Exploring the Wnt enhancer landscape in the mammary gland

Heijmans, N.

Publication date
2021

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Transcriptomic analysis reveals spatiotemporal changes in gene expression in the postnatal mammary gland

Nika Heijmans*, Katrin E. Wiese*, Renée van Amerongen

Section of Molecular Cytology and Van Leeuwenhoek Centre for Advanced Microscopy, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands.

*contributed equally
Abstract

Although we know there is a link between the reproductive cycle and breast cancer development, the molecular basis of this risk is not well understood. To unravel the effect of hormone fluctuations on homeostasis of the breast, we investigated the morphological changes of the mouse mammary epithelium and performed a transcriptomic analysis of different mammary gland cell types during puberty and different stages of the adult virgin estrous cycle. Our data show that proliferative changes that have been reported to occur during the estrous cycle, don’t occur in every mouse during every cycle. We show that the main changes in gene expression in different stages occur in adipocytes and stromal cell populations. Lastly, we present a comprehensive overview of the complex expression patterns of individual Wnt genes in different mammary gland cell types in pubertal and adult mice. This transcriptomic analysis provides a useful resource that will help contribute to our understanding of the subtle and dynamic changes in gene expression in different cell types of the mammary gland due to fluctuating hormone levels.
Introduction

One of the risk factors for breast cancer is an increased number of years of menstrual cycling, especially for every year younger at menarche. The foundation of this increased risk remains unknown. The mouse mammary gland provides a unique model to study breast development and physiology. Under the influence of steroid hormones, the mammary gland of mice undergoes morphological changes during puberty and during the adult reproductive cycle. A better comprehension of the molecular mechanisms underlying these dynamic changes in the mouse mammary gland could lead to an improvement of prevention and personalized treatment of breast cancer in premenopausal women.

Unlike most organs, the mouse mammary gland develops mostly postnatally. The rudimentary tree of ductal epithelial cells present in the mammary fat pad at birth, remains predominantly quiescent until the onset of puberty. Throughout puberty, the epithelial ducts expand into the mammary fat pad in a process called ductal morphogenesis, which is stimulated by estrogen. During the reproductive life of female mice, the mammary gland can undergo many rounds of expansion and regression. The remarkable regenerative potential of the mammary gland is most evident during pregnancy, when the mammary gland is preparing for lactation. Throughout pregnancy, extensive side-branching occurs in response to progesterone. In combination with prolactin, progesterone is also responsible for the formation of lobuloalveolar structures. These secretory alveoli establish the milk supply for the newborn offspring during the lactation stage. At weaning, a reduction in demand for milk will induce involution: an apoptotic process in which the secretory epithelium is removed and the mammary gland is restored to a pre-pregnancy state.

It has been reported that more subtle morphological changes of the epithelial ducts can occur during the estrous cycle, which repeats itself every 4 to 5 days. This short cycle length makes the mouse mammary gland an ideal model to investigate changes that occur due to hormonal fluctuations during the reproductive cycle. The mouse estrous cycle is divided into four stages: proestrus, estrus, metestrus and diestrus. The proestrus/estrous stages and metestrus/diestrus stages correspond to the follicular and luteal phases of the human menstrual cycle, respectively. During the proestrus stage there is an increase of circulating estrogen and these levels decline during the estrus stage. A small surge of estrogen levels occurs in metestrus and during the diestrus stage the estrogen levels are relatively low, until they increase again towards proestrus. Progesterone levels show a small increase during the proestrus/estrous phase, rise during metestrus and reach their maximal peak in diestrus.

Fluctuations in hormone levels are thought to cause cycles of growth and regression of lobuloalveolar structures in the mammary epithelium. However, the molecular basis of the morphological changes that occur during the estrous cycle is poorly understood and discrepancies exist between the results of studies investigating these proliferative changes. Some studies observe an increase of epithelial cells mainly during late proestrus/estrus and apoptosis at late metestrus/diestrus. Other studies have reported that outgrowth of lobuloalveolar structures mainly...
occurs in diestrus compared to other stages\textsuperscript{12–14}. These studies also show that apoptosis takes place in the diestrus stage\textsuperscript{12,13}. In addition, a more recent study shows that proliferative expansion of the epithelial ducts does not occur in every cycle in every mouse, highlighting the complexity of proliferative heterogeneity during the estrous cycle\textsuperscript{15}. In conclusion, proliferative changes may not occur every cycle, and when they occur, the timing of lobuloalveolar outgrowth is not completely clear.

![Figure 1: Hormonal fluctuations during the mouse estrous cycle.](image)

The outgrowth and regression of epithelial ducts that has been reported, is presumably controlled by steroid hormones via mammary stem cell (MaSC) regulation\textsuperscript{13,17–19}. One of the candidate genes for controlling mammary stem cell behavior downstream of steroid hormones is \textit{Wnt4}\textsuperscript{13,20,21}. \textit{Wnt4} is a member of the \textit{Wnt} gene family, which is highly conserved and encodes for ligands that play a pivotal role in stem cell self-renewal and tissue homeostasis in several types of tissue\textsuperscript{22}. Multiple studies have indicated the importance of the WNT signaling pathway in mammary gland homeostasis\textsuperscript{13,17,21,23–30}. However, the mechanism and role of hormone induced WNT signaling during the estrous cycle is not well understood.

Studies investigating the link between steroid hormones and Wnt genes or MaSC activity are often performed using ovariectomized mice receiving treatment with progesterone and/or estrogen pellets or injections\textsuperscript{13,17,20,31}. Therefore, there is a need for studies conducted under physiological conditions to examine the direct molecular consequences of hormonal fluctuations in the mammary gland. Furthermore, as most studies that try to elucidate the underlying transcriptional changes either focus on epithelial cells or do not clearly separate different mammary gland cell types\textsuperscript{20,31,32}, the contribution of adipocytes and other stromal cells has been largely overlooked so far.

To investigate the hormone-driven transcriptional response during the estrous cycle, we established a comprehensive overview of gene expression in mammary glands of wild-type mice during different developmental time points. In addition to pubertal mammary glands, we focused on the different stages of the estrous cycle of adult mice, to study whether estrous cycle stages are correlated with specific changes in mRNA levels. To be able to examine cell type specific
gene expression, we isolated different cell types of the mammary gland during each of the different developmental stages. In this study we especially focused on Wnt genes and investigated which members of the Wnt gene family are differentially expressed during the estrous cycle. Finally, we show a comprehensive overview of the expression patterns of individual Wnt genes in the mammary gland. This work contributes to understanding the effects of physiological hormone fluctuations on gene expression and the molecular mechanisms underlying dynamic morphological changes in the breast during the reproductive cycle.

Results

**Isolation of pubertal and adult estrous-staged mammary glands**

To investigate gene expression in cell populations of pubertal and adult estrous-staged mammary glands, tissues needed to be isolated from mice at specific time points. For the isolation of mammary glands of pubertal mice, we selected female FVB/N mice of 34-36 days old, since at this time point the mammary epithelium is actively undergoing branching morphogenesis. For each day, the 3rd and 4th glands of 4 individual mice from 3 different litters were isolated, pooled per mouse and processed to single cells for further analysis (Table 1).

