Who watches the watchmen?

WNT responsive stem cells and the regulation of their niche ligands

van de Grift, Y.B.C.

Publication date

2023

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.
'Alas, poor Yorick!'

Hamlet
Chapter 1

General introduction
WNT/CTNNB1 signalling

Extensive cell-to-cell communication is a prerequisite to build and maintain complex tissues. Cells communicate via so-called signal transduction pathways, including WNT/CTNNB1 signalling. The WNT signalling pathway controls a myriad of biological processes throughout development and adult life in multicellular organisms. The mammalian genome harbours 19 WNT genes that encode 19 different WNT proteins. Fundamentally, WNT proteins (ligands) are secreted short-ranged hydrophobic growth stimulatory factors that lead to proliferation and other processes in recipient cells. These WNT ligands bind their target receptors, Frizzled (FZD), of which 10 different genes are encoded in the mammalian genome. The intracellular signalling cascade that is initiated in response to WNT/FZD binding has been extensively studied.

Briefly, binding of the WNT protein results in the dimerization of FZD and the LRP5/6 coreceptor. This interaction is promiscuous, as WNT proteins can bind multiple different FZDs. Like many signalling pathways, ligand binding induces a conformational change of the WNT receptors followed by phosphorylation of key target proteins. An essential step in downstream WNT/CTNNB1 signalling is the binding of AXIN to the phosphorylated cytoplasmic tail of LRP6 post activation. This prevents the APC destruction complex from ubiquitinating and degrading CTNNB1. CTNNB1 is ubiquitously expressed and translated, as it has an important dual function as both a WNT signalling co-factor and as an essential component of the E-cadherin complex. The APC destruction complex continuously degrades CTNNB1 to prevent cytoplasmic accumulation and nuclear translocation. Inhibition of the APC destruction complex by WNT signalling activation causes CTNNB1 levels to rise, allowing it to act as a transcriptional co-activator in the nucleus, where it binds to T-cell factor/lymphoid enhancer factor (TCF/LEF) to activate a WNT-driven, tissue specific transcriptional program (Fig1).
Chapter 1

**Figure 1: Schematic representation of canonical WNT/CTNNB1 signalling in the WNT-responsive stem cell niche.** Niche cells produce WNT ligands that are palmitoylated and secreted as short-ranged signals that activate downstream canonical WNT/CTNNB1 signalling in WNT-responsive stem cells. Binding of WNT ligands to LRP and FZD triggers a cascade that sequesters the APC destruction complex, thereby preventing the degradation of CTNNB1. This allows CTNNB1 to accumulate and translocate to the nucleus where it acts as a transcription factor and activates a WNT specific transcription program in collaboration with TCF/LEF.

**Aim of this thesis**

WNT/CTNNB1 signalling has been shown to be essential for stem cell maintenance across a variety of adult tissues. During adult tissue homeostasis, stem cell fate decisions are tightly controlled by extrinsic cues from the microenvironment, the so-called ‘stem cell niche’, as well as intracellular signalling cascades. In general, proliferation of WNT-responsive stem cells depends on the availability of WNT ligands that are secreted by local ‘niche’ cells that control the stem cell population. WNT/CTNNB1 signalling in stem cells has been extensively studied, however, it remains largely unknown which signals control WNT ligand expression in ‘niche’ cells and thus mediate spatiotemporal control of stem cell activation and population size.

The aim of this thesis was two-fold. First, to develop novel mouse models to study endogenous WNT signalling and stem cell interactions *in vivo* and second, to unravel the epigenetic regulation of WNT ligands that possibly control (stem) cell proliferation in the mammary gland. Before describing the outline of this thesis, more background will be provided on WNT signalling and stem cell identity, maintenance of the adult mammary gland, WNT-responsive mammary gland stem cells, WNT gene expression in the mammary gland and the basics of 3D genome organisation and epigenetic regulation of spatiotemporal gene expression.

