aim

The aim of my PhD research was to gain a better understanding on how the expression of individual Wnt genes is regulated in the mammary gland. As WNT ligands are critical regulators of stem cell behavior, it is imperative that the expression of Wnt genes is tightly controlled in both time and place. Surprisingly, despite many years of WNT research, very little is known about how expression of these genes is regulated. In this thesis we dive into this knowledge gap and investigate the molecular mechanism behind cell type specific Wnt expression at the chromatin level.

Outline of this thesis

Above (chapter 1) the development and maintenance of the mammary gland, and the role of WNT/CTNNB1 signaling in these processes are introduced. In chapter 2, we performed a transcriptomic analysis on different time points and cell populations of the mammary gland. We present a comprehensive overview of Wnt gene expression, which showed specific expression patterns of Wnt genes in different cell populations. Generally speaking, specific gene expression patterns are thought to be established by regulatory elements that are scattered throughout the genome. Chapter 3 starts with an introduction of these genomic elements and describes the challenges regarding their identification. We present a three-step plan on the identification of enhancers, which constitute a subcategory of regulatory elements that have the capacity to induce transcription of one or more genes. In chapter 4 we use our workflow to find enhancers that control Wnt4 expression in the mammary gland. This resulted in the identification of an active enhancer hub, located upstream of the Wnt4 gene, and a model of how Wnt4 expression is regulated in the mammary gland. The goal of chapter 5 was to find enhancers that induce Wnt2 expression in the mammary gland stroma. This resulted in the identification of a specific enhancer element that is located 15.4 kb upstream of the Wnt2 promoter. Chapter 6 provides a summary of the described research in this thesis and a discussion of the findings in the context of gene regulation.
References


Chapter 1