To isolate mammary glands from female adult FVB/N mice at different stages of the estrous cycle, we monitored progression through the estrous cycle in individual mice over time. The stage of the estrous cycle can be determined by collecting vaginal swabs and scoring the relative proportion of nucleated epithelial cells, cornified epithelial cells and leukocytes in the sample. In proestrus, mostly nucleated epithelial cells are present, together with cornified epithelial cells. A small number of leukocytes may be detected in early proestrus stage. When the female mouse is in estrus, mostly cornified epithelial cells will be present, together with a small number of nucleated cells (in early estrus) or some leukocytes (in late estrus). In metestrus, a mixture of cornified epithelial cells, nucleated epithelial cells and leukocytes is present. Swabs containing mostly leukocytes indicate that the mouse is in diestrus (see Figure 2B for representative images).

We tracked the estrous cycle of 32 adult (14-16 weeks old) female FVB/N mice every day for at least 10 days to make sure that the selected mice were stably cycling (examples in Figure 2C). Based on the ratio of cell types present in the samples, we determined the stage of the estrous cycle. When a smear did not allow us to assign a clear stage, it was scored as a transition smear (e.g. proestrus to estrus), also taking into account the smear of the preceding day for that particular mouse. Mice that were undergoing regular estrous cycles were sacrificed at a defined stage. The 3rd and 4th mammary glands were isolated, pooled per mouse and processed to single cells. Out of the 32 tracked FVB/N mice, 7 showed an irregular cycle and were therefore excluded from further analysis (for examples, see Figure 2D). In total, we isolated mammary glands from 5 mice that were in proestrus, 5 in estrus, 4 in metestrus and 4 in diestrus (Table 1).
To confirm the interpretation of the estrous cycle stage at which the mammary glands were isolated, we additionally performed histological evaluations on H&E stained sections of paraffin embedded vaginal tissue taken at the time of sacrifice (Figure 3). During the estrous cycle the vaginal epithelium shows different characteristics for each of the four stages. The proestrus stage can be recognized by a relatively thick epithelial layer and an outer layer consisting of nucleated cells. In estrus the epithelial layer is at its thickest and the outer later is now comprised of cornified cells. The metestrus stage is characterized by thinner epithelium, the loss of the cornified cell layers and the transepithelial migration of leukocytes. Diestrus samples show the thinnest layer of epithelium, solely containing non-cornified epithelial cells. Our analysis of the estrous cycle stage by assessing the vaginal epithelium in tissue sections corresponded with our staging based on vaginal smears. Supplementary Figure 1 depicts an overview of cytology and histology images of every mouse that showed a regular estrous cycle.

Figure 2: Determining the mouse estrous cycle stage by vaginal cytology. A) Illustration depicting the ratio of cell types present at each stage of the estrous cycle. X-axis represents a rough estimate of the duration of each stage. Total cycle takes 4-5 days. Dashed lines represent transition into the next stage. Relative proportions of each cell type are based on descriptions from the literature. B) Brightfield microscopy images of vaginal swabs after staining with Giemsa. Images in this figure are from the day of mammary gland isolation. Per stage one example is depicted. Scale bar = 100 µm. C) Monitoring of the estrous cycle. Per stage one example is depicted, corresponding to the same mice in B). D) Graphs depicting two examples of irregular estrous cycles. These mice were excluded from further analysis.
Transcriptomic analysis of the postnatal mammary gland

A

Proestrus

Estrus

Metestrus

Diestrus

4-5 days

N Nucleated Epithelial Cells

C Cornified Epithelial Cells

L Leukocytes

B

Proestrus

Estrus

Metestrus

Diestrus

C

Proestrus

Estrus

Metestrus

Diestrus

D
Cell numbers and relative proportions are similar throughout estrous cycles

To investigate gene expression in different mammary gland cell types we isolated luminal cells, basal cells, adipocytes and other stromal mammary cells. We collected the adipose tissue fraction of the fat pad, referred to as adipocytes, by separating it from other fractions by centrifugation. Using Fluorescence-Activated Cell Sorting (FACS) we isolated luminal (lin/EpCAM$^{high}$/CD49$^{med}$), basal (lin/EpCAM$^{med}$/CD49$^{high}$) and stromal (lin-/EpCAM/CD49f) cells (Figure 4A). Supplementary Figure 2 shows the complete gating strategy for sorting the different mammary gland cell populations.

We did not measure consistent differences in epithelial cell numbers between different estrous cycle stages (Figure 4B). Furthermore, the percentages of basal, luminal and stromal cells were comparable across different estrous cycle stages (Figure 4C). These data fit with the idea that expansion of the epithelial network does not occur during every estrous cycle in every mouse.
Figure 4: Isolation of different mammary gland cell populations. A) FACS plot that shows gating for luminal, basal and stromal cells. This example is from an adult mouse in proestrus. To separate the mammary gland cell types, an EpCAM-PE/CD49f-FITC staining was used. B) Cell numbers of sorted populations. For every replicate for the pubertal samples 4 mice were pooled. For the adult samples every replicate is 1 mouse (all adult mammary glands were sorted separately). Error bars represent mean with SD. Lin- cells = lineage negative cells. C) Same samples as B), but here the percentages of cell types per sort are plotted.
To further investigate the hypothesis that epithelial side branching does not occur during every cycle, we visualized the morphology of epithelial ducts in mammary glands of stably cycling mice by carmine staining. As we used all of the available mammary gland tissue from previously monitored mice for FACS, we checked the estrous cycle of a second cohort of FVB/N mice using vaginal cytology (Supplementary Figure 3). A total of 6 mice were used for this histological analysis, of which 3 were sacrificed in estrus and 3 in diestrus. No clear difference was visible in epithelial morphology between estrus and diestrus, which is in agreement with the idea that outgrowth and regression do not occur every cycle\textsuperscript{15} (Figure 5A). From these same mice, we used another gland for RNA isolation and qRT-PCR. Expression levels of the progesterone receptor ($\text{Pgr}$) were increased in diestrus compared to estrus (Figure 5B). This fits with earlier studies that showed expression of $\text{Pgr}$ to be induced by progesterone treatment and the higher levels of progesterone that are known to be present in diestrus\textsuperscript{13,16,40}. $\text{Wnt4}$ expression levels between estrus and diestrus were similar. This does not fit with studies stating that $\text{Wnt4}$ expression is progesterone responsive\textsuperscript{13,20,21,41}. The results of Figure 5 are in agreement with the cell numbers we measured in Figure 4, as we also don't observe clear differences in epithelial cell numbers between estrus and diestrus after sorting. To conclude, after careful monitoring of the estrous cycle stages, our results show that a heightened proliferative state of epithelial cells does not have to occur every estrous cycle, which has been previously reported\textsuperscript{15}. However, our results are not in agreement with studies that show that $\text{Wnt4}$ expression is progesterone responsive, as progesterone levels differ between estrus and diestrus. We hypothesize that this discrepancy may be caused by the use of ovariectomized mice receiving treatment with progesterone and/or estrogen\textsuperscript{13,20} versus physiological conditions.