**WNT signalling and stem cell identity**

Stem cells are defined as cells that have the capacity to both self-renew and generate more differentiated progeny. This ability is immediately obvious in pluripotent embryonic stem cells, which can be maintained in culture indefinitely, while also giving rise to all tissues of the developing embryo. In the last decades, it has become clear that these features are not restricted to development, and that most adult tissues are maintained by uni-, bi-, or multipotent stem cell populations that generate cell-type restricted progeny. The first adult stem cells were identified in the bone marrow. These hematopoietic stem cells (HSCs) were initially described as ‘radiation sensitive cells’. These HSCs can be subdivided in long-term (LT-HSC), intermediate (IT-HSC) and short-term populating HSCs (ST-HSC) which differ
in their self-renewing ability. HSCs are quiescent stem cells that divide sparingly and give rise to progenitor cells that continue to divide rapidly and differentiate to lineage restricted cells. The HSCs exist at the top of a differentiation roadmap that yields all mature blood cells. The current consensus is that the ST-HSC mostly contributes to native haematopoiesis, however, the ratio in which the HSC populations contribute to haematopoiesis in different biological contexts is still not entirely clear. This model provides an ‘one-way ticket’, cell identity can only move in one direction: towards differentiation. However, stem cells that have been identified in other tissues behave much more dynamic. These stem cells are often non-quiescent and stem cell identity is determined by extrinsic signals from the environment post cell division. Cell identity is much more plastic in these cell populations, depending on signals from the microenvironment, differentiated daughter cells can revert back to a stem cell state. This has for example been shown upon ablation of the stem cell crypt in the intestine where multiple populations of differentiated cells along the crypt-villus axis revert back to a stem cell state when these cells relocate to the crypt and upon wound repair in the skin where differentiated Gata6 expressing cells in the sebaceous duct of the hair follicle translocate to the epidermis to act as self-renewing stem cells. This highlights the general importance of extrinsic local niche signals for stem cell identity and cell identity in general.

**Maintenance of the adult mammary gland**

The mammary gland is a highly dynamic tissue whose morphology changes regularly during the reproductive life. Several dynamic processes shape the mammary gland postnatally. Mice are born with a rudimentary tree that rapidly begins to expand across the fat pad at the onset of puberty (around three weeks) in response to ovarian hormones like oestrogen. At approximately seven weeks of age the ductal tree occupies the entire mammary fat pad. These ductal tubes comprise of an inner layer of cuboidal shaped luminal cells, flanked by an outer layer of highly elongated basal cells (Fig2).

In the adult gland, short(er) tertiary branches can form, and regress, in response to the estrous cycle which in mice is repeated every four to five days. The estrous cycle consists of four phases: proestrus, estrus, metestrus and diestrus during which the levels and ratio of estrogen and progesterone vary. Previous studies have shown that during the estrous cycle outgrowth and regression of lobuloalveolar epithelium can occur in response to fluctuations in steroid hormone levels. However, when outgrowth occurs is not entirely clear, some studies indicate that this happens during the proestrus/estrus, whereas other studies report that the...
proliferative phase happens during the diestrus \(^{73, 75, 78}\). Regression by apoptosis occurs mainly at the diestrus phase \(^{73, 75, 77}\).

This heightened proliferative state, however, does not occur every estrous cycle \(^{79}\). During pregnancy, the mammary gland is extensively remodelled. In response to progesterone, extensive side-branching and the formation of alveolar buds is initiated in early pregnancy \(^{80}\). Together with prolactin, progesterone promotes the outgrowth of these alveolar buds into large lobuloalveolar structures that produce milk during lactation \(^{81}\). At the end of lactation, post-weaning, the process of involution is initiated which reverts the mammary gland back to its pre-pregnancy state \(^{72, 82}\).

Figure 2: Tissue structure of the mammary gland. A) Schematic overview of macrostructure of the mouse mammary gland. The mammary gland consists of an epithelial ductal network embedded in the mammary fat pad. This fat pad harbours the surrounding stromal tissues and mostly consists of fat cells, but also contains fibroblasts, immune cells and blood vessels. B) The epithelial duct consists of two epithelial cell types: elongated basal cells (K5+) on the outside and large cuboidal luminal cells on the inside (K8+).

