Pituitary stem cell regulation: who is pulling the strings?

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Abstract

The pituitary gland plays a pivotal role in the endocrine system, steering fundamental processes of growth, metabolism, reproduction and coping with stress. The adult pituitary contains resident stem cells, which are highly quiescent in homeostatic conditions. However, the cells show marked signs of activation during processes of increased cell remodeling in the gland, including maturation at neonatal age, adaptation to physiological demands, regeneration upon injury and growth of local tumors. Although functions of pituitary stem cells are slowly but gradually uncovered, their regulation largely remains virgin territory. Since postnatal stem cells in general reiterate embryonic developmental pathways, attention is first being given to regulatory networks involved in pituitary embryogenesis. Here, we give an overview of the current knowledge on the NOTCH, WNT, epithelial–mesenchymal transition, SHH and Hippo pathways in the pituitary stem/progenitor cell compartment during various (activation) conditions from embryonic over neonatal to adult age. Most information comes from expression analyses of molecular components belonging to these networks, whereas functional extrapolation is still very limited. From this overview, it emerges that the 'big five' embryonic pathways are indeed reiterated in the stem cells of the 'lazy' homeostatic postnatal pituitary, further magnified *en route* to activation in more energetic, physiological and pathological remodeling conditions. Increasing the knowledge on the molecular players that pull the regulatory strings of the pituitary stem cells will not only provide further fundamental insight in postnatal pituitary homeostasis and activation, but also clues toward the development of regenerative ideas for improving treatment of pituitary deficiency and tumors.

Key Words
- pituitary
- stem cells
- regulation
- homeostasis
- regeneration
- tumor
- NOTCH
- WNT
- Hedgehog
- epithelial–mesenchymal transition
- Hippo
- SOX2

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Pituitary stem cells: purported players in pituitary housekeeping

The pituitary gland acts as the governor of the endocrine system, integrating inputs from the central hypothalamus and from peripheral grounds to adjust the output of endocrine glands to hormonal needs of the body. The pituitary consists of two major parts, the adenohypophysis and the neurohypophysis. The adenohypophysis contains the hormone-producing cells, located in the anterior pituitary (AP) or the intermediate lobe (IL). The AP harbors the cells responsible for the production of growth hormone (GH), prolactin (PRL), adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH) and luteinizing hormone (LH)/follicle-stimulating hormone (FSH). The IL encompasses the cells that produce melanocyte-stimulating hormone (MSH),

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a protein also derived from the ACTH precursor molecule proopiomelanocortin (POMC). In addition to the endocrine cells, the adenohypophysis contains non-hormonal cells including endothelial cells that form the sinusoidal capillary network, supportive mesenchymal cells and folliculostellate cells that have multiple functions (Allaerts & Vankelecom 2005, Susa *et al.* 2012).

Embryogenesis of the pituitary starts with invagination of the oral roof ectoderm, which expresses the paired-like homeodomain transcription factor 1 (PITX1) and PITX2 and bulges toward the neural ectoderm of the ventral diencephalon (for extensive reviews, see Rizzoti & Lovell-Badge 2005, Zhu *et al.* 2007, Kelberman *et al.* 2009, Vankelecom & Gremeaux 2010, Vankelecom 2012). This invagination, known as Rathke's pouch (RP), is mainly composed of progenitor cells marked by expression of sex determining region Y-box 2 (SOX2) and later of Prophet of PIT1 (PROP1). RP then detaches from the oral ectoderm, to further expand and generate the different hormonal cell lineages under the influence of opposing gradients from morphogens (like fibroblast growth factor (FGF) and bone morphogenetic protein (BMP)) that drive the spatio-temporal expression of specific transcriptional regulators of hormonal lineage specification. For instance, PROP1 is essential for the formation of the cell lineage marked by POU domain, class 1, transcription factor 1 (POU1F1), also known as the PIT1 lineage, which consists of somatotropes (producing GH), lactotropes (PRL) and thyrotropes (TSH). Eventually, the AP and IL are formed, still separated by a lumen remaining within the inflating RP and forming the later cleft (Rizzoti & Lovell-Badge 2005, Zhu *et al.* 2007, Kelberman *et al.* 2009, Vankelecom & Gremeaux 2010, Vankelecom 2012). In humans, the cleft between the adeno- and neurohypophysis is still discernible but the IL is not clearly demarcated anymore, and MSH-expressing melanotropes and RP remnants are found dispersed within the AP (McNicol 1986, Garcia-Lavandeira 2009, 2012).

After birth, the pituitary further expands in size and cell number by an active neonatal growth wave, completed around postnatal day (P) 21 in mice (Carbajo-Pérez & Watanabe 1990, Taniguchi *et al.* 2002). Following that period and throughout adulthood, the gland is fairly static with very low turnover during homeostasis. However, cell remodeling is regularly (re-)activated according to the endocrine needs. Adaptation of the cellular composition is observed during puberty (increase in somatotropes) and during pregnancy and lactation (expansion of lactotropes). Mechanisms underlying this pituitary plasticity, as well as the homeostatic turnover, are still uncertain (Melmed 2003). The increased number of a precise group of endocrine cells may be generated by mitosis of existing hormonal cells, by transdifferentiation between different hormonal cell lineages or by differentiation from resident stem cells.

Although the existence of stem cells in the pituitary was regularly hypothesized in the last 50 years (Yoshimura *et al.* 1969, Melmed 2003), the cells flew under the radar for a long time. In 2005, a side population (SP) of cells, distinguished by efflux capacity for Hoechst33342 dye, was identified in the mouse adult AP and found to encompass cells possessing self-renewal and multipotent differentiation capacity, both fundamental hallmarks of stem cells (Chen *et al.* 2005). Further refinement pinpointed a SP subfraction (referred to as the stem cell-side population or SC-SP) highly enriched in cells with stem cell characteristics and expressing stemness markers, in particular SOX2 and SOX9 (Chen *et al.* 2009). These and other markers (including E-cadherin, glial cell line derived neurotrophic factor family receptor alpha 2 or GFRA2, nestin and PROP1) were found in the cells of the marginal zone bordering the cleft (Fauquier *et al.* 2008, Gleiberman *et al.* 2008, Chen *et al.* 2009, Garcia-Lavandeira *et al.* 2009, Yoshida *et al.* 2011, 2014), with a similar expression and organization picture in human pituitary (Garcia-Lavandeira *et al.* 2009, 2012). In addition, SOX2$^+$ cells were also found within the gland's parenchyma, mostly occurring in clusters and also expressing SOX9 and E-cadherin (Fauquier *et al.* 2008, Gremeaux *et al.* 2012, Chen *et al.* 2013, Rizzoti *et al.* 2013, Nantie *et al.* 2014, Zhu *et al.* 2015). The marginal zone and parenchymal clusters are proposed to represent plural stem cell niches in the pituitary to enable swift adaptation during (subtle) cell remodeling processes (Vankelecom 2012, Vankelecom & Chen 2014). The different niches appear physically interconnected in a 3D network (Gremeaux *et al.* 2012, Mollard *et al.* 2012, Vankelecom & Chen 2014, Vankelecom 2016). However, whether the stem cells at the different locations exhibit different properties, regulation and function is not clear. Some reports point to molecular distinctions, including differential expression of PROP1 and extracellular matrix components (Yoshida *et al.* 2011, 2014, 2016*a*).

Once pituitary stem cells were identified, the search started for unraveling their functional responsibilities in the gland. In general, stem cells in adult tissues are involved in tissue turnover, repair after injury and/or pathogenesis (like tumorigenesis) when their regulation goes awry. A pituitary injury model was developed in which somatotropes are destroyed by diphtheria toxin (DT)

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injection of GH-Cre/iDTR mice expressing the DT receptor (DTR) under control of the GH promoter (Fu *et al.* 2012). A first remarkable observation in this study was the considerable restoration of the GH-expressing (GH$^+$) cells after 4–5 months, for the first time showing that the adult pituitary is capable of cell regeneration. Moreover, the stem cells were found to respond to the pituitary injury by expanding and expressing GH, thus strongly supporting their involvement in the regenerative reaction. A similar process was observed in another pituitary injury model, i.e. the PRL-Cre/iDTR mouse in which lactotropes were killed by DT (Fu & Vankelecom 2012). The studies together point to a more general regenerative competence of the pituitary and its stem cells (Willems & Vankelecom 2014). This capability, however, quickly disappears with aging, coinciding with a decrease in stem cell number and fitness (as probed in the stem cell-driven sphere-forming assay) (Willems *et al.* 2016). Moreover, regeneration was not observed anymore after prolonged injury impact (i.e. 10 days of DT injection instead of 3 days), which may be due to exhaustion of the repeatedly activated stem cells during the extended injury impact (Willems *et al.* 2016). Genome-wide expression analysis identified several embryonic pathways in the stem cell compartment in these models, which may underlie the success or failure of the pituitary's regeneration (Willems *et al.* 2016), as further discussed below.

Whether pituitary stem cells are also involved in the gland's early-postnatal maturation and in the adult homeostatic turnover has recently been investigated. During the neonatal growth wave, pituitary stem cells are more abundant and more proliferative as compared to the stem cells in the adult gland and show higher expression of several components of key embryonic pathways like sonic hedgehog (SHH) and wingless-type MMTV integration site (WNT) (Yoshida *et al.* 2011, Gremeaux *et al.* 2012). In accordance, lineage tracing studies showed a larger contribution of the pituitary SOX2$^+$ and SOX9$^+$ stem cells to endocrine cells at young age than at adult age when pituitary turnover is very low, indicating that the stem cells are (more) active in early-postnatal maturation but largely quiescent under adult homeostatic conditions (Andoniadou *et al.* 2013, Rizzoti *et al.* 2013). However, endocrine changes triggering pituitary plastic adaptations seem to involve reactivation of the dormant stem cells in the adult gland. For instance, adrenalectomy causes a transient surge in ACTH-producing corticotropes of which 20% is derived from SOX9$^+$ stem cells (Rizzoti *et al.* 2013). In addition, gonadectomy or *in vivo* estradiol treatment elicits a (SOX2$^+$) stem cell reaction showing higher proliferative activity (Rizzoti *et al.* 2013).

Although the functional position of the pituitary stem cells is being unveiled step by step, not much is known about the molecular regulation of the cells' activity during the execution of their several roles. In the first part of this review, we summarize what has presently been described regarding major regulatory pathways in the pituitary stem cell compartment during the different conditions of embryonic and neonatal development, homeostasis, adaptation and regeneration. In the second part, we discuss what is known about these pathways in pituitary tumorigenesis. We describe the pathways in a 'downstream' logic, i.e. from ligands to receptors to intracellular signals.

NOTCH pathway: contained *Janus*-type regulator of the pituitary stem cells?