**RNA-sequencing confirms accurate isolation of cell types**

To obtain an unbiased overview of changes in gene expression across different developmental time points, estrous cycle stages, and cell types, we performed genome wide expression analyses.

Samples from pubertal and adult mice that were sorted by FACS were pooled for RNA-seq analysis according to Table 1. Per stage, we pooled the adult mice into two groups: early and late stage. This division was based on our combined interpretation of vaginal cytology and histology sections (Supplementary Figure 1). In case we did not observe a clear difference between early or late stages we used the cell numbers of the different cell populations as a second criterium. For RNA-seq analysis we ultimately ended up with 2 replicates per estrous cycle stage, each containing cells from 2 or 3 mice. For the puberty samples we also selected 2 replicates, based on RNA quality (see methods).
Transcriptomic analysis of the postnatal mammary gland

Figure 5: Mammary glands in estrus and diestrus show similar epithelial morphology and Wnt4 expression levels. A) Images of carmine stained whole mounts of staged 3rd mammary glands from 6 different mice (3 in estrus and 3 in diestrus). Every image is from a different mouse. Scale bar = 1 mm. Data from monitoring the estrous cycle of these 6 mice can be found in supplementary figure 3. Numbers 1, 2, and 3 from this figure correspond to the same numbers in supplementary figure 3. B) Graph showing Wnt4 and Pgr mRNA expression levels. These results were obtained by qRT-PCR from whole mammary gland samples (3 in estrus and 3 in diestrus). Samples belong to the same mice as in A. Ct values of Wnt4 and Pgr were normalized to Krt8 expression, which is a marker for luminal cells. Line represents the mean.

Two independent RNA sequencing experiments were performed, one for puberty and one for adult samples. To measure the level of similarity between samples we conducted a multi-dimensional scaling (MDS) analysis (Figure 6A). As expected, the different samples cluster by cell type and epithelial and non-epithelial cells are separated in the first dimension. With the exception of pubertal basal cells, the two replicates from corresponding time points do not consistently cluster together within each cell type. To conclude, MDS analysis reveals major differences in gene expression between mammary gland cell types, but not between the selected developmental or estrous cycle stages.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Puberty</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P34</td>
<td>P35</td>
</tr>
<tr>
<td># of mice</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 1: Mammary glands of different mice were pooled per stage. P34, P35 and P36 in the pubertal samples represent different days of mammary gland isolation. P, E, M and D are different estrous cycle stages. For the adult samples, “1” and “2” indicate early and late estrous cycle stage, respectively.
To validate correct isolation of luminal, basal, adipocytes and stromal cells, we verified cell type specific marker expression in the different replicates (Figure 6B). The keratins Krt18 and Krt14 are epithelial cell markers for luminal and basal cells, respectively. Adipoq is an adipocyte cell marker and expression of Col1a1 marks fibroblasts. Together, these data confirm that we correctly isolated defined populations of luminal cells, basal cells, adipocytes and stromal cells of the mammary gland.

**Figure 6: Clustering of different mammary gland cell populations.** A) MDS plot of all RNA-sequencing samples (4 cell types each from 2 pools of puberty mice and 2 pools of mice for each of the 4 adult estrous cycle stages). Colors represent different cell types and shapes represent different time points. Distances on the plot correspond to the biological coefficient of variation (BCV) between samples. B) Expression of the cell type specific marker genes in every sample in Reads Per Kilobase per Million mapped reads (RPKM). Different cell types can be distinguished by color and different time points by shape (same as A). Genes are markers for the following cell types: Krt18-luminal, Krt14-basal, Adipoq-adipocytes, Col1a1-fibroblasts. Error bars represent mean with SD.

**Differential gene expression during the estrous cycle mainly occurs in non-epithelial cells**

To compare the different stages of the estrous cycle, we performed a differentially expressed gene analysis (Figure 7A). Contrary to our expectations, gene expression changes in epithelial cell types were limited. Although hormone receptor positive cells are located in the luminal cell layer, this
cell type contains the smallest number of differentially expressed genes in pairwise comparisons of two different estrous cycle stages. In adipocytes and stromal cells however, some comparisons show more than 1000 differentially expressed genes. In adipocytes, all pairwise comparisons involving diestrus show >1000 upregulated genes. In stromal cells, the estrus stage stands out the most, with most differentially expressed genes being downregulated. These results suggest that the non-epithelial cell populations are also hormone responsive, either directly or indirectly.

Functional gene ontology (GO) classification of differentially expressed genes show diverse cellular components (CC) and biological processes (BP) GO terms for which the differentially expressed gene list of each cell type is enriched (Figure 7B)⁴⁶,⁴⁷. Whereas differentially expressed genes in adipocytes are mostly linked to cell membrane and cell adhesion, the differentially expressed gene list in stromal cells is enriched for genes associated with nucleus and regulation of transcription. The most prominent enrichment for certain GO terms is observed in basal cells, namely genes related to chromosome, cell cycle, cell division and mitotic nuclear division. To highlight some of these differentially expressed genes, we selected the ones with the highest fold change (logFC of >|5|) in basal cells, that have a logCPM of >1 (Table 2). These genes are all downregulated in diestrus compared to estrus. Amongst the differentially expressed genes with the highest fold change in expression between different stages in basal cells, are genes that are directly linked to the cell cycle and mitosis, which validates our GO term analysis. However, considering that these genes are positive regulators of cell division, their downregulation in diestrus versus estrus is not intuitive, since we did not observe more basal cells in estrus than in diestrus (Figure 4), unless the basal cells are in mitotic arrest in estrus, which would explain the expression of mitotic markers.

To conclude, the highest number of differentially expressed genes between different stages in the estrous cycle were found in adipocytes and stromal cells, but functional enrichment analysis showed most prominent enrichment for genes linked to the cell cycle in basal cells, which does not translate to the number of cells after sorting different cell types of different estrous cycle stages.
Figure 7: Most differentially expressed genes during estrous cycle in adipocytes and stromal cells. A) Graph depicting the number of differentially expressed genes for each pairwise comparison between different stages of the estrous cycle (FDR <0.05). Yellow bars represent upregulated genes and dark blue bars represent downregulated genes. On the X-axis are different pairwise comparisons of different estrous cycle stages. P = proestrus, E = estrus, M = metestrus, D = diestrus. B) Graphs depicting functional enrichment analysis of all differentially expressed genes (DE genes) in basal cells, adipocytes and stromal cells (FDR <0.01) in DAVID (version 6.8). Per cell type the top 10 most enriched GO terms are shown. Color intensity indicates Benjamini corrected p-value. CC = cellular component, BP = biological process, MF = molecular function.
Transcriptomic analysis of the postnatal mammary gland