**WNT responsive mammary gland stem cells**

The regenerative potential of the mammary gland, as illustrated by the many cycles of growth and regression throughout life during both the estrous cycle and pregnancy, hints at the presence of stem cells in the mammary epithelium. Stem cell like behaviour in the mammary gland was first described decades ago by transplantation assays in the late 1950s and early 1960s \(^{83, 84}\). Evidence of a mammary stem cell population (MaSCs) was first demonstrated in 2006, when cell populations enriched for MaSCs could be enriched by Fluorescence-Activated Cell Sorting (FACS) \(^{85, 86}\). Serial transplantsations showed that these MaSCs have the capacity to self-renew and that both luminal and basal cells were derived from a
single transplanted multipotent cell \(^{85, 86}\).

Several recent studies highlight that WNT ligands can act as niche factors that control MaSCs in the postnatal mammary gland \(^{25, 30, 87-90}\). WNT3A is needed to maintain and expand MaSCs in vitro and to keep their regenerative potential in transplantation assays \(^{87}\). Mammary epithelial cells that express Axin2, a canonical WNT target gene, are enriched for MaSCs that can reconstitute the mammary gland upon transplantation in a cleared fat pad \(^{25}\). Lineage tracing with an \(Axin2^{CreERT2}\) mouse model confirms the presence of WNT-responsive stem cells in vivo. Furthermore, lineage tracing with an \(Lgr5^{CreERT2}\) driver reaffirmed the presence of active WNT/CTNNB1 signalling in the mammary gland \(^{30, 88}\). At this stage however, it is unclear to what extent \(Lgr5\) marks WNT/CTNNB1 responsive stem cells in the mammary gland \(^{30, 88}\). Unlike \(Lgr5\), a different WNT target gene, \(Procr\), has been shown to specifically mark MaSCs \(^{89, 90}\). \(Procr^{+}\) MaSCs display high regenerative capacity in transplantation assays and lineage tracing experiments showed that \(Procr^{+}\) cells are bipotent, thus able to generate progeny that differentiate to both the luminal and basal lineage \(^{89, 90}\). Taken together, several studies provide evidence for the presence of WNT-responsive stem cells in the mouse mammary gland, however, it is unclear to what extent the \(Axin2\), \(Procr\) and \(Lgr5\) positive populations overlap in the mammary gland.

**WNT expression in the mammary gland**

Several members of the \(Wnt\) gene family are expressed throughout various stages of mammary gland development \(^{67}\). During puberty, \(Wnt4\), \(Wnt5a\), \(Wnt5b\), \(Wnt6\), \(Wnt7b\) and \(Wnt2\) are expressed in the epithelium, of which \(Wnt2\), \(Wnt5a\) and \(Wnt7b\) expression is restricted to the terminal end buds (TEB) \(^{91, 92}\). \(Wnt2\) and \(Wnt5a\) are also detected in the surrounding stroma \(^{93}\). In the adult mammary gland, \(Wnt4\) and \(Wnt7b\) are exclusively expressed in the luminal epithelium \(^{94-96}\). \(Wnt5a\) is broadly expressed in both the stroma, luminal and basal cells, whereas \(Wnt5b\) is only detected in mammary epithelial cells \(^{97}\). Expression of \(Wnt10a\) is restricted to basal cells \(^{97}\), whereas \(Wnt11\) is detected in both basal cells and the stroma \(^{97}\). During pregnancy, \(Wnt2\), \(Wnt4\), \(Wnt5a\), \(Wnt5b\), \(Wnt6\), \(Wnt10b\) and \(Wnt7b\) are expressed \(^{91, 93, 94, 96, 99}\). \(Wnt5b\) and \(Wnt6\) during lactation \(^{91, 93, 98}\), and in the involution phase \(Wnt2\), \(Wnt5a\), \(Wnt5b\) and \(Wnt7b\) have been detected \(^{91, 93}\) (Table 1).
Table 1: **WNT expression in the mouse mammary gland.** This table provides an overview of which WNT ligands are expressed in the mouse mammary gland at which stage of development and by which cell type adopted from Yu QC et al. 67. qRT-PCR, RNA in situ hybridisation, northern blot and microarray base gene expression profiling were used to detect the expression of Wnt gene mentioned in this table. Table was adapted from data previously used in Heijmans N, Exploring the Wnt enhancer landscape in the mammary gland (2021) 100.

