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## **Development of the pituitary gland**

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### **Running Head**

Pituitary development

## **Abstract**

The development of the anterior pituitary gland occurs in distinct sequential developmental steps, leading to the formation of a complex organ containing five different cell types secreting six different hormones. During this process the temporal and spatial expression of a cascade of signalling molecules and transcription factors plays a crucial role in organ commitment, cell proliferation, patterning and terminal differentiation. The morphogenesis of the gland and the emergence of distinct cell types from a common primordium are governed by complex regulatory networks involving transcription factors and signalling molecules that may be either intrinsic to the developing pituitary or extrinsic, originating from the ventral diencephalon, the oral ectoderm and the surrounding mesenchyme. Endocrine cells of the pituitary gland are organized into structural and functional networks that contribute to the coordinated response of endocrine cells to stimuli; these cellular networks are formed during embryonic development and are maintained or may be modified in adulthood, contributing to the plasticity of the gland. Abnormalities in any of the steps of pituitary development may lead to congenital hypopituitarism that includes a spectrum of disorders from isolated to combined hormone deficiencies including syndromic disorders such as septo-optic dysplasia. Over the last decade, the acceleration of next generation sequencing has allowed for rapid analysis of the patient genome to identify novel mutations and novel candidate genes associated with hypothalmo-pituitary development. Subsequent functional analysis using patient fibroblast cells, and the generation of stem cells derived from patient cells, is fast replacing the need for animal models whilst providing a more physiologically relevant characterization of novel mutations. Furthermore, CRISPR-Cas9 as the method for gene editing is replacing previous

laborious and time-consuming gene editing methods that were commonly used, and thus yielding knock-out cell lines in a fraction of the time.

## **Introduction**

The pituitary gland is a central regulator of growth, metabolism, reproduction and homeostasis. In mice the mature gland consists of three lobes, the anterior, posterior and intermediate lobes, the latter of which involutes during embryogenesis in humans. The pituitary gland has a dual embryonic origin: the anterior and intermediate lobes derive from the oral ectoderm, whilst the posterior lobe originates from the neural ectoderm.

The development of the pituitary gland occurs in distinct and sequential developmental steps, leading to the formation of a complex organ containing five different cell types that populate the anterior pituitary, secreting six different hormones: somatotrophs (growth hormone; GH), thyrotrophs (thyrotrophin or thyroid stimulating hormone; TSH), lactotrophs (prolactin; PRL), gonadotrophs (follicle-stimulating hormone; FSH and luteinizing hormone; LH) and corticotrophs (corticotrophin or adrenocorticotrophic hormone; ACTH). The intermediate lobe contains the melanotrophs which secrete proopiomelanocortin (POMC), which is a major precursor to endorphins and melanocyte-stimulating hormone (MSH). The posterior pituitary or neurohypophysis consists of the axonal terminals of magnocellular neurons from the supraoptic and paraventricular nuclei of the hypothalamus secreting arginine vasopressin and oxytocin respectively.

The release of the specific anterior pituitary hormones is under the control of the hypothalamic parvocellular neurosecretory system that consists of neurons secreting

thyrotrophin releasing hormone (TRH) stimulating secretion of TSH and prolactin, corticotrophin releasing hormone (CRH) that acts to stimulate the secretion of ACTH, gonadotrophin releasing hormone (GnRH) that stimulates release of FSH and LH; growth hormone releasing hormone (GHRH) that stimulates the secretion of GH, somatostatin (SS) which negatively regulates GH secretion, and dopamine that inhibits secretion of prolactin. These hypothalamic factors are rapidly transported via the hypophyseal portal blood system to the anterior pituitary where they regulate endocrine cell proliferation, hormone synthesis and release from all the pituitary cell types (Kelberman et al., 2009; Davis et al., 2013).

The development of the pituitary gland and hypothalamus are closely linked; both are derived from the anterior neural plate, their development is conserved across vertebrates and, throughout development, the sustained contact between the oral ectoderm and the neuroepithelium is critical for the induction, shaping and patterning of Rathke's pouch, from which the anterior and intermediate lobes of the anterior pituitary are derived (Zhu et al., 2007b; Kelberman et al., 2009).