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Comparison</th>
<th>Gene</th>
<th>LogFC</th>
<th>LogCPM</th>
<th>FDR</th>
<th>Function</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>D vs E</td>
<td>Iqgap3</td>
<td>-6.874</td>
<td>2.144</td>
<td>1.13E-03</td>
<td>Regulates cell proliferation through Ras/ERK pathway</td>
<td>50</td>
</tr>
<tr>
<td>Basal</td>
<td>D vs E</td>
<td>Kif18b</td>
<td>-6.234</td>
<td>1.285</td>
<td>1.43E-03</td>
<td>Mediator of mitotic spindle stability</td>
<td>51</td>
</tr>
<tr>
<td>Basal</td>
<td>D vs E</td>
<td>Ncapg</td>
<td>-6.183</td>
<td>1.453</td>
<td>3.25E-03</td>
<td>Subunit of condensing I complex, which regulates chromosome condensation and segregation during mitosis</td>
<td>52</td>
</tr>
<tr>
<td>Basal</td>
<td>D vs E</td>
<td>Pbk</td>
<td>-6.084</td>
<td>1.828</td>
<td>1.43E-03</td>
<td>Mitotic regulator, promotes cytokinesis, active during mitosis</td>
<td>53</td>
</tr>
<tr>
<td>Basal</td>
<td>D vs E</td>
<td>Knl1</td>
<td>-5.102</td>
<td>1.099</td>
<td>5.24E-03</td>
<td>Plays a crucial role in spindle assembly checkpoint activation</td>
<td>54</td>
</tr>
<tr>
<td>Basal</td>
<td>D vs E</td>
<td>Pclaf</td>
<td>-5.234</td>
<td>2.486</td>
<td>9.00E-03</td>
<td>PCNA-associated protein, essential for DNA replication and cell cycle progression</td>
<td>55</td>
</tr>
</tbody>
</table>

Table 2: Differentially expressed genes with highest logFC in basal cells are directly linked to the cell cycle. Genes with a LogFC of >|5| and LogCPM of >1 were selected from the list of differentially expressed genes in basal cells. These six genes (from the total list of differentially expressed genes in basal cells, from all comparisons) fulfill these criteria. All six genes are downregulated genes in diestrus compared to estrus. LogFC (= log2 fold change) is the log-ratio of gene expression between diestrus and estrus. LogCPM (= log2 count per million) is the average expression taken over all samples within basal cells. FDR (= false discovery rate) is the expected proportion of false positive errors.

Six Wnt genes are differentially expressed during the estrous cycle

Next, we focused on the expression of individual Wnt genes in the mammary gland. Besides the earlier mentioned progesterone-induced Wnt4 expression in the mammary gland\[^{13,20,21}\], there are other studies that link Wnt gene expression to steroid hormones in different tissues. Wnt4, Wnt5a and Wnt7a were shown to be differentially expressed in the mouse uterus during the estrous cycle\[^{56}\]. Another study reported that these same Wnt genes were downregulated in the rat reproductive tract after administration of a potent estrogen receptor agonist\[^{57}\]. Also in the pituitary gland, expression of Wnt4 and Wnt5a was modulated in estrogen-treated ovariectomized female rats\[^{58}\]. In the Ishikawa cell line, which is established from a human endometrial adenocarcinoma, WNT7A mRNA levels decreased after treatment with 17b-estradiol\[^{59}\]. Overall, several studies have shown that steroid hormones can modulate the expression of various Wnt genes in different tissues.

We investigated whether Wnt genes are differentially expressed in different mammary gland cell types in different stages of the estrous cycle. To our surprise, the epithelial cell types did not show differential expression of any Wnt gene (FDR <0.05) (Table 3). We also did not observe differential expression of Wnt4 in luminal cells, even though Wnt4 expression has been reported to occur downstream of progesterone in this cell population\[^{13,20,21}\]. We also did not observe differential expression of Wnt7a, despite its previously described link to steroid hormones\[^{56,57,59}\]. We did, however, observe differential expression of 6 Wnt genes in adipocytes or stromal cells. In adipocytes, Wnt7b and Wnt5b are upregulated in diestrus compared to other stages. Wnt4 and Wnt10a are also upregulated in adipocytes in both diestrus compared to proestrus and diestrus compared to metestrus. In stromal cells, Wnt6 and Wnt5a are downregulated in diestrus compared to estrus and metestrus, respectively.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Comparison</th>
<th>Wnt gene</th>
<th>logFC</th>
<th>logCPM</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipocytes</td>
<td>D vs P</td>
<td>Wnt7b</td>
<td>3.724</td>
<td>5.529</td>
<td>4.90E-04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wnt5b</td>
<td>2.362</td>
<td>6.136</td>
<td>9.42E-04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wnt4</td>
<td>2.016</td>
<td>4.145</td>
<td>8.10E-03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wnt10a</td>
<td>3.490</td>
<td>3.167</td>
<td>1.20E-02</td>
</tr>
<tr>
<td></td>
<td>D vs E</td>
<td>Wnt5b</td>
<td>2.268</td>
<td>6.136</td>
<td>1.39E-03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wnt7b</td>
<td>2.800</td>
<td>5.529</td>
<td>3.55E-03</td>
</tr>
<tr>
<td></td>
<td>D vs M</td>
<td>Wnt7b</td>
<td>3.475</td>
<td>5.529</td>
<td>1.28E-03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wnt4</td>
<td>2.395</td>
<td>4.145</td>
<td>4.10E-03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wnt10a</td>
<td>3.486</td>
<td>3.167</td>
<td>1.78E-02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wnt6b</td>
<td>1.458</td>
<td>6.136</td>
<td>2.60E-02</td>
</tr>
<tr>
<td>Stromal</td>
<td>D vs E</td>
<td>Wnt6</td>
<td>-1.630</td>
<td>1.450</td>
<td>4.42E-02</td>
</tr>
<tr>
<td></td>
<td>D vs M</td>
<td>Wnt5a</td>
<td>-1.504</td>
<td>5.603</td>
<td>4.11E-02</td>
</tr>
</tbody>
</table>

Table 3: Differentially expressed Wnt genes during estrous cycle. Only in comparisons of adipocytes and stromal cells differentially expressed (FDR <0.05) Wnt genes were present. Column “Comparison” shows the different pairwise stage comparisons which showed differentially expressed Wnt genes. P = proestrus, E = estrus, M = metestrus, D = diestrus. LogFC (= log2 fold change) is the log-ratio of Wnt expression between two different stages. LogCPM (= log2 count per million) is the average expression taken over all samples within a cell type. FDR (= false discovery rate) is the expected proportion of false positive errors.