The NOTCH pathway is a well-known regulatory system essential for embryonic development of many organs, particularly regarding cell fate decisions during organogenesis. NOTCH signaling is activated by binding of the membrane-tethered ligands Jagged (JAG1, 2) and Delta-like (DLL1, 3, 4) to the NOTCH1–4 receptors on adjacent cells (Baladrón *et al.* 2005) (Fig. 1A). Binding induces cleavage of the intracellular domain of the NOTCH receptor (NICD) by γ-secretases. The released NICD subsequently migrates into the nucleus to combine with the core NOTCH transcription factor recombination signal-binding protein for immunoglobulin kappa J region (RBP-J) and co-transcriptional activators like Mastermind-like. The formed complex activates the expression of various downstream target genes such as the basic helix-loop-helix hairy and enhancer of split-1 (HES) and HES-related with YRPW motif 1 (HEY) transcription factor family (e.g. *Hes1*, *Hey1*) (Braune & Lendahl 2016). NOTCH signaling induces divergent cell fates starting from an initially uniform cell pool through a process called 'lateral inhibition' (Sprinzak *et al.* 2010). Neighboring cells inhibit each other to coexpress within the same cell both NOTCH receptors and ligands. This mechanism eventually generates a mosaic pattern in which NOTCH-receptive cells alternate with ligand-expressing cells, thereby inducing different cell fates within a previously identical population (Sprinzak *et al.* 2010). Applied to stem cells, NOTCH activation in general keeps the cells undifferentiated through the HES/HEY

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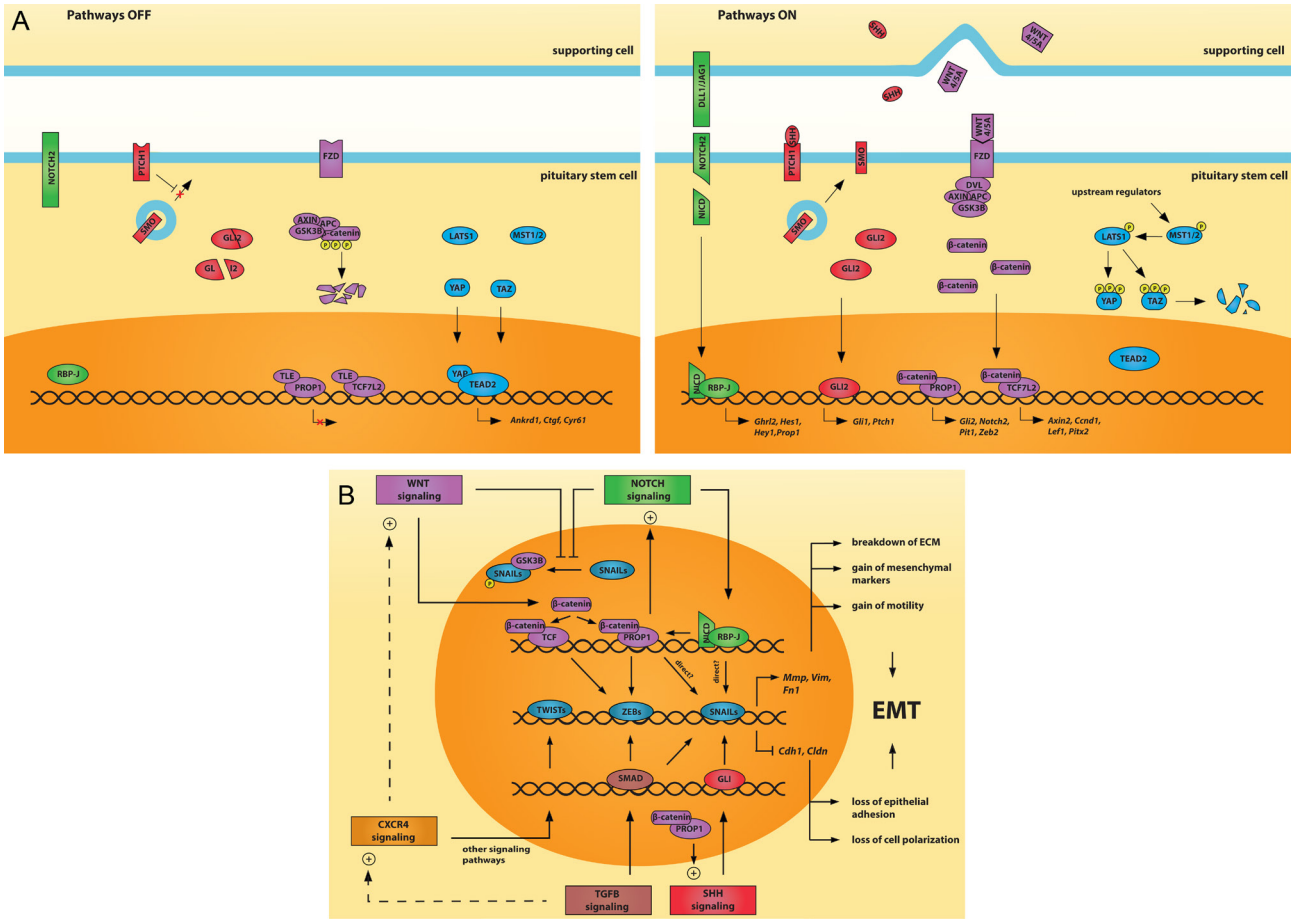

Figure 1
Overview of the signaling pathways potentially involved in pituitary stem cell regulation. (A) Schematic presentation of the putative pituitary stem cell niche, encompassing the stem cells and the stem cell-supporting cells. The NOTCH (green), SHH (red), WNT (purple) and Hippo (blue) pathways are shown in their 'off' (left) and 'on' status (right). Interaction of the NOTCH transmembrane receptor with DLL/JAG ligands on adjacent cells initiates cleavage of the Notch intracellular domain (NICD), which translocates to the nucleus to associate with the transcription factor RBP-J, thereby inducing expression of NOTCH target genes. Binding of SHH to PTCH receptor releases SMO from endocytic vesicles (light blue circle), preventing GLI degradation and thereby enabling GLI migration to the nucleus where it regulates SHH target gene expression. In the presence of WNT ligands, interaction with the FZD receptor inhibits degradation of β-catenin which then in the nucleus converts transcriptional repressors (e.g. TCF7L2) to activators or activates transcription by PROP1, resulting in WNT target gene expression. When Hippo signaling is 'on', a phosphorylation cascade involving the LATS and MST kinases is activated, leading to YAP/TAZ phosphorylation, thereby inhibiting downstream gene expression. DVL, Disheveled. For further details and abbreviations, see text. (B) Schematic model of the EMT network potentially unfolding in the pituitary stem cells. During EMT, epithelial markers (e.g. E-cadherin) are downregulated and mesenchymal markers (e.g. VIM) are upregulated. A central role is played by three families of transcription factors, i.e. TWISTs, ZEBs and SNAILs (blue), which repress transcription of the *Cdh1* gene (coding for E-cadherin). Induction of matrix metalloprotease (*Mmp*) allows the eventual mesenchymal cell to move by breakdown of the surrounding extracellular matrix. EMT regulation involves a complex interplay between several pathways. TGFB signaling (brown) promotes transcription of *Zebs* and *Snails* through SMAD complexes and may enhance CXCR4 signaling (dashed line). CXCR4 signaling (orange) upregulates expression of *Twists* via ERK and PI3K/AKT signaling, leading to progression of EMT. CXCR4 may also activate WNT signaling (dashed line). The WNT pathway (purple) inhibits the phosphorylation of SNAILs by GSK3B, thereby increasing the stability of SNAILs, and can stimulate the expression of *Zebs* via TCF. The WNT-associated PROP1 increases expression of ZEBs and may also directly influence the expression of SNAI2. The involvement of PROP1 in EMT becomes even more elaborate by its stimulatory effect on the expression of NOTCH and SHH components, and its dependence on NOTCH activation. SHH signaling (red), through its transcription factor GLI1, enhances *Snai1* expression which (further) induces EMT. NOTCH signaling (green) increases the stability of SNAI1 through disrupting GSK3B-SNAI1 interaction, and may also enhance the expression of *Snails* in a direct way. ECM, extracellular matrix. For further details and abbreviations, see text.

transcriptional repressors while allowing the neighboring cells to differentiate.

Also in the pituitary, NOTCH activity in the embryonic progenitor cells is essential for maintenance of the undifferentiated state (Zhu *et al.* 2006, 2015, Nantie *et al.* 2014), with HES1 inhibiting progression toward hormonal cells (Zhu *et al.* 2006). In analogy, enforced persistent activation of NOTCH signaling in the more committed cells obstructs their final differentiation during embryogenesis (Zhu *et al.* 2006,

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Goldberg *et al.* 2011). NOTCH activity also affects the stem cells of the adult pituitary. *In vitro* stimulation by soluble JAG1 ligand triggers a proliferative expansion of the SC-SP (see above), whereas inhibition with DAPT shrinks the stem cell population (Chen *et al.* 2006, Tando *et al.* 2013).

Regarding the NOTCH pathway ligands, *Dll1* is especially expressed around the cleft during pituitary embryogenesis (Raetzman *et al.* 2004, Zhu *et al.* 2006). *Jag1* expression is comparable but largely extinguishes around the cleft from embryonic day (E) 12.5 onwards, while persisting in some parenchymal areas of the nascent AP (Zhu *et al.* 2006). In contrast, *Dll3* is detected in developing corticotropes during embryogenesis and in melanotropes before and after birth (Hrabě de Angelis *et al.* 1997). Knockout of *Dll3* did not affect pituitary cell specification, implying a non-essential or redundant role of *Dll3* in the developing pituitary. *Dll1*$^{-/-}$ mice showed early embryonic death prior to pituitary development (Hrabě de Angelis *et al.* 1997). Conditional deletion specifically in the pituitary has not yet been performed. *Prop1*$^{df/df}$ embryonic pituitaries, displaying virtual absence of *Notch2*, showed higher *Dll1* expression, which is in line with the principle of lateral inhibition within the NOTCH concept (Raetzman *et al.* 2004, Sprinzak *et al.* 2010).

In the adult resting pituitary, NOTCH ligands are higher expressed in the non-stem cell populations (Chen *et al.* 2009, Vankelecom 2010), also in agreement with the principle of lateral inhibition with NOTCH receptors and ligands being expressed in different (neighboring) cells, i.e. stem cells and niche-supportive cells, respectively (Fig. 1A). Additional identification of the ligand-exposing cells may further portray the pituitary stem cell niche(s). Remarkably, a recent study showed co-expression of NOTCH2 and JAG1 in the same pituitary stem cells (Batchuluun *et al.* 2017) which is not in line with the model of lateral inhibition. Moreover, in pituitary stem cell-activated conditions of neonatal maturation and somatotrope ablation injury (GH-Cre/iDTR model), expression of the ligands *Jag1* and/or *Dll1* was found upregulated in the isolated stem cell populations (Gremeaux *et al.* 2012, Willems *et al.* 2016) (Table 1), suggesting that NOTCH activation is unfolding between the cells of the stem cell compartment in these activated states. In addition, the atypical Delta-like 1 homolog (DLK1) ligand, typically proposed to inhibit NOTCH signaling, is mainly expressed in somatotropes (Cheung *et al.* 2013), and elimination of these cells in the GH-Cre/iDTR mice through the DT-mediated somatotrope ablation may thus lead to a disinhibition of NOTCH signaling.