Spatial gene expression of some, but not all WNT proteins is maintained in the human breast (Fig3). It should be noted that no expression data from pregnancy is available and that most human expression data is derived from what is likely post-menopausal tissue. Nevertheless, we can appreciate that the expression of WNT2, WNT4, WNT5A, WNT5B and WNT7B in particular is conserved in adult mammary gland tissue between mouse and human (Fig3).

<table>
<thead>
<tr>
<th>STAGE</th>
<th>CELL TYPE</th>
<th>WNT GENES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PUBERTY</strong></td>
<td>TEB epithelium</td>
<td>Wnt2, Wnt4, Wnt5a, Wnt5b,</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><strong>ADULT</strong></td>
<td>Luminal cells</td>
<td>Wnt4, Wnt5a, Wnt5b, Wnt7b</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><strong>PREGNANCY</strong></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, Wnt10b</td>
</tr>
<tr>
<td><strong>LACTATION</strong></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><strong>INVOLUTION</strong></td>
<td>Whole mammary gland</td>
<td>Wnt2, Wnt5a, Wnt5b, Wnt7b</td>
</tr>
</tbody>
</table>
Figure 3: WNT expression in the human breast. Based on human breast scRNA-seq data from the Tabula Sapiens Consortium. This dot-plot illustrates the expression of all 19 WNT genes in the human breast across different cell types. The colour scale bar indicates the mean expression in each group, whereas the dot size illustrates the fraction of cells that express a WNT gene within that group. Bars indicate the total number of cells detected for each group within this scRNA-seq dataset.

For most individual WNT ligands the specific function in the mammary gland is far from clear, the exception being Wnt4 and Wnt2. Wnt4 has been linked to the maintenance of stem cells in various tissues, including the mammary gland. WNT4 maintains outgrowth and side-branching during early pregnancy, in part by maintaining and controlling the MaSC population. Outgrowth of the mammary epithelium in serial transplantation assays is impaired in Wnt4−/− mammary tissue compared to wildtype tissue. Wnt4 expression is restricted to hormone responsive mature luminal cells and is reported to be directly controlled by progesterone signalling in adult and pubertal mammary glands. This means that Wnt4 expression is possibly dependent on the estrous cycle. WNT4 secretion by mature luminal cells activates downstream WNT/CTNNB1 signalling in neighbouring Axin2+ basal cells. Several observations indicate that Wnt4 expression can also be regulated independent of the progesterone receptor (PR). For example, outgrowth of Wnt4−/− tissue is more severely impaired than Pr−/− tissue in serial transplantation assays, implying that WNT4 can act as a MaSC regulator independent of PR. Moreover, perinatal Wnt4 expression has been reported to be independent from both the progesterone and oestrogen receptor. Taken together, this implies that hormone responsive mature luminal cells convert a systemic...
endocrine signal into a localized proliferative growth signal in the form of WNT4. However, how PR-independent *Wnt4* expression is regulated, and what co-factors control *Wnt4* expression beyond PR is currently unknown.

*Wnt2* is expressed by fibroblasts cells at the stromal-epithelial border. Two recent studies show that growth factors, including WNT2, secreted by fibroblasts at the stromal-epithelial interface are required for WNT/CTNNB1 activation and side-branching in the mammary epithelial compartment. Somewhat surprisingly, *Wnt2* gene expression appears to be regulated by WNT/CTNNB1 signalling and is expressed in *Axin2* fibroblasts, suggesting the existence of a positive feed forward loop. This transcriptional program is activated by WNT ligands secreted from local endothelial cells. It has also been shown that *Wnt2* gene expression in mammary fibroblasts is dependent on GLI2 and that this GLI2-WNT2 axis is needed for mammary epithelial regeneration. Taken together, these two studies show that stromal-epithelial crosstalk is needed for mammary epithelial cell expansion, and that *Wnt2*, expressed in *Gli2*/*-Axin2* fibroblasts at the stromal-epithelial border, is one of the growth factors involved.

**3D genome organisation, epigenetic regulation and spatiotemporal control of gene expression**

Precise spatiotemporal gene expression across tissues is mediated by the activation of distal enhancers that communicate with promotors via 'enhancer-promotor' looping, in other words physical proximity, in a context dependent manner. To achieve this, the regulatory landscape of the genome folds in 3D nuclear space to form complex structures that influence enhancer-promoter interactions and thereby gene expression.