It should be noted, however, that we have to be careful when interpreting these fold changes. The cell types in which the Wnt genes are differentially expressed show low absolute expression levels of those Wnt genes (Figure 8). In adipocytes, for example, expression of individual differentially expressed Wnt genes does not exceed 4 RPKM, except Wnt5b in diestrus. Also in stromal cells, Wnt6 and Wnt5a expression are <4 RPKM, which is close to background levels. Wnt expression is not inherently low; Wnt4, Wnt5a, Wnt5b and Wnt7b are all decently expressed (between 8.0 and 106.4 RPKM) in luminal cells, but in these cells no Wnt genes were differentially expressed in pairwise stage comparisons. Similarly, basal cells express decent levels (between 3.7 and 29.2 RPKM) of Wnt5a and Wnt5b, but also in this cell type no Wnt genes met our cut off (FDR <0.05). Future research is needed to investigate whether the observed changes in Wnt gene expression across different stages of the estrous cycle in adipocytes and stromal cells are biologically relevant.
Figure 8: Expression levels of differentially expressed Wnt genes in different mammary gland cell types during the estrous cycle. RNA-sequencing results from samples from different mammary gland cell types and different stages of the estrous cycle. This figure shows the expression of Wnt genes that were found to be differentially expressed (FDR <0.05) in pairwise comparisons of different estrous cycle stages (Table 3). RPKM = Reads Per Kilobase per Million mapped reads. Colors represent different cell types and symbols represent different stages of the estrous cycle.
Wnt genes show an intricate expression pattern in the mammary gland

Although we did not observe differential expression for most Wnt genes across different stages of the estrous cycle (Table 3), there was a clear difference in Wnt expression between different cell types (Figure 8). To further analyze cell type specific Wnt expression, we checked the relative mRNA levels of all Wnt genes in the different cell types of the mammary gland, for all pubertal and estrous cycle staged samples (Figure 9).

Samples from the same cell types clustered together according to expression of different Wnt genes. In luminal, basal and stromal cells, also the puberty samples cluster together. In pubertal mammary glands, mostly Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b, and Wnt10a are expressed in epithelial cells. Wnt5a and Wnt5b are expressed in both basal and luminal cells, while Wnt4 and Wnt7b expression is restricted to luminal cells, and Wnt6 and Wnt10a are only expressed in the basal cells. This corresponds to an earlier study that showed expression of Wnt4, Wnt5a, Wnt5b, Wnt6 and Wnt7b in the epithelium of pubertal mammary glands using microarray gene expression analysis \(^6^0\). In this study, mature epithelial ducts were separated from terminal end buds (TEBs), which are epithelial structures, characteristic for pubertal mammary glands. The authors showed that Wnt2 is expressed in TEBs, while we did not observe Wnt2 expression in epithelial cells in our dataset. RNA in situ hybridization experiments have shown that Wnt2 is also expressed in the stroma surrounding epithelial ducts in pubertal glands\(^6^0,6^1\). This fits with our data, which shows that Wnt2 is mainly expressed in the pubertal stromal population. In addition to Wnt2, we also observed expression of Wnt2b, Wnt6, Wnt9a, Wnt9b, and Wnt16 in the pubertal stroma. In adipocytes of pubertal mammary glands Wnt expression was relatively low, although replicates showed some expression of Wnt2, Wnt9b, Wnt10b, and Wnt16.

In Figure 9, we distinguished the different estrous cycle stages of the adult samples. With the exception of some replicates from adipocytes, replicates from the same stage generally did not cluster together within the different cell type clusters. This was not unexpected, given the overall lack in differential Wnt gene expression described above (Figure 8). Similar to pubertal mammary glands, in adult glands mainly Wnt4, Wnt5a, Wnt5b and Wnt7b are expressed in the luminal population. This fits with published microarray and scRNA data of adult mammary epithelium that show expression of these Wnt genes in luminal cells\(^6^2,6^3\). Other studies using RNA in situ hybridization or qRT-PCR experiments have also previously reported Wnt4, Wnt5a, Wnt5b and/or Wnt7b expression in the luminal epithelium of adult mammary glands\(^2^0,2^1,2^7,6^4,6^5\). In the epithelium, expression of Wnt6 and Wnt10a was restricted to the basal layer. This corresponds to previous studies that have also shown high expression of Wnt6 and Wnt10a in basal cells compared to luminal cells\(^6^2,6^3\). Besides Wnt6 and Wnt10a, also Wnt5a, Wnt5b, Wnt9a, Wnt10a and Wnt11 show expression in basal cells. Expression of these Wnt genes was also observed in the basal cluster from published scRNA data\(^5^9\). Adipocytes are distinguished by expression of Wnt2, Wnt10b and Wnt16. Adipocytes share the expression of these Wnt genes with stromal cells, which in addition also express Wnt2b, Wnt4, Wnt6, Wnt9a, Wnt9b, and Wnt11. Most genome wide expression analyses so far have focused on mammary epithelial cells, frequently omitting adipocytes and stromal cells.
However, expression of \textit{Wnt2} and \textit{Wnt6} in stromal cells has been reported before\textsuperscript{65,66}. In conclusion, \textit{Wnt7b} and \textit{Wnt10a} are exclusively expressed in luminal and basal cells, respectively. Other \textit{Wnt} genes are expressed in multiple cell types. Summarizing, expression patterns of individual \textit{Wnt} genes in mammary epithelial cells vary between cell types rather than developmental time points and our results confirm observations from earlier published datasets of adult mammary epithelium.

\textbf{Figure 9: RNA-sequencing reveals complex spatiotemporal expression patterns of individual \textit{Wnt} genes.} Hierarchical clustering of \textit{Wnt} gene expression in different mammary gland cell types and different time points. Labels for different samples: * = puberty samples, \(P\) = proestrus, \(E\) = estrus, \(M\) = metestrus, \(D\) = diestrus. Numbers "1" and "2" in the names of adult samples refer to early and late estrous cycle stage, respectively. RPKM = Reads Per Kilobase per Million mapped reads and these values were normalized for each gene across all samples using Z-scores.
Discussion

In this study we analyzed gene expression in luminal cells, basal cells, adipocytes and stromal cells of pubertal and estrous cycle stage specific adult mammary glands. We extensively monitored the estrous cycle of adult FVB/N mice to be able to isolate mammary glands at defined stages of the estrous cycle. Using enzymatic digestion and FACS we isolated different cell types and we measured mRNA levels by performing RNA-sequencing.

One specific aim of this study was to obtain a comprehensive overview of mRNA level fluctuations in different estrous cycle stages, to better understand the transcriptional response of changing hormone levels in the mammary gland. Given its role in tissue maintenance and previously reported link to MaSC maintenance and steroid hormone responsiveness\textsuperscript{13,17,21,23–30}, we mapped Wnt gene expression in different cell types of the mammary gland.