Regarding NOTCH receptor expression, *Notch2* and *Notch3* were preferentially found in the SC-SP of the adult pituitary (Chen *et al.* 2009) (Fig. 1A and Table 1). Interestingly, the same NOTCH receptor subtypes are expressed in the embryonic pituitary progenitor cells, particularly around the cleft (Raetzman *et al.* 2004, Zhu *et al.* 2006), and *Notch2* remains predominantly expressed around the cleft in the postnatal pituitary (Nantie *et al.* 2014). It is not known yet how NOTCH2 and NOTCH3 functionally overlap in the pituitary. In *Prop1*-mutant mice exhibiting a dwarf phenotype (*Prop1*$^{df/df}$), expression of NOTCH2 protein is absent, while *Notch3* gene expression is not affected (Raetzman *et al.* 2004), positioning NOTCH2 but not NOTCH3 downstream of PROP1. Mice with Cre-mediated conditional knockout of *Notch2* under the control of *Foxg1*, a factor expressed in RP cells at E9.5 (Wang *et al.* 2010), showed no obvious defects during pituitary embryonic development, suggesting that other NOTCH receptors like NOTCH3 may take over or provide sufficient NOTCH signaling during embryogenesis (Nantie *et al.* 2014). However, the *Notch2* mutants showed a reduction in postnatal AP volume, together with a decrease in PIT1 lineage (in particular, GH$^+$ and TSHβ$^+$) cells and an increase in POMC$^+$ cells. Moreover, SOX2$^+$ and SOX9$^+$ cells were less abundant in the marginal zone of the *Notch2* mutant pituitary. SOX2$^+$ cell clusters were still present throughout the parenchyma and also at reduced abundance, whereas SOX9$^+$ cell clusters were not observed anymore (Nantie *et al.* 2014), suggesting that maintenance or development of the stem cell population(s), and particular of the SOX9$^+$ cells, is dependent on NOTCH signaling. The decreased number of postnatal stem cells may explain the reduced AP volume. Taken together, NOTCH2-mediated signaling is required for stem cell maintenance as well as appropriate differentiation (i.e. needed for the PIT1 lineage while repressing the corticotrope POMC lineage) during embryonic and postnatal pituitary development.

Taking out NOTCH activation by deleting the central mediator *Rbp-J* showed a profound effect on progenitor cell maintenance and differentiation in the embryonically nascent pituitary (Zhu *et al.* 2006, 2015). Conditional deletion of *Rbp-J* using Cre under control of *Pitx1* (already expressed in the oral ectoderm) diminished pituitary progenitor cell proliferation from E12.5 and resulted in a differentiation shift from PIT1 toward corticotrope lineage, again showing that proper NOTCH signaling is essential to induce the PIT1 lineage and suppress the corticotrope fate in the developing pituitary. Postnatal development could not be studied in these *Pitx1-Cre/Rbp-J*$^{fl/fl}$ mice because of

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Table 1 Genes from regulatory pathways upregulated in the stem cell compartment of neonatal, adult, regenerating and tumorigenic pituitary.

Pathway[a]	Neonatal pituitary (SC-SP vs adult SC-SP)[b]	Adult pituitary (SC-SP vs non-SC-SP)[b]	Adult pituitary ($SOX2^{eGFP+}$ vs $SOX2^{eGFP-}$ population)	Regenerating pituitary (SC-SP after damage vs control SC-SP)[b]	Human pituitary tumors (SP vs non-SP)[c]
NOTCH					
Ligands	*Dll1*, *Jag1*			*Dll1*	*DLL1*, *JAG1*
Receptors	*Notch2*	***Notch2***, *Notch3*	***Notch2***	*Notch2*	*NOTCH1*, ***NOTCH2***
Transcription factors	*Rbpj*				
Transcriptional targets	*Hey1*, *Prop1*	***Hey1***, *Hey2*, *Heyl*	***Grhl2***, *Hey1*, *Hey2*, *Heyl*, ***Prop1***	*Hes1*, *Hey1*	*HES1*, *HEY2*, *HEYL*
WNT					
Ligands	*Wnt2b*, *Wnt4*, *Wnt5a*		*Wnt4*, *Wnt7b*, *Wnt9a*, *Wnt10a*		*WNT5A*
Receptors	*Fzd1*, *Fzd2*, *Fzd3*	*Fzd2*, *Fzd7*	*Fzd1*, *Fzd3*, *Fzd4*, *Fzd6*, *Fzd7*	*Fzd6*	*FZD1*, *FZD10*
RSPO/LGR	*Rspo1*	*Lgr5*	***Lgr4***, *Lgr5*	*Rspo2*	*LGR5*
Intracellular cascade components			*Csnk1a1*	*Csnk1g2*	
Transcription factors	*Prop1*, *Tcf7l1*, *Tcf7l2*	*Lef1*, *Tcf7*	***Prop1***, *Tcf7*, *Tcf7l2*	*Lef1*	*LEF1*, *TCF7L1*, *TCF7L2*
Transcriptional co-factors	*Aes*, *Tle3*			*Tle1*	*TLE3*
Transcriptional targets	*Axin2*, *Myc*	*Lef1*	*Ccnd1*, *Myc*, *Pitx2*	*Axin2*, *Lef1*	*LEF1*, *MYC*
Non-canonical WNT components	*Ptk7*		*Ankrd6*, *Celsr1*, *Celsr2*, *Fzd3*, *Ror1*, *Vangl2*		*PRICKLE1*, *ROR1*
EMT					
Ligands	*Cxcl12*	*Cxcl12*, *Tgfb2*	*Tgfb2*	*Cxcl1*, *Cxcl9*, *Cxcl12*, *Tgfb1*, *Tgfb2*	*CXCL1*, *CXCL12*, *TGFB1*, *TGFB2*
Receptors		*Cxcr4*	*Tgfbr1*	*Cxcr7*, ***Tgfbr2***	*CXCR4*, *CXCR7*, *TGFBR2*
Transcription factors	*Foxc1*, *Foxc2*, *Twist1*, *Twist2*, *Zeb2*	*Foxc2*, *Prrx1*, *Prrx2*, *Twist1*, *Twist2*, *Zeb2*	***Prrx1***, ***Prrx2***	*Foxc2*, *Klf8*, ***Snai1***	*FOXC1*, *SNAI1*, ***SNAI2***, *ZEB1*, ***ZEB2***
Downstream effectors	*Mmp2*, *Fn1*, *Vim*	*Acta2*, *Fosl1*, *Fosl2*, *Mmp2*, *Mmp3*	*Acta2*, *Fosl1*	*Fosl1*, *Mmp2*, *Mmp3*, *Vim*	*ACTA2*, ***FN1***, *FOSL2*, *MMP1*, ***VIM***
SHH					
Ligands	*Hhip*	*Hhip*			
Receptors	*Ptch1*, *Smo*		*Ptch1*, *Smo*		
Transcription factors	*Gli1*, *Gli2*, *Gli3*			*Gli1*, *Gli2*	*GLI1*
Transcriptional targets	*Ptch1*		*Ptch1*		*PTCH1*
Hippo					
Upstream kinases	*Lats1*		*Nf2*, *Wwc1*	*Lats2*, *Stk4*	*LATS2*
Transcription (co-) factors	*Tead2*, *Yap1*				*TEAD4*, *YAP1*
Transcriptional targets			*Cyr61*, *Ankrd1*	*Ctgf*, *Cyr61*	*CTGF*, *CYR61*
FGF/EGF					
Receptors	*Egfr*, *Fgfr1*, *Fgfr2*	*Egfr*, *Fgfr1*	*Egfr*, *Fgfr1*, *Fgfr2*	***Egfr***, *Fgfr1*	*EGFR*, *FGFR1*, *FGFR3*

[a]Selection of genes at least 1.5-fold upregulated, as identified by microarray analyses in our previous studies (Chen *et al.* 2009, Vankelecom 2010, Gremeaux *et al.* 2012, Mertens *et al.* 2015, Willems *et al.* 2016) and from $SOX2^{eGFP}$ RNA-sequencing data (B Cox, H Roose and H Vankelecom, unpublished observations). Underlined: genes ≥5-fold upregulated. Bold: genes validated using other techniques in studies of our or other groups (see text). [b]More details on the definition of these populations can be found in Chen *et al.* (2009), Vankelecom (2010), Gremeaux *et al.* (2012) and Willems *et al.* (2016). In short, SC-SP represents the SP depleted from cells expressing stem cell antigen-1 (SCA1) at a high level; non-SC-SP refers to the remaining SP ($SCA1^{high}$ SP) and the MP. [c]More details on the definition of these populations can be found in Mertens *et al.* (2015). In short, SP here stands for the SP depleted from $CD31^+$ endothelial and $CD45^+$ immune cells, and non-SP for the MP depleted from $CD31^+$ endothelial and $CD45^+$ immune cells.

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perinatal lethality. Conditional deletion of *Rbp-J* using a *Prop1-Cre* driver resulted in a milder hormonal phenotype with timely formation of the PIT1 lineage (Zhu *et al.* 2015). PITX1 expression is initiated in the developing pituitary before PROP1 (E9.0 and E11.5, respectively), and the earlier blockade of NOTCH signaling may explain the more severe phenotype in the above described *Pitx1-Cre/Rbp-J*$^{fl/fl}$ mice (Olson *et al.* 2006, Zhu *et al.* 2006). Moreover, PROP1 expression failed to peak in *Pitx1-Cre/Rbp-J*$^{fl/fl}$ mice at E12.5 (Zhu *et al.* 2006), appointing *Prop1* as a target gene of NOTCH signaling, which may further contribute to the fiercer phenotype as *PROP1* deficiency is well known to result in combined pituitary hormone deficiency (Abrão *et al.* 2006). During embryogenesis, *Prop1-Cre/Rbp-J*$^{fl/fl}$ mice showed a reduction in proliferating SOX2$^+$ cells, both in the developing AP and IL (Zhu *et al.* 2015). After birth, stem cells lining the cleft and parenchymal stem cell clusters were largely missing, as indicated by the absence of SOX2, SOX9, E-cadherin and PROP1 immunoreactivity (Zhu *et al.* 2015). Accordingly, sphere formation was largely reduced. This important impact of NOTCH signaling on pituitary stem cell functionality was further supported by the virtual abrogation of sphere formation when adult (wild-type) AP cells were treated with the NOTCH inhibitor DAPT. Of note, RBP-J is not only able to bind the promoters of *Sox2* and *Prop1* but also of genes encoding various transcriptional effectors of the WNT, Hippo and SHH pathways (Zhu *et al.* 2006, Li *et al.* 2012*a*, Nantie *et al.* 2014) (see below).