This review will focus on the known genetic factors involved in pituitary development that have been elucidated from studies on transgenic animal models, functional assays, and the detailed phenotypic and genetic studies of patients with hypopituitarism.

## **Overview of pituitary development**

### **Morphogenesis of the gland**

The development of the anterior pituitary is dependent upon the sequential temporal and spatial expression of a cascade of signalling molecules and transcription factors that play a crucial role in organ commitment, cell proliferation, patterning and terminal differentiation.

The development of the pituitary gland has been studied extensively in the mouse and it

seems that, to a large extent, pituitary development in humans mirrors that in rodents (Sheng and Westphal, 1999). The anterior pituitary develops from the hypophyseal or pituitary placode, one of the six cranial placodes that appear transiently as localised ectodermal thickenings in the prospective head of the developing embryo. The pituitary placode appears at embryonic day (E) 7.5, located ventrally in the midline of the anterior neural ridge (ANR) and, even at this earliest stage, it is in continuity with the future hypothalamo-infundibular region, which is located posteriorly, in the rostral part of the neural plate (Rizzoti and Lovell-Badge, 2005). By E8.5, the neural tube bends at the cephalic end and the placode appears as a thickening of the roof of the primitive oral cavity. At E9.0, the placode invaginates and forms the rudimentary Rathke's pouch, from which the anterior and intermediate lobes of the anterior pituitary are derived (Takuma et al., 1998; Rizzoti and Lovell-Badge, 2005). Concomitant with these events, the hypothalamic primordium becomes morphologically evident in the neural ectoderm at E9.5 with hypothalamic neurogenesis commencing at E10, at the same time as the highest level of expression of genes important for the regional patterning of hypothalamic progenitor cells, such as *Sim1*, *Sim2*, *Arx* and *Nr5a1* (Shimogori et al., 2010). Hypothalamic neurogenesis is complete by E16 although expression of hypothalamic terminal differentiation markers peak postnatally (Blackshaw et al., 2010; Shimogori et al., 2010). By E10.5 the definitive Rathke's pouch is formed, whilst the neural ectoderm at the base of the developing diencephalon evaginates to give rise to the posterior pituitary. The continuing apposition and contact between the neural ectoderm and the developing pituitary is essential for successful organogenesis of the pituitary gland and is maintained throughout this process (Treier and Rosenfeld, 1996; Treier et al., 1998). Between E10.5 and E12, the pouch

epithelium continues to proliferate and separates from the underlying oral ectoderm at E12.5. The progenitors of the hormone-secreting cell types proliferate ventrally from the pouch between E12.5-17.5 and populate what will form the anterior lobe (Dasen et al., 2001; Ward et al., 2006). The remnants of the dorsal portion of the pouch will form the intermediate lobe, whilst the lumen of the pouch remains as the pituitary cleft, separating the intermediate from the anterior lobe (Rizzoti and Lovell-Badge, 2005).

### **Endocrine cellular differentiation and organisation in the developing pituitary gland**

In the developing pituitary gland, the differentiated hormone-producing cells arise in a temporally and spatially regulated manner and are characterised by the expression of terminal differentiation markers. Progenitor cells divide around the lumen of Rathke's pouch and relocate ventrally, away from the lumen, as they differentiate; this ventral re-localisation is associated with exit from the cell cycle (Davis et al., 2013; Drouin et al., 2010). The first cells to exit the cycle progress to a transitional stage of non-cycling precursor cells, expressing both cyclin-dependent kinase inhibitor 1C (*p57Kip2*) and cyclin E, at the boundary between the lumen and the forming anterior lobe (Bilodeau et al., 2009). These cells are restricted both spatially and temporally and may represent a mixed population of cells, including uncommitted progenitors and cells that are engaged in a particular fate and may undergo lineage differentiation. As they form the anterior lobe they lose expression of *p57Kip2* and *cyclin E* and express cyclin-dependent kinase inhibitor 1B (*p27Kip1*) and intermediate markers of differentiation, for example *TBX19* (also known as *TPIT*) in corticotrophs, before they differentiate into hormone producing cells (Drouin et al., 2010). Between E11.5 and E18.5, cells in the developing pituitary evolve from