Determining estrous cycle stage by vaginal cytology

Different methods exist for assessing the stage of the estrous cycle of a female adult mouse. For example, it can be done by assessing the overall appearance of the vaginal opening\textsuperscript{67}. However, it is difficult to distinguish the metestrus and diestrus stages using this method. The recommended way of determining the stage, is to take vaginal cytology samples and check the ratio of different cell types in these smears\textsuperscript{68}. This non-invasive method has also proven to be accurate in our project, as our estrous cycle stage determination by vaginal cytology was confirmed by histological analysis of the vaginal epithelium (Supplementary Figure 1). We also observed that 7 out of 32 mice were not cycling reliably (for examples, see Figure 2D). This points out the importance of monitoring the cycle for multiple days: only collecting swabs on the day of mammary gland isolation or collecting samples only once or twice a week will not be sufficient to determine the precise stage and progression of the estrous cycle\textsuperscript{32}. As shown in previous studies, to properly follow the estrous cycle, samples should be collected every day, at the same time, for at least one week\textsuperscript{12–14,69}. By monitoring the estrous cycle every day for at least 8 days, we were able to identify irregular estrous cycles and exclude these mice from further analysis.

Epithelial outgrowth does not occur in every mouse in every cycle

Studies investigating morphological changes of the mammary epithelium during the estrous cycle have shown variability\textsuperscript{9–15}. Although results concerning the timing of apoptosis are consistent (diestrus\textsuperscript{9–13}), it remains unclear whether outgrowth of side branches occurs during proestrus/estrus\textsuperscript{9–11} or diestrus\textsuperscript{12–14}. Importantly, another study shows that outgrowth and regression of the mammary epithelium does not occur during every cycle\textsuperscript{15}. Our results are in agreement with this latter study, as we did not observe consistent differences in cell numbers between different stages after careful monitoring of the estrous cycle (Figure 4B). Moreover, when visualizing the epithelial ducts of whole mount mammary glands, we did not observe a clear difference in morphology between the different stages either (Figure 5A). The origin of the discrepancies
between the different studies is unknown. Apparently, proliferation of epithelial cells during the estrous cycle displays heterogeneous patterns across different mice and this phenomenon is not as black and white as reported in some cases.

It remains challenging to unravel the complex and heterogeneous proliferative phenotypes when it comes to specific time points in the estrous cycle. Most available techniques to compare cell numbers between different stages of the estrous cycle (e.g. FACS), visualize the morphology of the epithelial ducts (e.g. carmine staining), or determine the expression of certain markers (e.g. Ki67 immunostaining) have in common that they require tissue sampling, thus resulting in an irreversible end point of the estrous cycle. A method that could shed more light on the dynamics of the mammary epithelium during the estrous cycle in real-time is intravital imaging of the mammary gland. Combining this with endogenous, fluorescently labeled, cell-type specific markers would show us the morphology changes throughout the estrous cycle in the same mouse.

**Gene expression fluctuations between different estrous cycle stages**

In our RNA-seq analysis, different cell types cluster together, indicating major differences in gene expression between these cells (Figure 6A). However, we had expected to find more differentially expressed genes between different estrous cycle stages within cell types (Figure 7). There are several possible explanations for this result.

First of all, we may have missed subtle differences in gene expression between cell types due to our experimental setup. The effect of fluctuations in steroid hormone levels on gene expression might be masked, since the estrogen and progesterone receptor (ER and PR) are only expressed in approximately 55% and 60% of the luminal cells of mammary glands of adult mice, respectively. A way to avoid this, is to isolate the hormone receptor positive cells separately. Unfortunately, because of the intracellular location of ER and PR, there is no straightforward staining protocol for specifically sorting non-fixed cells using FACS. However, strategies exist to increase the proportion of ER and PR positive cells in sorted populations. Furthermore, single-cell sequencing would facilitate gene expression analysis of individual cells, allowing one to easily distinguish and analyze mRNA levels of hormone receptor expressing cells specifically.

It is also possible that the rapid changes in morphology that have previously been observed during the estrous cycle are not caused by major changes in gene expression. In fact, processes other than transcription of certain genes might enable a faster and more dynamic way of transferring signals that induce these morphological changes. One of these mechanisms is alternative splicing of mRNA transcripts, resulting in functionally distinct splice variants. Several studies have shown the importance of alternative splicing in tissue development and homeostasis in vertebrates. Changes to the poly(A) tail of mRNA can also play a regulatory role by, for example, changing translation efficiency, mRNA stability and degradation. In addition to the diversity of the transcriptome, modifications of the proteome can add another level of regulation of cellular homeostasis. Post translational modifications such as phosphorylation, acetylation or ubiquitination can change protein functionality and can be analyzed using mass spectrometry.
This could yield valuable information about changes in cell behavior, which are not primarily controlled by fluctuations in gene expression.

Lastly, the fact that we did not observe a change in cell numbers or morphology throughout the different stages can also be an explanation for the small number of differentially expressed genes in epithelial cells (Figure 5 and Figure 7). We cannot expect major fluctuations in gene expression that hypothetically would cause expansion of the epithelial ducts, when we did not observe such changes morphologically.

**Non-epithelial cells should be included in studies of mammary gland biology**

Differential expression analysis between different estrous cycle stages revealed that, interestingly, most differentially expressed genes were found in the adipocytes and stromal cells and not in the epithelial cell populations (Figure 7A). The fact that these cells show differentially expressed genes during the estrous cycle is not surprising, since hormone receptor positive cells are also located in cell types surrounding the mammary epithelium. However, the difference in absolute numbers of differentially expressed genes between the epithelial and non-epithelial mammary cell types that we observed, is remarkable. This adds to the evidence that molecular cues that control epithelial cell behavior during the estrous cycle are not only coming from the epithelial cells itself, but also from adipocytes and stromal cells.

At the same time, some caution is warranted when interpreting these results. The adipocytes were isolated using differential centrifugation, possibly resulting in less pure samples than the cell types isolated using FACS. We took great care of properly digesting the mammary glands according to established protocols in the field, but we have to consider possible contaminations in the adipocyte samples. We also have to keep in mind that the sorted stromal population is not well defined. Setting up the sorting gates of the FACS, we made sure to exclude doublets, dead cells, haematopoietic cells and endothelial cells (Supplementary Figure 2). However, sorting the stromal cells of the mammary gland is not based on the presence of specific stromal markers, but the absence of epithelial markers (Figure 4A). As fibroblasts are the main component of the mammary stroma and we did not exclude them in any step of the preparation for FACS, we expect them to also be the main component of the stromal cells we sorted. This is supported by the specific expression of Col1a1 in the sorted stromal population (Figure 6B).

**Wnt genes show defined spatial gene expression patterns in different mammary cell populations**

We did not find a link between hormone status and Wnt4 gene expression in epithelial cells. In our data, Wnt4 is not significantly differentially expressed throughout the estrous cycle in luminal cells. This was surprising, as previous studies suggest that Wnt4 expression in the mammary gland is regulated by progesterone. However, in some of these studies, Wnt4 mRNA was measured after treating ovariectomized mice with 17b-estradiol and progesterone. As we measure Wnt4 levels under physiological steroid hormone conditions, this might be an explanation for the observed
differences, since physiological changes are likely to be much smaller\textsuperscript{13,20}. Another explanation is that changes in \textit{Wnt4} levels, like the proliferative phenotype of the mammary epithelium, are heterogenous amongst different mice. Our observation that \textit{Wnt4} levels are not higher in diestrus compared to other estrous cycle stages could thus be directly linked to the fact that we did not measure an increase in basal or luminal cells in the diestrus stage either.