Among the other NOTCH downstream effectors, HES1 has been shown important to sustain proliferation of the RP progenitor cells by regulating cell cycle inhibitors and to suppress the expression of genes encoding pro-differentiation transcription factors like *Mash1*, *Tbx19* and *NeuroD1* (Zhu *et al.* 2006, Monahan *et al.* 2009). *Hes1* knockout mice exhibit premature corticotrope differentiation, similar to *Pitx1-Cre/Rbp-J*$^{fl/fl}$ mice. In contrast, the PIT1 lineage is properly induced in the *Hes1*$^{-/-}$ animals, suggesting the involvement of dissimilar transcriptional targets of RBP-J (e.g. HES1 for corticotrope and PROP1 for PIT1 lineage) (Himes & Raetzman 2009). In the postnatal pituitary, expression of both *Hes1* and *Hey1* is detected in the cells lining the cleft (Nantie *et al.* 2014). In the stem cell population (SC-SP) of the adult pituitary, *Hey1* seems to be the main NOTCH pathway target component (Chen *et al.* 2009) (Table 1), whereas *Hes6*, in general, involved in NOTCH-driven differentiation (Bae *et al.* 2000), is higher expressed in the non-SP 'main population' (MP) (Chen *et al.* 2006). Recently, grainyhead-like 2 (*Grhl2*) has been advanced as another direct NOTCH downstream target gene in the pituitary (Edwards *et al.* 2016). Its expression is mainly restricted to SOX2$^+$ cells, both in the marginal zone and in the parenchyma. In agreement, we detected higher *Grhl2* gene expression in pituitary SOX2^{eGFP+} stem cells as purified by FACS based on their *Sox2*-driven enhanced green fluorescent protein (eGFP) expression (Table 1). GHRL2 is also co-localized with the pituitary stem cell indicator E-cadherin and both markers concomitantly go down in *Notch2* conditional knockout mice (Edwards *et al.* 2016). In other tissues, GHRL2 has been found to induce the expression of *Cdh1* (encoding E-cadherin) (Werth *et al.* 2010, Chen *et al.* 2016), which may explain their colocalization in pituitary stem cells. In return, E-cadherin is also needed for proper NOTCH signaling as inhibition of E-cadherin function using antibodies decreases the proportion of NOTCH2$^+$ cells with HES1 immunopositivity (Batchuluun *et al.* 2017). Of note, another pathway with NOTCH-like juxtacrine makeup recently described in the pituitary stem cell niche is the ephrin–ephrin receptor system (Vankelecom 2010, Yoshida *et al.* 2014, Cheung *et al.* 2017). Together with NOTCH, ephrin–ephrin receptor signaling may operate to manage the stem cell niche and prevent the niche cells from intermingling with other cells (Batlle *et al.* 2002).

Taken together, the summarized findings support the existence of the NOTCH pathway as regulatory system contained within the pituitary stem cell niche(s), plausibly acting in a *Janus* 'two-headed' manner to govern on the one side stem/progenitor cell proliferation and maintenance, and on the other side, differentiation into particular cell lineages. So far, this functional role has particularly been studied in the embryonic pituitary. Although expression data point to a same picture in the postnatal gland, thorough functional validation is still awaited.

WNT pathway: broad pituitary regulatory system with tentacles twisting in and out the stem cell compartment?

The WNT pathway represents another regulatory system essential for development, already impacting on the earliest phases following egg fertilization. The pathway is composed of WNT ligands that bind to Frizzled (FZD) receptors, often in complex with low-density lipoprotein receptor-related protein (LRP), RAR-related orphan receptor (ROR) and/or protein tyrosine kinase 7 (PTK7), which all function as co-receptors (Niehrs 2012). In contrast to NOTCH ligands, WNTs are secreted although

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their action remains limited to short distances (Farin *et al.* 2016). In the absence of WNT ligand/receptor interaction, the downstream effector β-catenin is phosphorylated by a molecular complex containing the catalytic subunit glycogen synthase kinase-3β (GSK3B) and other proteins including AXIN, adenomatosis polyposis coli (APC) and casein kinase 1 (CK1) (Fig. 1A). β-catenin phosphorylation targets the protein for ubiquitination by binding to β-transducin repeat containing protein (BTrCP) and subsequent degradation by the proteasome. However, when WNT ligands bind to FZD receptors, β-catenin phosphorylation and degradation are inhibited, allowing the protein to accumulate and translocate to the nucleus (Fig. 1A). Classically, stabilization of β-catenin has been attributed to the recruitment of several components of the destruction complex to the WNT/receptor configuration, causing a disintegration of the destruction complex. More recently, it has been suggested that ubiquitination of β-catenin is inhibited because of the destruction complex indeed moving to the membrane, however, remaining intact (Clevers & Nusse 2012, Li *et al.* 2012*b*) (Fig. 1A). After translocation to the nucleus, β-catenin converts several transcriptional repressors, such as T-cell factor (TCF) 7 (previously called TCF1), TCF7-like 1 (TCF7L1, previously called TCF3), TCF7L2 (previously called TCF4) and lymphoid enhancer factor (LEF) 1, to activators by competing with corepressors like amino-terminal enhancer of split (AES) and transducin-like enhancer of split (TLE). The subsequent expression of downstream target genes like *Axin2*, *Myc*, *Lef1* and *CyclinD1* is often used as readout for the activation status of this canonical WNT/β-catenin pathway (Andoniadou *et al.* 2013, Chambers *et al.* 2013).

In addition, WNT-to-FZD binding can also influence other downstream signaling routes, together referred to as non-canonical WNT pathways. Best defined are the planar cell polarity (PCP) and the WNT-Ca^{2+} pathways. PCP involves small GTPases such as RAS homolog family member A (RHOA), and c-JUN N-terminal kinase (JNK), which can directly affect the cytoskeleton or activate transcription factors such as c-JUN and activating transcription factor 2 (ATF2) (Niehrs 2012). The WNT-Ca^{2+} pathway stimulates the production of inositol-1,4,5-triphosphate, leading to Ca^{2+} release from intracellular stores into the cytoplasm and activation of Ca^{2+}-responsive effectors such as calcineurin, Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase C (Niehrs 2012).

Last but not least, WNT activation is boosted by the R-spondin (RSPO)/leucine-rich repeat-containing G-protein-coupled receptor (LGR) system. Binding of RSPO1–4 ligands to LGR4–6 receptors inhibits the degradation of FZD receptors, thereby amplifying both canonical and non-canonical WNT signaling (Glinka *et al.* 2011, Hao *et al.* 2012). Although specific WNT ligands and receptors have been assigned to canonical or non-canonical signaling, it is rather the particular combination of ligand, receptor and co-receptor, which determines this nature in a context-dependent way (Niehrs 2012, Fu *et al.* 2016).

Various WNT ligands and FZD receptors have been detected in the pituitary depending on the stage of development (Potok *et al.* 2008). WNT4 and WNT5A, mainly assigned to the non-canonical WNT pathway but also involved in some canonical actions (Potok *et al.* 2008, Bernardi *et al.* 2011, Fu *et al.* 2016), play important roles during pituitary embryogenesis. WNT4 is detected adjacent to the cleft during embryogenesis and is required for differentiation of the PIT1 lineage, suggesting a role for WNT4 in activating PROP1 (Treier *et al.* 1998, Potok *et al.* 2008). WNT5A is faintly expressed in RP while its prime expression site is within the neighboring ventral diencephalon. Knockout of *Wnt5a* leads to a dysmorphic gland, but endocrine cell specification is almost not affected (Cha *et al.* 2004, Potok *et al.* 2008). FZD2 is also expressed in the RP progenitor cells (E12.5), which renders these cells sensitive to the prevailing WNT ligands (Douglas *et al.* 2001, Potok *et al.* 2008). *Wnt4* and *Fzd2* continue to be upregulated in the neonatal stem cell compartment as compared to the adult SC-SP, which may be associated with the activated status of these cells during neonatal maturation (Gremeaux *et al.* 2012) (Table 1). In the adult gland, *Fzd2* remains higher expressed in the SC-SP as compared to the non-stem cell populations (Chen *et al.* 2009, Vankelecom 2010) (Table 1). In the adult pituitary's $SOX2^{eGFP+}$ stem cells, we found upregulated expression of *Wnt4* (and *7b*, *9a*, *10a*) and *Fzd3* (and *1*, *4*, *6*, *7* but not *Fzd2*) when compared to the GFP^{-} cells (Table 1). Interestingly, *Lgr4 and Lgr5* are also upregulated in the pituitary stem cell compartment of adult mice (Chen *et al.* 2009) (Table 1). These findings are in agreement with the observation that LGR proteins mark adult stem cells in a variety of epithelial tissues (Barker *et al.* 2007, 2010, Ren *et al.* 2014). Moreover, LGR4-driven β-galactosidase expression has been detected in the adult gland before pituitary stem cells were identified, with $LGR4^{lacz+}$ cells almost exclusively found around the cleft (Van Schoore *et al.* 2005). The LGR ligand *Rspo2* is upregulated in the pituitary SC-SP in response to damage (Willems *et al.* 2016) and *Rspo1* is higher expressed in the SC-SP during neonatal maturation (Gremeaux *et al.* 2012)

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(Table 1), thereby suggesting that WNT signaling in the pituitary stem cells is further magnified by RSPO/LGR activation during these activation conditions. Together, it appears that both ligands and receptors of the WNT pathway, at least certain members, are preferentially found within the pituitary stem cell compartment. Yet, WNT5A immunopositivity has been reported to occur throughout the adult pituitary parenchyma (Treier *et al.* 1998, Potok *et al.* 2008), suggesting a broader territory of unfolding of the WNT pathway.

Regarding downstream components of WNT signaling, several factors have been detected in the pituitary. The WNT pathway corepressors *Aes* and TLE1 are expressed in the nascent pituitary gland but are extinguished by E16.5 (Dasen *et al.* 2001, Brinkmeier *et al.* 2003). HESX1, an early (E11.5) marker and essential regulator of pituitary development, forms a complex with TLE1 in the RP progenitor cells and is expressed in a mutually exclusive manner with PROP1 (Dasen *et al.* 2001, Carvalho *et al.* 2010). WNT pathway activation results in nuclear β-catenin that forms a complex with PROP1 (E12.5–13.5), which represses *Hesx1* transcription, being essential for pituitary development to progress, and induces the expression of differentiation-driving factors like *Pou1f1*/*Pit1* (Dasen *et al.* 2001, Olson *et al.* 2006, Pérez Millán *et al.* 2016) (Fig. 1A). In agreement, conditional deletion of the *Ctnnb1* gene (encoding β-catenin) leads to the absence of PIT1 expression and reproduces the phenotype of the *Prop1*-mutant (*Prop1*$^{df/df}$) mouse, thereby underscoring the importance of the β-catenin/PROP1 interaction for pituitary development to advance (Andersen *et al.* 1995, Olson *et al.* 2006). In accordance, activation of WNT signaling by inhibiting β-catenin degradation using GSK3B inhibitors promotes the (*in vitro*) differentiation of pituitary progenitor cells toward hormone-expressing cells (Suga *et al.* 2011, Yoshida *et al.* 2016*a*).