proliferating to mostly differentiating cells, and at E13.5 there is a clear division in cell-cycle state between the dorsal side of the anterior lobe containing proliferating cells, and the ventral side where the first differentiated cells appear (Bilodeau et al., 2009; Drouin et al., 2010). The earliest marker of differentiation in the anterior pituitary is the expression of *Cga*, encoding the alpha-glycoprotein subunit ( $\alpha$ GSU), in the thyrotrophs and gonadotrophs at E12.5. The thyrotrophs also express the transcription factor Islet-1 (*Isl1*) and will subsequently initiate the expression of *Tshb* (thyroid stimulating hormone subunit- $\beta$ ). However, this represents an early and transient cell population that disappears at birth (Ericson et al., 1998; Kelberman et al., 2009). In the presence of *Lhx3*, *Cga* expression is a concomitant marker of differentiation with both *Foxl2* and *Lh $\beta$*  in gonadotrophs (Sheng et al., 1996; Ellsworth et al., 2008).

Corticotroph cells start to differentiate at E12.5 in an intermediate domain, just dorsal to the previously mentioned prospective thyrotrophs. They are defined by the expression of *Pomc*, which is also expressed by melanotrophs of the intermediate lobe from E14.5. At the same time (E14.5) the definitive thyrotrophs are also detected and characterised by the expression of *Tshb* in a restricted number of cells within the anterior lobe (Kelberman et al., 2009; Zhu et al., 2007a).

The expression of Growth Hormone (*Gh*) and Prolactin (*Prl*) by E15.5 is the hallmark of the differentiation of somatotroph and lactotroph lineages respectively. Somatotrophs appear in the anterolateral wings of the developing gland, following the appearance of thyrotrophs and corticotrophs (Japon et al., 1994). The appearance of differentiated somatotrophs is followed by a dramatic increase in their number and their migration by E18.5 throughout the central and lateral parts of the anterior lobe, whilst lactotrophs remain

localized to a more restricted medial zone adjacent to the ventral surface of the intermediate lobe (Le Tissier et al., 2012; Bonnefont et al., 2005). The gonadotrophs are the last cell type to emerge, beginning at E16.5 with the onset of expression of hormone-specific beta subunit of LH (*Lhb*), followed by FSH-beta subunit (*Fshb*) a day later (Kelberman et al., 2009). A number of transcription factors have been shown to determine the gonadotroph cell fate (such as *Gata2*, *SF1*, *Egr1*, *Pitx1*, *Pitx2*, *Prop1*), resulting in mature cells expressing terminal cell differentiation markers GnRHR (GnRH receptor), LH $\beta$  and FSH $\beta$  (Weltzien et al., 2014; Thackray, 2014; Ciccone and Kaiser, 2009).

Although the classic description of cell differentiation is based on the sequential appearance of differentiating markers, birthdating studies imply that endocrine cells may be specified earlier and migrate some distance before they can be characterized by their differentiated markers. In fact, most of the hormone expressing cell types appear to differentiate between E11.5 and E13.5, denoting a broader range of specification rather than a sequential pattern of discrete times (Davis et al., 2011). In this model, the distribution of specific cell types within areas of the gland is not related to their exit from the cell cycle, but other mechanisms may be involved including active or passive cell movements, lateral inhibitory factors, and network formation (Davis et al., 2011; Hodson et al., 2010).