As far as we know, \textit{Wnt} gene expression across luminal cells, basal cells, adipocytes and stromal cells during puberty and the four stages of the estrous cycle has not been examined before in such well-defined specific spatiotemporal groups. However, transcriptomic analyses have been performed in which a distinction was made based on the hormone receptor status of luminal cells\textsuperscript{62,63}. Data from these analyses show that \textit{Wnt4}, \textit{Wnt5a} and \textit{Wnt7b} are mainly (but not only) expressed in hormone receptor positive cells of the luminal population. This information is valuable when studying gene expression during the estrous cycle. However, practical limitations (including the amount of RNA that can be isolated from freshly sorted mammary gland cells) prevented us from performing more detailed analyses in the present study. For further research it will be informative to make a distinction between hormone positive and negative cells, also for non-epithelial cell types in the mammary gland.

**General conclusion**

This dataset provides a useful resource for future studies investigating gene expression patterns in different cell types of the mammary gland across different developmental timepoints. Also for studies concerning reproductive cycle-related breast cancer risks our data can provide a solid foundation for checking fluctuations in expression for genes of interest. Evidently, as correlation does not imply causality, further research is needed to analyze the role of differentially expressed genes in the estrous cycle.

Lastly, we would like to highlight the differences in \textit{Wnt} gene expression between different cell types of the mammary gland. The question remains how the expression of \textit{Wnt} genes, which in our study could not be linked to steroid hormone fluctuations, is regulated in the mammary gland. In the next chapters of this thesis our research is described in which we aim to unravel the mechanisms behind the cell-type specific \textit{Wnt} expression by exploring the \textit{Wnt} enhancer landscape in the mammary gland.

**Materials and methods**

**Mice**

Wildtype, inbred FVB/NHan\textsuperscript{®}Hsd mice (referred to as FVB/N in the main text) used in this study were purchased from Envigo. Breeding and estrous cycle monitoring was performed in-house. All mice were maintained under standard housing conditions in open cages, with 12 hour light/dark cycle and \textit{ad libitum} access to food and water. Experiments were performed according to
institutional and national guidelines and regulations. Experiments were approved by the Animal Welfare Committee of the University of Amsterdam. Pubertal mice of 34-36 days old were used and for each day 4 mice from 3 different litters were pooled to obtain sufficient cells for RNA-sequencing. Adult mice of 14-16 weeks old were sacrificed at the appropriate time point in the estrous cycle.

**Vaginal cytology and histology**

Vaginal swabs were collected using plastic Pasteur pipettes and PBS. From the tip of the vaginal opening, the vagina was flushed 2-3 times with a few drops of PBS. The sample was transferred to a glass slide, air-dried at 37°C, stained with Giemsa (Sigma-Aldrich cat. #48900) for 30 seconds and rinsed with PBS. For histology, vaginal tissue samples were fixed in 4% PFA for 24 hours, dehydrated through ascending grades of ethanol, cleared in Histo-Clear II (National Diagnostics cat. #HS-200) and embedded in paraffin. Five µm thick tissue sections were cut and mounted on glass slides. Slides were baked at 60°C for 45 minutes, deparaffinized in Histo-Clear II, rehydrated through descending grades of ethanol, stained with 50% Mayer’s Hematoxylin (Sigma-Aldrich) for 30 seconds, rinsed for 5 minutes in tap water, washed in PBS for 3 minutes and 70% ethanol for 5 minutes, stained with Eosin Y (Sigma-Aldrich, cat. #HT110132) for 2 minutes, dehydrated (through 70% ethanol, 100% ethanol and 100% isopropanol), cleared in Histo-Clear II (National Diagnostics cat. #HS-200) and mounted with a coverslip. Under bright field illumination, images of the cytology and histology samples were taken at 20x magnification using a Zeiss Axio Vert. A1 microscope.

**Mammary gland digestion**

The 3rd and 4th mammary glands of FVB/N mice were isolated, minced and enzymatically digested in an orbital shaker for 2 hours at 37°C in the following digestion mix: 9.2 ml DMEM/F12, 5% FCS, 1% Penicillin/Streptomycin, 25 mM HEPES (Gibco, cat. #15630056) and 300 U/ml Collagenase IV (Gibco, cat. #17104019) (10 ml per mouse with 4 glands per mouse). The fat fraction, referred to as adipocytes, was obtained from the top layer after centrifugation, transferred to TRIzol LS (Invitrogen, cat. #10296028) and stored at -80°C. Cell pellets were resuspended in HBSS (Gibco, cat. #11540476 supplemented with 2% FBS (Gibco, cat. #11573397) and ACK solution (Gibco, cat. #A1049201) (1:3) and incubated at room temperature (RT) for 5 minutes to lyse red blood cells. To dilute and inactivate the ACK buffer, 13 ml of HBSS was added and cells were spun down for 5 minutes, 1000 rpm, 4°C, and brake set to 1. Cell pellets were resuspended in 2 ml pre-warmed 0.05% Trypsin-EDTA (Gibco, cat. #11590626) and incubated for 5 minutes at 37°C, after which 3 ml of pre-warmed serum-free DMEM and 1 µg/ml DNaseI was added to the solution. After mixing well, 8 ml of DMEM with 10% FCS was added to stop trypsinization. Cells were filtered through a 40 µm mesh into a fresh tube.
Antibody staining and FACS

Cells were resuspended in 200 µl HBSS supplemented with 10% FBS and a cocktail of the following antibodies: EpCAM-PE (1:100, eBioscience, 12-5791-82, clone G8.8), CD49f-FITC (1:100, eBioscience, 11-0495-82, clone GoH3), CD45-Bio (1:100, eBioscience, 13-0451-82, clone 30-F11), CD31-Bio (1:100, eBioscience, 13-0311-81, clone 390), Ter119-Bio (1:100, eBioscience, 13-5921-81, clone TER-119). After incubating in the dark on ice for 20 minutes, cells were washed twice with HBSS supplemented with 2% FBS and incubated in 200 µl HBSS supplemented with 10% FBS containing Streptavidin-APC (1:200, eBioscience, 17-4317-82). After antibody staining, cells were stained with DAPI (1:5000 (Invitrogen cat. #D1306) and filtered through a 50 µm mesh. Cells were sorted using a BD FACS Aria III. FITC was excited with a 488 nm laser and emission was filtered using a 530/30 nm bandpass filter. PE was measured using a 561 nm laser and 582/15 nm bandpass filter. DAPI was excited with a 407 nm laser and emission was filtered using a 450/50 nm bandpass filter. APC was measured using a 633 nm laser and 660/20 nm bandpass filter. Cells were sorted with a plate voltage of 2500 V using the 4-Way Purity precision mode. Sorted cells were collected in TRIzol LS (Invitrogen, cat. #10296028). Post-sort purity checks were performed after every sort and were always >90%. Samples were stored in TRIzol LS at -80°C.