While the most important β-catenin actions during pituitary embryogenesis may be mediated by PROP1 activation, additional β-catenin-responsive transcription factors are also detected in the developing pituitary like PITX2, LEF1, TCF7L1 and TCF7L2. PITX2 plays a crucial role in pituitary embryogenesis and is expressed in the oral ectoderm already before RP formation (Gage *et al.* 1999, Suh *et al.* 2002). PITX2 is also dependent on complexing with β-catenin for its function (Kioussi *et al.* 2002). LEF1 is temporally detected in the developing pituitary at E9.0, and reappears at E13.5, but no aberrant pituitary phenotype was observed in *Lef1*$^{-/-}$ mice during embryonic development (Olson *et al.* 2006). In contrast, conditional *Tcf7l1* knockout affects pituitary development, but coexisting effects at the level of the ventral diencephalon complicate the interpretation of the pituitary phenotype (Gaston-Massuet *et al.* 2016). TCF7L2 may be involved in the repression of *Prop1* in embryonic pituitary progenitor cells (Brinkmeier *et al.* 2003, 2007). Several of these components of the WNT transcriptional machinery remain upregulated in the pituitary stem cell compartment during the neonatal maturation phase (*Prop1*, *Tcf7l1*, *Tcf7l2*) as well as during adult life (*Lef1*, *Prop1*, *Tcf7*, *Tcf7l2*) (Chen *et al.* 2009, Garcia-Lavandeira *et al.* 2009, Vankelecom 2010, Gremeaux *et al.* 2012) (Table 1). In contrast, in addition to WNT5A (Treier *et al.* 1998, Potok *et al.* 2008; see above), TCF7L2 and nuclear β-catenin immunostaining have been reported to exist throughout the adult gland (Brinkmeier *et al.* 2007), supportive of WNT signaling also outside the stem cell compartment. In the stem cell niches of the adult resting pituitary, β-catenin was detected as a membranous signal (Garcia-Lavandeira *et al.* 2009), suggesting rather a role in epithelial cell–cell adhesion by connecting the membranous E-cadherin (as also found in the stem cells) to the cytoskeleton (El-Bahrawy & Pignatelli 1998). Along the same line, SOX2 is able to sequester β-catenin, thus creating a higher threshold for canonical WNT/β-catenin signaling to occur within the pituitary stem cells (Kelberman *et al.* 2008, Alatzoglou *et al.* 2011). On the other hand, the β-catenin chaperone PROP1 is primarily detected in the SOX2^{+} (stem) cells in the adult gland, although its exact location is still debated, being either in the parenchymal SOX2^{+} cell clusters (as reported in rat) (Yoshida *et al.* 2011, 2014) or in the SOX2^{+} cells lining the cleft (as reported in mouse) (Garcia-Lavandeira *et al.* 2009). The downstream WNT target genes *CyclinD1* (*Ccnd1*), *Lef1*, *Myc* and *Pitx2* are upregulated in the adult pituitary stem cell compartment (Vankelecom 2010) (Table 1). Interestingly, *Axin2* is upregulated in the SC-SP of the neonatal pituitary and of the regenerating adult gland after somatotrope ablation injury (Gremeaux *et al.* 2012, Willems *et al.* 2016) (Table 1), pointing to (further) canonical WNT activation in the stem cell compartment during the vivid early-postnatal growth phase and upon damage. This specific activation may be needed for stem cell expansion, which is in line with the role of WNT signaling in many other tissues (Sato *et al.* 2009). In the context of the somatotrope regeneration process, WNT activation may further be needed for stem cell differentiation toward the PIT1 lineage by derepressing PROP1 function.

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In addition to this mostly canonical perspective on pituitary WNT signaling, non-canonical aspects should also be considered, including a potential PCP-like organization of the marginal zone as suggested by polarized staining patterns (e.g. of GFRA2 and β-catenin, see Garcia-Lavandeira *et al.* 2009). Moreover, several non-canonical components (e.g. *Ankrd6*, *Celsr1*, *Fzd3*) are found upregulated in the adult pituitary SOX2^{eGFP+} stem cell population (Table 1).

Taken together, WNT signaling plays an important role during pituitary embryogenesis with impact on the early progenitor cells, both at the level of their maintenance and their differentiation. The presence of multiple components in the pituitary after birth also points to WNT activity in the postnatal gland. WNT signaling does not seem to be restricted to the stem cell compartment, but occurring all over the gland in the basal, homeostatic situation. Its activity may be boosted in the stem cells when these cells are brought in activation modus for neonatal maturation or regeneration. WNT signals may sprout from the endocrine parenchymal cells, but may reversely also deploy from the stem cells toward the endocrine cells in a forward signaling manner, together suggesting a complex interplay between endocrine AP cells and stem cells regarding WNT/LGR signaling. Taken together, WNT appears to 'blow' softly all over the postnatal '(g)land' under basal conditions, but may become more 'stormy' in the stem cell compartment in the damage- and neonatally activated pituitary.

EMT: preparing the pituitary stem cells for a stroll?

Epithelial–mesenchymal transition (EMT) is a highly conserved cellular process in which epithelial cells, typically polarized in apical–basal direction and tightly linked to the basal membrane, are converted into motile non-polarized mesenchymal cells. The epithelial cells gradually lose cell–cell adhesion and polarization to progressively acquire mesenchymal properties, including the ability of invasion and migration. EMT, together with the reverse process of mesenchymal–epithelial transition, plays a pivotal role in the formation of organs and tissues during embryonic development, in wound repair and in cancer pathogenesis and progression.

Central facets of the EMT process are the (gradual) disappearance of the cell–cell adhesion protein 'epithelial cadherin' (E-cadherin) and the emergence of mesenchymal markers like vimentin (VIM) and fibronectin (FN) (Fig. 1B). Several factors repress the transcription of the *Cdh1* gene, encoding for E-cadherin. SNAI1 (also known as SNAIL1), a zinc finger transcription factor, downregulates *Cdh1* expression by direct interaction with the *Cdh1* promoter. The EMT-inducing activity of other transcriptional repressors such as SNAI2 (also known as SLUG), zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2 (also known as SIP1), TCF3 and Krüppel-like factor 8 (KLF8) relies not only on direct inhibition of *Cdh1* gene transcription but also on simultaneous repression of genes encoding other junctional proteins such as claudins (CLDN) and desmosomal junction components (e.g. desmoglein, desmoplakin) (Fig. 1B). TWIST1 (Yang *et al.* 2004), the embryonic transcription factor forkhead box protein C1 (FOXC1) and FOXC2 (Mani *et al.* 2007), transcription factor 4 (TCF4, also known as E2–2) (Sobrado *et al.* 2009) and Goosecoid (Hartwell *et al.* 2006) seem to trigger EMT without binding directly to the *Cdh1* promoter, but detailed mechanisms remain unclear. Increased expression of proteases like matrix metallopeptidase 3 (MMP3) promotes EMT, not only by clearing the way for cell movement but also by modulating signal transduction leading to the stabilization of EMT (De Craene & Berx 2013) (Fig. 1B).

EMT is also closely intertwined with the fundamental signaling networks of WNT, NOTCH, SHH and transforming growth factor-β (TGFB) (Fig. 1B). Both WNT and NOTCH signaling can prevent phosphorylation of SNAI1 and SNAI2 (collectively referred to as SNAILs) by GSK3B, thereby increasing their stability and availability for the EMT process (Lamouille *et al.* 2014, Frías *et al.* 2015). Moreover, WNT signaling activates the transcription of *Zeb1* and *Zeb2* (collectively referred to as *Zebs*), while NOTCH activation can trigger the expression of SNAILs depending on the cellular context (Xu *et al.* 2009*a*, Lamouille *et al.* 2014) (Fig. 1B). In addition, the transcription factor PROP1, which promotes EMT (see below), relies on both NOTCH and WNT signaling for its expression and activation (as mentioned previously; Olson *et al.* 2006, Zhu *et al.* 2006, Pérez Millán *et al.* 2016). TGFB signaling stimulates the expression of *Zebs* and *Snails* by the activation of SMAD transcriptional complexes (Lamouille *et al.* 2014) (Fig. 1B). SHH pathway activation can also promote EMT by increasing *Snai1* expression (Lamouille *et al.* 2014). Another important regulator of EMT-associated cell migration is the chemokine (C-X-C motif) receptor 4 (CXCR4) and its ligand stromal cell-derived factor 1 (SDF1), also called CXCL12. This signaling system positively influences the expression of *Twist1* and *Twist2* (collectively referred to as

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Twists), mediated by activation of the extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase/thymoma viral proto-oncogene (PI3K/AKT) pathways (Yao *et al.* 2016). Moreover, other reports show a positive influence of the TGFB pathway on CXCR4 signaling, while the CXCR4 pathway can enhance WNT signaling (Bertran *et al.* 2009) (Fig. 1B).

During pituitary embryogenesis, the RP progenitor cells that are located around the cleft need not only to proliferate but also to colonize the ventral region in order to grow the developing anterior lobe. The observed morphological changes of the progenitor cells, from tightly packed columnar-type cells into more loosely distributed cells, are reminiscent of an EMT program. PROP1 has regularly been proposed to regulate the progenitor cell migration process during pituitary organogenesis (Ward *et al.* 2005, Himes & Raetzman 2009). *Prop1*$^{df/df}$ mice exhibit a dysmorphic embryonic pituitary with enlarged progenitor zone and small ventral part, purportedly due to a failure of progenitor cells to leave the proliferative zone in the absence of PROP1. In agreement, *Prop1*$^{df/df}$ mice lose SLUG (SNAI2) expression in most parts of the embryonic pituitary, which may explain the absence of EMT and its accompanying motility (Himes & Raetzman 2009). The resulting accumulation of progenitor cells finally leads to increased apoptosis and pituitary hypoplasia. Also human patients with mutations in *PROP1* eventually show dysmorphic, hypoplastic pituitaries with underdevelopment of one or multiple hormone-producing cells, leading to (combined) pituitary hormone deficiency (Abrão *et al.* 2006). PROP1 signal, being co-expressed with SOX2, does not only peak during early embryogenesis but also during the early-postnatal wave of pituitary growth (Pérez Millán *et al.* 2016), supporting the idea that stem cell EMT also plays a role during this transient neonatal growth phase. In accordance, genes involved in EMT are found to be more prominently expressed in the neonatal pituitary SC-SP when compared to the adult SC-SP (e.g. *Fn1*, *Mmp2*, *Prop1*, *Twist1*, *Twist2*, *Vim*, *Zeb2*) (Gremeaux *et al.* 2012) (Table 1). The link between PROP1 and EMT during early-postnatal life is further clear from the downregulation of EMT-related genes (such as *Zeb2*, *Cxcl12* and *Mmps*) and the upregulation of epithelial genes (like *Cdh1* and *Cldn23*) in adherent colonies formed *in vitro* from pituitary (stem) cells of 2-week-old *Prop1*$^{df/df}$ mice (Pérez Millán *et al.* 2016). PROP1 directly regulates *Zeb2* transcription by binding to its promoter, and *Zeb2* knockdown blocks EMT as tested in wild-type pituitary-adherent colonies (Pérez Millán *et al.* 2016). In addition, components of the major signaling pathways (NOTCH, WNT, SHH and TGFB), fundamentally also linked to EMT (Fig. 1B), are differently expressed in the absence of PROP1, implying a central role for PROP1 in their regulation. Among others, PROP1 directly binds to the promoter of *Notch2* and of the SHH mediator *Gli2* (for SHH pathway, see below) (Pérez Millán *et al.* 2016) (Fig. 1B). It should be noted that expression of the mesenchymal marker N-cadherin did not change between wild type and *Prop1*$^{df/df}$ pituitary colonies (Pérez Millán *et al.* 2016), suggestive of a partial but not complete EMT that is unfolding (Leroy & Mostov 2007, Savagner 2010). Cell cycle regulators (like CyclinD1 and CyclinE) are also lower expressed in the embryonic and neonatal pituitary of *Prop1*$^{df/df}$ mutant vs wild-type mice, proposing that PROP1 not only regulates the exit process of the stem/progenitor cells from the niche, but also their proliferation during the growth phase (Pérez Millán *et al.* 2016).