Enhancers are fundamentally defined as non-coding blocks of sequence that can precisely control spatiotemporal gene expression, even of distant genes (for example up to 1 Megabase (Mb) away). Nevertheless, not all enhancers can activate all promotors, thus different enhancers likely use different mechanisms to specify if and how they activate a specific gene. Since enhancers can act over long genomic (linear) distances and do not always activate the nearest gene, identifying enhancers and dissecting how they communicate with their correct target gene is still a major challenge. Candidate enhancers can be identified by a distinct epigenetic signature that includes chromatin accessibility, H3K4me1 and H3K27ac. However, these features do not guarantee that a given region functions as a cis or trans-regulatory element. They also offer no indication of its intended target gene. Thus, a candidate enhancer always needs to be further validated experimentally.
The genome is organised in large Megabase size domains called Topologically Associating Domains (TADs). These domains are separated by topological boundaries that favour interactions between enhancers and genes within said domain over interactions with genes and enhancers in neighbouring domains. Thus, the 3D organisation of the genome into TADs adds an operational limit to the regulatory landscape of genes and disruption of TAD boundaries can lead to the formation of de-novo enhancer-promoter interactions that result in gene dysregulation and disease. (Fig4). For example, rearrangements of the TAD boundaries in the WNT6/IHH/EPHA4/PAX3 locus results in the misexpression of these developmental genes, which subsequently leads to human limb malformations. This illustrates that although the effect on gene expression likely differs between loci, the 3D organisation of the genome has direct functional consequences in facilitating enhancer-promoter interactions and thereby gene expression.

TAD formation and function is instituted by the combined action of the COHESIN complex and the CCCTC-binding transcription factor (CTCF) via loop-extrusion. In vertebrates, the most common COHESIN barrier is formed by two convergent CTCF binding sites. Beyond a role in TAD boundary formation, recent studies highlight a novel function for CTCF in promoting long distance enhancer-promoter interactions (>100kb). For example, CTCF and COHESIN facilitate a 1Mb spanning contact loop between the Shh promoter and the ZRS enhancer.
Figure 4: Schematic overview of 3D genome organisation. A) Schematic representation of a Hi-C contact map and TAD organisation. Colouration in a contact map is representative for interaction frequency between two points and can be used to visualize 3D physical loops between two points in the genome. B) Cartoon that illustrates the folding of the genome in the region represented in A) based on the Hi-C contact map. Adapted from Robson MI et al. 2019 "111.

Outline of this thesis

There is a clear knowledge gap in our understanding of stem cell regulation and WNT signalling in general, its spatiotemporal regulation (or: the ‘when’ and ‘where’ of its activity), and what this means for tissue homeostasis. This thesis addresses this outstanding question from different angles. In chapter 2, we characterized a novel mouse model that allows spatiotemporal imaging of the endogenous expression of \textit{Axin2}, a canonical WNT reporter gene, \textit{in vivo} and \textit{in vitro}. In combination, we developed a multicolour lineage tracing mouse model to better study stem cell dynamics and directly visualize cell-cell interactions, which we describe in chapter 3. Finally, in chapters 4-7 we provide an in-depth framework for the epigenetic regulation of several WNT ligands in the mammary gland. Given that dynamic yet tight patterns of WNT gene expression have been observed, equally tight regulatory control mechanisms must exist \textsuperscript{67, 127}. This process is poorly understood, yet is critical in the spatiotemporal control of WNT responsive stem cell activation and population size. Taken together, this thesis aims to provide a more holistic view on the regulation of the WNT-responsive stem cell ‘niche’.
References

1. Nusse, R. & Clevers, H. Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. Cell 169, 985-999 (2017).


Chapter 1


75. Fata, J. E., Chaudhary, V. & Khokha, R. Cellular turnover in the mammary gland is correlated with systemic levels of progesterone and not 17beta-estradiol during the estrous cycle. Biol. Reprod. 65, 680-688 (2001).

76. H. A. C. The Mammary Gland of the Mouse, During the (Estrous Cycle, Pregnancy and Lactation.