Carmine staining

Freshly isolated 3rd mammary glands of estrous cycle monitored mice were flattened between two glass slides, incubated on a nutator at RT for 4 hours in a 50 ml tube containing 12.5 ml 100% EtOH and 12.5 ml acetic acid. Glands were removed from the glass slides, incubated in 70% EtOH for 1 hour on a nutator at RT and rinsed in demi-water and stained O/N in a carmine solution (1 g carmine (Sigma, cat. #C1022), 2.5 g aluminum potassium sulphate (Merck, cat. #101047), 500 ml water, boiled for 20 minutes and filtered). After staining, glands were washed with 100% EtOH for 4 hours. Glands were cleared and stored in Histo-Clear II at RT. Images were taken on a Leica stereomicroscope at 5x magnification.

qRT-PCR

RNA from the 4th mammary glands was isolated from TRIzol LS (Invitrogen, cat. #10296028) according to manufacturer’s guidelines. RNA was isolated according to the manufacturer’s protocol. After DNase treatment, cDNA synthesis was performed from 4 µg RNA using SuperScript IV Reverse Transcriptase (Invitrogen, cat. #18090200) and Random Hexamers (Invitrogen, cat. #N8080127) according to manufacturer’s guidelines. During the reverse transcriptase reaction, Ribolock RNase Inhibitor (Thermo Scientific, cat. #EO0328) was added. After completion of cDNA synthesis, samples were diluted 10x for further steps. qRT-PCR reactions were performed using a QuantStudio 3 Real-Time PCR System (Applied Biosystems). For each reaction, 5 µl of diluted cDNA was added to a mix of 4 µl 5X HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis Biodyne, cat. #08-24-00008), 1 µl primers (0.5 µl forward and 0.5 µl reverse, from a 10 µM stock) and 10 µl nuclease-free water. Reactions were performed in triplicate in a
96x0.2 ml plate (BIOplastics, cat. #AB17500). Thermal cycling reactions included the following stages: 2 minutes at 50.0°C and 15 minutes at 95.0°C, then 40 cycles of 15 seconds at 95.0°C and 1 minute at 60.0°C, followed by the melting curve stage. The following primers were used: Wnt4 forward: ACTGGACTCCCTCCCTGTCT, Wnt4 reverse: TGCCCTTGTCACCTCCACTGAAA, Pgr forward: TGCACCTGATCTAATCCTAAATGA, Pgr reverse: GGTAAGGCACAGCGAGTAGAA, Krt8 forward: AGTTGCCTCTCTTCCATTGAC, Krt8 reverse: GCTGCAACAGGCTCCACT, Ctbp1 forward: GTGCCCTGATGTACCATAACCCA, Ctbp1 reverse: GCCAATTCCGACGATATTCTA.

RNA-sequencing
RNA extraction, purification and sequencing was done at the NKI Genomics Core facility, as well as data processing until read count calculation. For the puberty samples, the following samples were used based on quality control: Replicate 1: Basal P34, Luminal P34, Stromal P34, Fat P34; Replicate 2: Basal P35, Luminal P35, Stromal P35, Fat P36. Adult samples were pooled according to Table 1. RNA was extracted from TRIzol LS and purified using the Qiagen RNeasy column purification kit. RNA quality was checked with a Bioanalyzer (Agilent), after which polyA+ stranded RNA library preparation was performed using the Illumina TruSeq stranded RNA prep kit. RNA-sequencing was performed on a HiSeq 2500 (Illumina) System at the NKI Genomics Core Facility. Single-end reads (65 bp) were aligned to reference sequence GRCm38/Mm10 with Tophat version 2.1 and Bowtie version 1.1.0. Expression values were determined by HTSeq-count. Raw gene-level count tables were processed further using edgeR (version 3.28.0) and limma (version 3.42.1) packages with R (version 3.6.2). A cutoff of CPM > 1 in at least 2 libraries was applied to filter out genes with low counts prior to trimmed mean of M-values (TMM) normalization. To visualize distances between gene expression profiles based on the biological coefficient of variation, the plotMDS function was used with method set to “bcv”. RPKM values were generated with the RPKM function after calculating exonic region lengths from non-overlapping exons per transcript ID (genome version Mm10). The edgeR glmQLFit function was used to estimate the dispersion trend before testing for differential expression between different estrous cycle stages. Unless otherwise noted, a false discovery rate (FDR) of 5% was considered as a threshold for significance. The heatmap of Wnt gene expression in different mammary gland cell types was created with the heatmap.2 function (gplots) with Z-score scaling along rows and dendrograms computed using default settings.

Acknowledgements
We would like to thank Jos Jonkers and the NKI Genomics Core facility for the RNA-sequencing experiments. We also would like to thank our colleagues at the section of Molecular Cytology at the Swammerdam Institute for Life Sciences for discussions and feedback during the project. This research was funded by the Netherlands Organization for Scientific Research (NWO ALW VIDI 864.13.002 to R.v.A.).
**Author contributions**

K.E.W., R.v.A., and N.H. conceived the study. K.E.W. and N.H. designed and performed all experiments. N.H. and K.E.W. performed the monitoring of estrous cycle stages and FACS. K.E.W. performed qRT-PCR, RNA-seq analysis, and the differentially expressed gene analysis. All authors analyzed and interpreted the data. R.v.A. supervised the study and N.H. wrote the manuscript with input from all authors.
Supplementary figures
Transcriptomic analysis of the postnatal mammary gland
Supplementary Figure 1: Estrous cycle monitoring of adult FVB/N mice. All mice that showed a stable estrous cycle and were selected for RNA-sequencing are shown in this figure. The left column shows estrous cycle
monitoring over several days. The middle column and right column depict vaginal cytology and histology samples respectively, from the day of mammary gland isolation. Scale bar = 100 μm.

**Supplementary Figure 2:** Gating strategy for sorting luminal, basal and stromal mammary cell populations. SSC-A/FSC-A, FSC-W/FSC-H and SSC-W/SSC-H plots show gating for living single cells and to exclude dead cells, debris and doublets. DAPI plotted against FSC-A was also used to exclude dead cells. In the APC/FSC-A plot we gate for Lin- cells that are negative for CD45, CD31 and Ter119. CD45 and Ter119 markers were used to exclude haematopoietic cells and CD31 to exclude endothelial cells. EpCAM-PE and CD49f-FITC staining allowed us to gate for mammary luminal, basal and stromal cells before sorting.
Supplementary Figure 3: Estrous cycle monitoring of adult FVB/N mice for whole mount carmine staining. Six stably cycling mice were selected for carmine stainings; 3 in estrus and 3 in diestrus. This figure shows the graphs of monitoring the estrous cycle for at least one week using vaginal cytology. The shown image is the cytology sample on the day of mammary gland isolation. Scale bar = 100 µm. Samples 1, 2, and 3 correspond to the same numbers in Figure 5.
References


Transcriptomic analysis of the postnatal mammary gland