EMT expression characteristics remain present in the stem cell compartment of the adult gland; EMT-associated markers (e.g. *Twist1*, *Twist2*, *Zeb2*) are upregulated in the SC-SP when compared to the MP (Chen *et al.* 2009, Vankelecom 2010) (Table 1). Moreover, paired-related homeobox (*Prrx*) 1 and *Prrx2*, mainly known as mesenchymal markers and potentially involved in EMT (Ocaña *et al.* 2012), are also upregulated in the adult pituitary stem cell population (SC-SP and SOX2^{eGFP+} cell compartment) (Chen *et al.* 2009, Vankelecom 2010) (Table 1). PRRX1 is expressed in SOX2^{+} cells of the embryonic and adult (rat) pituitary and in a subset of all hormonal cell types in the adult pituitary (as analyzed at P30) (Susa *et al.* 2012). In contrast, PRRX2 only emerges after birth from P30 in a subset of the SOX2^{+} cells (Higuchi *et al.* 2014). Another mesenchymal marker, VIM, is found co-localized with coxsackie and adenovirus receptor (CAR) in some cells just beneath the marginal cell layer, particularly at neonatal age (Chen *et al.* 2013). CAR has been identified as an additional marker of the (rat) pituitary stem cells in the marginal zone as well as the parenchymal clusters throughout life (Chen *et al.* 2013). The VIM^{+}/CAR^{+} cells observed at neonatal age may represent the transitional state of the stem cells undergoing EMT and preparing to move (Yoshida *et al.* 2016*b*). Intriguingly, CAR is typically present at the apical side of the marginal zone cells, whereas a multicellular layer of 'depolarized' CAR^{+} cells is observed in the marginal zone during the neonatal maturation phase (Chen *et al.* 2013). Moreover, nestin^{+} cells from the postnatal (rat) AP start to express mesenchymal markers (like VIM) and to adopt migratory activity when seeded in culture, further

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linking pituitary stem cells to EMT (Krylyshkina *et al.* 2005). Together, the data described previously support the idea that (partial) EMT is involved in pituitary stem cell regulation, in particular in migration toward the 'point of interest' during or after which the cells differentiate. Moreover, upregulation of EMT-associated genes (such as *Cxcl12*, *Mmp2*, *Mmp3*, *Snai1*, *Tgfb2*, *Vim*) has been found in the reacting SC-SP of the regenerating pituitary (Willems *et al.* 2016) (Table 1), likely needed to fulfill the increased demand for hormonal (in this case, GH$^+$) cells.

In addition to epithelial (stem) cells adopting a mesenchymal state through (partial) EMT, authentic mesenchymal cells may also be present in the stem cell niche, functioning as stem cell-supporting cells (as found in niches of many tissues). A vimentin$^+$ layer of cells has been observed adjacent to the marginal zone in the postnatal rat and mouse pituitary (Krylyshkina *et al.* 2005, Garcia-Lavandeira *et al.* 2009, Vaca *et al.* 2016). However, these cells may also represent stem cells that have undergone EMT and have started to move (as described in the previous paragraph). The exact functional position, being either differentiating stem cells, stem cell-supportive cells or both, still has to be clarified.

Taken together, EMT appears involved in pituitary stem cell regulation not only during the embryonic phase, but also in postnatal life during neonatal maturation, adult homeostasis and regeneration in response to injury.

Sonic Hedgehog: regulator of adult pituitary stem cell proliferation?

The Hedgehog (HH) pathway is one of the protagonists of embryogenesis and crucial for the development of many organs. Mutations in this pathway cause severe developmental disorders (like holoprosencephaly; Cohen 2010). The pathway includes the secreted ligands Indian Hedgehog, Desert Hedgehog and SHH, which all bind to Patched (PTCH) receptors in the cell membrane. PTCH exerts inhibitory activity on SHH signaling by holding the smoothened (SMO) receptor imprisoned in endocytic vesicles (Fig. 1A). Binding of SHH to PTCH releases SMO, which inhibits the cytosolic degradation of the SHH downstream effectors GLI1–3, otherwise exerted by a complex including protein kinase A (PKA), GSK3B and CK1 (Tempé *et al.* 2006, Cohen 2010, Yavropoulou *et al.* 2015*a*). These GLI transcription factors are then able to enter the nucleus and activate (GLI1 and GLI2) or repress (GLI3) different transcriptional targets. *Gli1* and *Ptch1* themselves are targets of the SHH pathway (Fig. 1A), but also ligands of the BMP, FGF and WNT pathway can be induced by the GLI factors (Mullor *et al.* 2001, Cohen 2010, Wang *et al.* 2010, Yavropoulou *et al.* 2015*a*).

During embryogenesis, SHH that is first expressed throughout the oral ectoderm, becomes excluded from the invaginating part that forms RP, while the adjacent zones and the ventral diencephalon keep producing SHH (Treier *et al.* 2001). The progenitor cells constituting RP express *Ptch1*, hence being receptive to these incoming SHH signals (Treier *et al.* 2001). Inhibition of the SHH pathway interferes with the separation of RP from the oral ectoderm and results in a severely hypoplastic pituitary with delayed PROP1 expression (Treier *et al.* 2001). GLI2 turns out to be a key player in pituitary embryogenesis since patients with *GLI2* mutation develop severe hypopituitarism (Bear *et al.* 2014, Arnhold *et al.* 2015). From mouse studies, it is known that GLI2 ensures proper proliferation of the RP progenitor cells, although also indirect effects originating from the ventral diencephalon are playing in this phenotype (Wang *et al.* 2010). Together, SHH signaling is essential for pituitary specification by driving the earliest stages of pituitary development and regulating the early progenitor cells. In agreement, development of pituitary-phenotypical cells from pluripotent stem cells invariably requires activation of the SHH pathway (Suga *et al.* 2011, Dincer *et al.* 2013, Ozone *et al.* 2016, Zimmer *et al.* 2016).

Addition of the SMO inhibitor cyclopamine to adult mouse pituitaries cultured as explants was reported to reduce levels of the downstream target *Gli1*, indicating that SHH is also basally active in the adult pituitary (Pyczek *et al.* 2016). The pathway receptors and/or targets *Ptch1* and *Smo* are detected in the adult pituitary, particularly in the stem cell compartment (Chen *et al.* 2009, Vankelecom 2010) (Table 1). Moreover, expression of several SHH pathway components, including *Gli2*, *Smo* and the target genes *Gli1* and *Ptch1*, are upregulated in the neonatal SC-SP and/or the adult SC-SP after pituitary injury, supportive of the pathway's association with the activated state of the stem cells in these conditions (Gremeaux *et al.* 2012, Willems *et al.* 2016) (Table 1).

Conditional, tamoxifen-induced knockout of *Ptch* during adulthood (using a ubiquitously expressed *CreERT* transgene), leading to the activation of the pathway in SMO-expressing cells of all organs, resulted in GLI1 detection in both pituitary stem cells and more committed hormonal cells, and in increased proliferation (as probed by BrdU incorporation) of the SOX2$^+$/SOX9$^+$ stem cells. The findings suggest that the SHH pathway regulates adult pituitary stem cell proliferation (Pyczek *et al.* 2016).

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However, there are some caveats to this recent study. BrdU incorporation was analyzed *in vitro* using pituitary explants. The explants from normal adult pituitaries showed a very high proportion of BrdU$^+$ cells within the SOX2$^+$ and SOX9$^+$ cell population (around 20%) even after only 48 h of BrdU addition, which is in clear contrast with the quiescent state of these cells as observed *in vivo* by several groups (being in line with the very low turnover rate of the adult gland; Garcia-Lavandeira *et al.* 2009, Fu *et al.* 2012, Andoniadou *et al.* 2013, Rizzoti *et al.* 2013, Zhu *et al.* 2015). *Ptch* deletion was ubiquitously induced, which affected the animal's health and restricted analysis to a limited period (no longer than 2 weeks after deletion). In addition, ubiquitous deletion likely leads to indirect effects since the SHH pathway is also active in neighboring structures of the pituitary like the hypothalamus. Pituitary-specific deletion is needed for more compelling conclusions here.

Together, the SHH pathway appears active in the postnatal pituitary stem cells and may regulate their proliferation although more data are needed to convincingly support this picture.

Hippo pathway: a new player in pituitary stem cell regulation?

Following the observation of tissue overgrowth in *Hippo*$^{-/-}$ *Drosophila* (Harvey *et al.* 2003, Udan *et al.* 2003, Wu *et al.* 2003) and similar findings in mammals (Dong *et al.* 2007, Lu *et al.* 2010, Heallen *et al.* 2011), the Hippo pathway has been advanced as a key regulator of organ growth and size. To exert this regulation, the pathway integrates several upstream inputs, such as scaffolding proteins forming activation complexes in close proximity to cell junctions (rendering Hippo sensitive to cell–cell contacts and cell polarity); signals from extracellular matrix and cytoskeleton and mechanical tension (reviewed in Yu & Guan 2013, Johnson & Halder 2014, Sun & Irvine 2016). The core of the mammalian Hippo signaling network comprises the kinases mammalian STE20-like protein kinase 1 (MST1), MST2, large tumor suppressor homolog 1 (LATS1) and LATS2 (Fig. 1A). The kinases are involved in a cascade ultimately phosphorylating yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), thereby marking these proteins for cytoplasmic retention and proteasomal degradation. When Hippo signaling is inactive, YAP and TAZ are not degraded and function as transcriptional co-activators that form complexes with TEA domain-containing sequence-specific transcription factors (TEAD), resulting in the expression of target genes that promote cell proliferation and survival such as connective tissue growth factor (*Ctgf*) and cysteine-rich angiogenic inducer 61 (*Cyr61*) (Johnson & Halder 2014) (Fig. 1A). When the Hippo pathway is activated, YAP and TAZ are degraded and their target gene expression stops, resulting in a halt of cell proliferation and start of differentiation and/or apoptosis. This way, the Hippo pathway controls the size of organs during development. In addition, Hippo signaling has recently also been identified in the regulation of tissue stem cells (reviewed in Yu & Guan 2013, Johnson & Halder 2014), cells reasonably involved in the processes of organ growth. Genetic deletion of the YAP/TAZ-phosphorylating *Mst1* and *Mst2* leads to the increased emergence of liver stem cells (so-called oval cells) (Lu *et al.* 2010). Moreover, transient YAP induction results in de-differentiation of various tissue cells into proliferating cells with stem/progenitor cell characteristics (Panciera *et al.* 2016). The Hippo network has also been implicated in tissue regeneration. Genetically enhanced YAP/TAZ activity in the adult heart stimulates cardiac regeneration after myocardial infarction (Heallen *et al.* 2013, Xin *et al.* 2013). In the intestine, YAP was found indispensable for regeneration of the epithelium by the intestinal stem cells after irradiation (Gregorieff *et al.* 2015).

The Hippo pathway has only recently been explored in embryonic and adult pituitary (Lodge *et al.* 2016, Willems *et al.* 2016). Expression of *Mst1*, *Mst2* and *Lats1* (but not *Lats2*) was detected during the entire period of pituitary embryonic development (Lodge *et al.* 2016). *Tead2* and *Yap1* were found highly expressed in the embryonic progenitor cell region and nuclear localization of YAP1 and TAZ was observed in RP. These data point to a role of the Hippo pathway in pituitary embryogenesis, likely concentrated in the progenitor cells. Also in the postnatal (3-week-old) pituitary, nuclear localization of YAP1 and TAZ was observed in the SOX2$^+$ stem cell niche, and some signals were found scattered in the parenchyma (Lodge *et al.* 2016). As SOX2 has been suggested to exert a stimulatory effect on YAP/TAZ activity in other tissues, directly by promoting *Yap1* gene transcription (Seo *et al.* 2013) and indirectly by repressing the Hippo pathway activators neurofibromin 2 (*Nf2*) and WW domain-containing protein 1 (*Wwc1*) (Basu-Roy *et al.* 2015), an upstream regulatory function of SOX2 in the Hippo cascade may also be assumed in the pituitary (stem cells), although this connection has still to be explored.

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Interestingly, elevated levels of YAP/TAZ target genes (like *Ctgf* and *Cyr61*) were observed in the SC-SP stem cell compartment after pituitary injury (Willems *et al.* 2016). The Hippo pathway may thus be implicated in the regenerative response of the pituitary stem cells, in agreement with its involvement in (stem cell-driven) repair in other tissues like the intestine (Gregorieff *et al.* 2015) and heart (Heallen *et al.* 2013, Xin *et al.* 2013).

Taken together, very recent data suggest that the Hippo pathway is involved in pituitary embryogenesis, homeostasis and regeneration, likely through its action in the stem/progenitor cell compartment, but further experimental elaboration is needed.

EGF/FGF: additional regulators of adult pituitary stem cell proliferation?

Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF, also known as FGF2) are two commonly used mitogens to stimulate stem cell growth in sphere and adherent cell cultures (Chen *et al.* 2005, Lepore *et al.* 2007, Gleiberman *et al.* 2008, Yoshida *et al.* 2016*a*). With the exception of FGF signaling from the ventral diencephalon during embryogenesis, both growth factor systems are not highly documented in the pituitary.

EGF and bFGF have been shown to activate pituitary stem cell proliferation. Both factors increase the proportion of SP cells in mouse adult AP cell aggregate cultures through stimulation of the cells' proliferation (Chen *et al.* 2006). In addition, bFGF-treated mouse RP explants exhibit higher BrdU incorporation (Ericson *et al.* 1998), while bFGF-treated aggregates of adult rat AP cells show a higher proportion of nestin$^+$ cells (Krylyshkina *et al.* 2005). bFGF may exert its proliferative effects through various mediators such as signal transducer and activator of transcription-3 (STAT3), ERK and AKT, as has been identified using a folliculostellate cell line, which may represent a certain pituitary stem cell subpopulation (Vlotides *et al.* 2009). Adult pituitary stem cells show prominent expression of the cognate receptors *Egfr* and *Fgfr1/2*, which make them highly receptive to EGF and FGF signals (Chen *et al.* 2009) (Table 1). Moreover, these receptors are further upregulated in the pituitary stem cell compartment upon injury and during the neonatal growth phase (Gremeaux *et al.* 2012, Willems *et al.* 2016) (Table 1). Taken together, both growth factor pathways may provide additional cues for stem cell proliferation in the (activated) adult pituitary.

Pituitary tumorigenesis: stem cell regulation going awry?

Like in other tissues, deregulation of pituitary stem cells may be associated with or foreshadow the development of disorders like tumorigenesis. Pituitary adenomas represent the most common intracranial tumors. Although largely benign, significant morbidity is present mainly due to hormone hypersecretion and/or compression of neighboring tissue. Pituitary tumors are classified as hormone-producing adenomas (prolactinomas, somatotropinomas, corticotropinomas, thyrotropinomas and plurihormonal adenomas) and non-functional pituitary adenomas (NFPA), which do not measurably secrete hormones (Melmed 2011). In addition, craniopharyngiomas occur in the pituitary (sellar/parasellar) region, representing rare epithelial tumors and categorized as adamantinomatous craniopharyngioma (ACP) and papillary craniopharyngioma (Gaston-Massuet *et al.* 2011, Garcia-Lavandeira *et al.* 2012). Whereas ACP represents the most common pediatric tumor of the pituitary, most probably originating from (ectopic) RP remnants, papillary craniopharyngiomas occur in adults (Esheba & Hassan 2015).

In general, the molecular and cellular mechanisms underlying pituitary tumorigenesis are largely unknown, and only less than 5% have been assigned to genetic mutations (Melmed 2011). Treatment through transsphenoidal resection, irradiation and/or medicines is not highly satisfactory, also because of therapy resistance and recurrence in a substantial proportion of the patients.

Recently, 'cancer stem cells' (CSC) have been identified in various cancer types (Sergeant *et al.* 2009, Wouters *et al.* 2009, Clevers 2011). CSC are defined as the subpopulation of the tumor's cells that drives initial and recurrent growth, invasion, metastasis and/or therapy resistance. The origin of CSC is still much debated and may be diverse, ranging from mature tissue cells that become transformed and de-differentiated thereby resuming stemness properties, to stem cells of which the tight regulation goes awry resulting in aberrant (tumorigenic) growth.

Recently, candidate CSC populations have been identified in pituitary adenomas using different approaches. Of note, since pituitary tumors are predominantly benign, the cells are more correctly referred to as 'tumor stem cells' (TSC). Human pituitary adenoma cells were found to generate sphere-like structures in culture showing some, although limited, differentiation capacity (Xu *et al.* 2009*b*). Pituitary adenoma cells expressing the stemness marker CD133

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showed self-renewal and also limited differentiation capacity in another study (Würth *et al.* 2016). However, these cells were sensitive to the antiproliferative effect of drugs (in this case, dopamine/somatostatin chimeric agonists), which does not fit with the general concept of therapy resistance of TSC. In another recent study, CD15$^+$ cells were identified in pituitary adenomas exhibiting higher sphere-forming capacity than the remaining cells, and upregulated expression of *SOX2* (Manoranjan *et al.* 2016). The latter finding is in agreement with an earlier report that showed SOX2 expression in a candidate TSC population as identified by SP efflux capacity (Mertens *et al.* 2015). The pituitary tumor SP was found to have self-renewal competence and *in vivo* tumorigenic dominance (Mertens *et al.* 2015), as well as higher resistance to drugs (H Roose, F Mertens & H Vankelecom, unpublished observations). Increased expression of SOX2 was also found in the mouse *Drd2*$^{-/-}$ pituitary tumor model (Mertens *et al.* 2015). Female *Drd2*$^{-/-}$ mice harboring a deletion of the dopamine receptor D2 (*Drd2*) gene eventually develop prolactinomas. *Drd2*$^{-/-}$ pituitaries were found to contain more SP and (proliferating) SOX2$^+$ cells than control pituitaries (Mertens *et al.* 2015). Whether this finding means that the SOX2$^+$ cells represent pituitary tumor-driving cells is not clear yet, although SOX2$^+$ CSC have been reported in other tumor types (e.g. squamous cell carcinoma; Boumahdi *et al.* 2014). In a mouse model of ACP (see below), it was observed that the SOX2$^+$ cells rather act as paracrine-regulatory cells of the developing tumors, promoting proliferation of the cells adjacent to the SOX2$^+$ cells (Andoniadou *et al.* 2013). However, it should be noted that ACP is different from the typical AP (non-)endocrine tumors. Both in the tumor SP and in the ACP model, activation of stem cell regulatory networks (as discussed previously) has been observed suggesting that dysregulation of stem cells may be associated with pituitary tumorigenesis. Below, we summarize the current literature on these particular pathways in pituitary tumors, and where available (but still scarce), in the candidate TSC population.

Dysregulation of NOTCH in pituitary tumorigenesis

Because of its key role in (stem) cell maintenance, differentiation, proliferation and apoptosis, NOTCH dysregulation is logically also implicated in tumorigenesis as supported in a variety of cancers (e.g. colorectal, ovarian, breast; see Park *et al.* 2006, Yamaguchi *et al.* 2008, Serafin *et al.* 2011).

Expression of NOTCH pathway components has also been detected in pituitary adenomas and appears different in different tumor subtypes. In NFPA, *NOTCH3* and *JAG1* expression was found significantly increased as compared to normal human pituitary tissue (Lu *et al.* 2013, Yavropoulou *et al.* 2015*a*,*b*). Activation of NOTCH signaling in NFPA is further supported by the decreased expression of *DLK1,* the inhibitor of canonical NOTCH signaling (Moreno *et al.* 2005). Strangely, the NOTCH target gene *HES1* was found downregulated (Yavropoulou *et al.* 2015*b*). Plurihormonal pituitary adenomas also show downregulated expression of *HES1* and *HES5* suggesting diminished NOTCH activity although also expression of *DLK1* was found repressed (Jiang *et al.* 2012). In prolactinomas, an increase in *NOTCH3* and decrease in *DLK1* has been described, suggestive of NOTCH pathway activation in these tumors (Evans *et al.* 2008). However, these results were not confirmed in a second study, while somatotropinomas show a significant reduction of *NOTCH3* and *JAG1* (Yavropoulou *et al.* 2015*b*). Together, the NOTCH pathway appears activated or repressed depending on the pituitary tumor subtype, although results so far are not consistent. Dysregulation may affect tumor initiation, growth, invasion and/or resistance as found in other cancers like colorectal, liver, ovarian and breast cancer (Park *et al.* 2006, Yamaguchi *et al.* 2008, Han *et al.* 2015, Zheng *et al.* 2015).

Regarding the proposed pituitary tumor TSC populations, knowledge on the NOTCH pathway is at present limited and only includes expression analysis. Upregulation of several different NOTCH pathway components has been reported. Pronounced expression of *DLL1*, *JAG1*, *HES1*, *NOTCH1* and *NOTCH2* was found in the pituitary tumor SP (Mertens *et al.* 2015) (Table 1) while other candidate TSC populations (i.e. tumorspheres and CD133$^+$ cell populations) showed upregulation of *DLL1*, *JAG2*, NOTCH1 and *NOTCH4* (Xu *et al.* 2009*b*, Würth *et al.* 2016), the latter NOTCH receptor subtype in general is more associated with endothelial cells.

Dysregulation of WNT in pituitary tumorigenesis

Involvement of the WNT pathway in tumorigenesis, through its effects on cell proliferation, fate, death, polarity and migration, has been described in multiple cancer types (e.g. hepatocellular carcinoma, pancreatic ductal adenocarcinoma, colon carcinoma; see Morin *et al.* 1997, Miyoshi *et al.* 1998, Zhang *et al.* 2013). Moreover, WNT-activated cells may represent CSC as has been

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reported for LGR5-expressing cells in intestinal adenomas and cancer (Schepers *et al.* 2012).

Increased expression of WNT4 has been observed in somatotropinomas, prolactinomas and thyrotropinomas when compared to normal pituitary tissue (Miyakoshi *et al.* 2008, Chambers *et al.* 2013). Since no change in subcellular β-catenin distribution was seen, it was proposed that the non-canonical WNT pathway is activated in these PIT1 lineage tumors, in agreement with WNT4 being a typical ligand in this pathway. However, other studies found nuclear accumulation of β-catenin, suggestive of a role of canonical WNT activity, in pituitary tumorigenesis (Nakashima *et al.* 2002, Hassanein *et al.* 2003, Campanini *et al.* 2010). The WNT target gene *PITX2* was found to be overexpressed in NFPA (in comparison with normal pituitary) (Acunzo *et al.* 2011). Transduction of a dominant-negative *PITX2* mutant in cultured human NFPA cells and in the mouse gonadotrope tumor cell line αT3-1 induced apoptosis, which may suggest an anti-apoptotic role for the WNT target PITX2 during NFPA tumorigenesis (Acunzo *et al.* 2011, Rostad 2012).

At present, involvement of WNT activation in pituitary tumorigenesis is most clear for ACP. Mutations in the *CTNNB1* gene-encoding β-catenin have been detected in a substantial number of ACP (Sekine *et al.* 2002, Hölsken *et al.* 2016). Several of these alterations render the β-catenin protein resistant to degradation, hence leading to its accumulation and WNT pathway activation. Microarray analysis of ACP showed increased expression of direct canonical WNT and TCF/LEF target genes (e.g. *LEF1*, *AXIN2*, *PITX2*) when compared to normal pituitary (Gaston-Massuet *et al.* 2011, Gump *et al.* 2015, Hölsken *et al.* 2016). In contrast, no upregulation of WNT pathway components was found in papillary craniopharyngioma (Garcia-Lavandeira *et al.* 2012, Esheba & Hassan 2015, Hölsken *et al.* 2016, Goschzik *et al.* 2017), but interestingly, expression of the stemness markers SOX2, SOX9, OCT4 and KLF4 was observed (Garcia-Lavandeira *et al.* 2012). In a mouse model of ACP, activation of the WNT pathway by targeted expression of degradation-resistant β-catenin in SOX2$^+$ pituitary stem cells triggered a (transient) proliferation of the latter cell population (Gaston-Massuet *et al.* 2011, Andoniadou *et al.* 2013). However, the mutated SOX2$^+$ cells did not directly give rise to the ACP tumor, but promoted the proliferation of the surrounding cells, probably through the secretion of SHH and WNT ligands (like *Wnt5a*, *Wnt6*, *Wnt10a*) in a forward signaling manner. Interestingly, tumorigenesis did not occur when expression of the degradation-resistant β-catenin was targeted to committed (PIT1$^+$) or mature (GH$^+$ and PRL$^+$) pituitary cells, providing supportive evidence that WNT dysregulation (here, activation) in the pituitary stem cells is a key event in ACP tumor development. Upregulation of WNT pathway components was also observed in the candidate TSC SP of human pituitary adenomas (in comparison with the bulk non-SP of the tumor) (e.g. *LEF1*, *LGR5*, *TCF7L1*, *WNT5A*) (Mertens *et al.* 2015) (Table 1). Of note, nuclear accumulation of β-catenin may also be correlated with a loss of interaction with membranous E-cadherin, which is associated with tumorigenesis and invasion in various tissues (El-Bahrawy & Pignatelli 1998) and which may represent a step in EMT.

EMT in pituitary tumorigenesis

EMT plays an important role in cancer pathogenesis, particularly in maintenance, resistance and progression toward locally invasive and/or metastatic conditions (Brabletz *et al.* 2005, Yang & Weinberg 2008, De Craene & Berx 2013). In addition, EMT appears implicated in senescence, which suppresses apoptosis and cell cycle progression and hence may (additionally) underlie therapy resistance (Browne *et al.* 2010). Interestingly, it has been shown that EMT promotes the generation and activity of CSC/TSC (Mani *et al.* 2008, Polyak & Weinberg 2009, Scheel & Weinberg 2012).

TGFB signaling is an important regulator of EMT (Fig. 1B), which is involved in its pro-oncogenic activity (Miyazono 2009). In apparent contrast, TGFB/SMAD signaling seems downregulated in human dopamine-resistant prolactinomas (Li *et al.* 2015). However, TGFB has dual effects in the progression of cancer: it is not only pro-oncogenic (e.g. by stimulating proliferation) but can also act as tumor suppressor by inhibiting cell proliferation (Pardali & Moustakas 2007, Santibãnza *et al.* 2011). EMT is inherently associated with downregulation of E-cadherin (Fig. 1B). In somatotropinomas, decreased expression of E-cadherin appears associated with increased tumor size and invasiveness and declined susceptibility to somatostatin analogs (Lekva *et al.* 2013). Gene expression profiling of human pituitary adenoma SP supports the occurrence of (partial) EMT in this candidate TSC population (Mertens *et al.* 2015) (Table 1). Epithelial markers (*CDH1*, *CLDN1*) were found downregulated (as compared to the bulk non-SP), whereas mesenchymal markers (*FN1*, *VIM*) and EMT mediators (*SNAI1*, *SNAI2*, *ZEB1*, *ZEB2*) were upregulated. Also other regulators of

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EMT were found to be prominently expressed in the pituitary tumor SP (e.g. *CXCR4*, *FOSL2*, *FOXC1*, *LEF1*, *MMP1*). Furthermore, inhibition of CXCR4 signaling reduced *in vitro* EMT-associated cell motility as well *in vivo* xenograft tumor growth of the mouse corticotrope AtT20 tumor cell line (Mertens *et al.* 2015). Taken together, candidate TSC shows EMT-linked molecular characteristics but the (functional) significance in pituitary tumorigenesis needs further investigation.

Dysregulation of SHH in pituitary tumorigenesis

Aberration of SHH signaling has been linked with tumorigenesis in various tissues, as for instance found in pancreas (Kasai 2016), bladder (Syed *et al.* 2016) and prostate (Peng & Joyner 2015). Similar to the NOTCH pathway, SHH activity appears to differ according to pituitary adenoma subtype. Although SHH pathway components are found downregulated in NFPA as compared to normal pituitary tissue, *GLI1* is overexpressed in somatotropinomas (Yavropoulou *et al.* 2015*b*). No significant differences were observed between prolactinomas and normal pituitary tissue. Another study observed SHH production and *GLI1* upregulation not only in somatotropinomas (and corticotropinomas), but also in prolactinomas (Pyczek *et al.* 2016). From the studies so far, it may appear that NOTCH and SHH signaling are regulated in opposite ways in the different tumor subtypes. It is not clear whether activation of the SHH pathway is associated with the tumorigenic process or rather reflects a compensatory (protective?) mechanism (Jiang *et al.* 2012, Yavropoulou *et al.* 2015*a*,*b*). Lower expression of SHH and *GLI1* in a small cohort of the rare malignant pituitary carcinoma type in comparison with various kinds of benign pituitary adenomas may rather point to a protective role (Pyczek *et al.* 2016). SHH signaling may also be activated in ACP based on the observed increase in *GLI1*, *GLI2*, *GLI3*, *PTCH1* and *SHH* expression (Gomes *et al.* 2015, Gump *et al.* 2015, Hölsken *et al.* 2016), but is not upregulated in papillary craniopharyngioma (Hölsken *et al.* 2016, Goschzik *et al.* 2017). As mentioned previously, transgenically induced ACP showed increased production of SHH by the β-catenin-activated SOX2$^+$ cells, which then may contribute to ACP development and tumor cell proliferation (Andoniadou *et al.* 2012, 2013). Some SHH-associated genes were also found upregulated in the SP of human pituitary tumors when compared to the bulk non-SP (Mertens *et al.* 2015) (Table 1), and GLI1 expression seems to go together with SOX2 expression as analyzed in a cohort of pituitary adenomas (Lampichler *et al.* 2015).

Dysregulation of Hippo in pituitary tumorigenesis?

Deregulation of the Hippo pathway leading to elevated levels of YAP/TAZ has been found in several types of cancer (Harvey *et al.* 2013). Hippo signaling (i.e. repressing YAP/TAZ) may thus exert tumor-suppressive activity. The Hippo system may also interact with WNT signaling since *Yap1* deletion blocked the development of adenomas in WNT-activated *Apc*$^{-/-}$ intestinal crypts (Gregorieff *et al.* 2015). Implication of Hippo in pituitary tumorigenesis is still uncharted although enrichment of various Hippo pathway components (e.g. *LATS2*, *TEAD4*, *YAP1*) and of the downstream targets *CTGF* and *CYR61* has recently been reported in the SP of human pituitary adenomas when compared to the bulk non-SP of the tumor (Mertens *et al.* 2015) (Table 1).

Dysregulation of EGF/FGF in pituitary tumorigenesis

EGF and its receptor EGFR play an important role in different types of tumors (Yarden & Pines 2012). Several studies have found the expression of EGF and/or EGFR in pituitary adenomas, sometimes associated with a more aggressive (invasive) phenotype (Lubke *et al.* 1995, LeRiche *et al.* 1996, Asa & Ezzat 2002, Onguru *et al.* 2004, Cooper *et al.* 2011, Fukuoka *et al.* 2011, del Pliego *et al.* 2013). In ACP, EGF signaling has been reported to play a role in tumor cell migration (Hölsken *et al.* 2011). FGFs have been detected at elevated levels in pituitary adenomas when compared to normal pituitary (Asa & Ezzat 2002). Increased expression of the growth factor receptors EGFR, FGFR1 and FGFR3 was detected in the candidate TSC SP population (Mertens *et al.* 2015), suggesting a role for the EGF/FGF pathway in their proliferation.

Taken together, knowledge on the involvement of NOTCH, WNT, EMT, SHH, Hippo and EGF/FGF signaling in pituitary tumorigenesis is still fragmentary and limited. Moreover, whether dysregulation of these pathways in the pituitary stem cells leads to generation of TSC, or whether TSC are simply regulated by these pathways, is at present also not clear. Unraveling the nature of these connections may reveal interesting and valuable therapeutic targets and lead to new approaches for treating pituitary tumors. For example, the SHH pathway may be inhibited by SMO antagonists, which has been shown as a promising perspective for treating ACP (Rimkus *et al.* 2016), and

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blocking CXCR4 signaling may impede pituitary tumor growth (Barbieri *et al.* 2014, Mertens *et al.* 2015).

Concluding remarks

Back to the future: regulatory pathways from pituitary embryogenesis reiterated in adult stem cells

The field of pituitary stem cells has made a substantial leap forward over the past decade, especially with respect to their identification. Although more and more studies address the question of their involvement in postnatal pituitary processes, knowledge remains limited, not only regarding their function but also certainly regarding their regulation. The stem cells are highly quiescent in the stationary adult pituitary, but present as more active particularly during early-postnatal maturation, injury-triggered regeneration and tumorigenesis. Core stem cell regulatory pathways like NOTCH, WNT, EMT and SHH seem activated in the (tumor) stem cell compartment during these conditions. Since these pathways also play essential roles during pituitary embryogenesis, activation of the stem cells may picture a flashback toward the past embryogenic events. In addition to the 'old faithful' pathways classically involved in embryogenesis and stem cell regulation, Hippo may gather increasing importance and further complement these pathways to an assembly of 'the big five' that pull the regulatory strings. It is clear that deciphering the networks regulating the pituitary (tumor) stem cells will cross-fertilize the search for their function in the postnatal gland. To this end, conditional transgenic models activating or deleting core factors of the different regulatory pathways specifically in the pituitary and/or its stem cells are essential. A further challenge will be to identify the cells providing the secreted regulatory factors, being produced by the stem cells to act in an auto- or juxtacrine manner, by the cells in the close vicinity, or more generally by the endocrine cells of the gland, potentially in a reciprocal interaction. This search will portray the composition of the stem cell niche(s) in the pituitary in further desired detail.

Last but not least, pituitary stem cells also gradually enter the new era of *in vitro* organ and disease modeling, for which knowledge on regulatory networks is of essential importance. Using patient-derived (pluripotent) stem cells, *in vitro* modeling may eventually lead to a better understanding of pituitary diseases and open perspectives on regenerative approaches in pituitary deficiencies.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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