The spatial distribution of the hormone-secreting cell types within the pituitary is by no means random, as cells are maintained in structural and functional homotypic networks that facilitate the co-ordinated physiological response to stimuli and contribute to the plasticity of the gland (Bonnefont et al., 2005; Budry et al., 2011; Hodson et al., 2012b; Lafont et al., 2010; Hodson and Mollard, 2012; Le Tissier et al., 2012). For example, the network organization of somatotrophs ensures the co-ordinated response to GHRH and



other inputs and the generation of GH pulses despite the difference in the timing of individual cell stimulation. The architecture and function of this network is plastic throughout life and is modified in response to altered demand for GH (Schaeffer et al., 2011; Bonnefont et al., 2005; Budry et al., 2011; Sanchez-Cardenas et al., 2010). Similarly, the network organisation of lactotrophs increases their functional connectivity, intercellular communication and tissue output in response to stimuli, whilst the maintenance and development of the functional connectivity patterns allows the development of plasticity and “memory”. In this case, repeat challenges with identical stimulus are met with an evolved network behavior and improvement in tissue function (Hodson et al., 2012b; Hodson et al., 2012a; Schaeffer et al., 2010).

In terms of the plasticity of the gland, the identification of pituitary progenitor/stem cells in the developing and adult pituitary is of particular significance (Fauquier et al., 2008; Chen et al., 2005; Vankelecom and Gremeaux, 2010; Castinetti et al., 2011b). These were identified as a population of Sox2 (+) cells that persist in the adult pituitary in the area lining the pituitary cleft, and maintain their ability to self-proliferate and form pituispheres *in vitro*, and can be induced to differentiate into each of the pituitary lineages (Fauquier et al., 2008). Further studies *in vivo* confirmed that the Sox2 (+) precursors can generate hormone-producing cells during embryonic development as well as in adulthood, contribute to pituitary homeostasis (Rizzoti et al., 2013; Gremeaux et al., 2012; Vankelecom, 2007), are implicated in the regenerative potential of the pituitary gland (Fu et al., 2012; Fu and Vankelecom, 2012), and are able to act in a non-cell-autonomous manner to induce oncogenesis. These results conclude that the pituitary may be used as a model for exploring how physiological changes may influence stem cell behaviour.

Furthermore, manipulation of endogenous pituitary stem cells may be a potential therapeutic strategy for pituitary deficiencies (Andoniadou et al., 2013; Rizzoti et al., 2013).

Perhaps one of the most significant advances of the last years was the generation *in vitro* of a functional three-dimensional anterior pituitary gland from mouse embryonic stem cells (Suga et al., 2011). These elegant experiments demonstrated that, through diverse culture conditions and application of induction factors, embryonic stem cells could be programmed to recapitulate the formation of Rathke's pouch and differentiate into anterior pituitary hormone-producing cell types. Most remarkably when corticotrophs were transplanted into the kidneys of hypophysectomised mice, treatment with corticotrophin-releasing hormone treatment resulted in increased ACTH concentration with increased locomotor activity and improved survival with rescue (Suga et al., 2011; Suga, 2014).

### **Genes important for early patterning of the gland**

#### **Bone morphogenetic proteins (BMP4)**

Bone morphogenetic protein 4 (BMP4) is the earliest secreted molecule detected in the prospective infundibulum at E8.5, at a time that coincides with the initial contact between the ventral diencephalon and oral ectoderm, and its expression is maintained until E14.5. It is therefore important for the initial induction of Rathke's pouch, whilst its continuing expression to E14.5 may play a role in the maintenance of pouch (Ericson et al., 1998; Davis and Camper, 2007; Takuma et al., 1998). BMP4 belongs to a family of twenty secreted molecules which bind to serine-threonine receptor kinases and transduce an intracellular activation cascade, with several members of the Bmp family being involved

in multiple events during embryogenesis. In mice, loss of *Bmp4* results in early embryonic lethality; those animals that survive to E10 do not show any signs of the pituitary placode or pouch formation (Takuma et al., 1998). Ectopic expression of the BMP4 antagonist *Noggin* within the oral ectoderm and Rathke's pouch results in the early arrest of pouch development at E10 with absence of all pituitary cell types (Treier et al., 1998), whilst knock-down of the BMP receptor (*Bmpr1*) in Rathke's pouch at E9.5 results in an underdeveloped structure at E10.5 and early lethality (Davis and Camper, 2007). In contrast, *Noggin* null embryos show expanded domains of BMP4 activity within the ventral diencephalon resulting in a range of Rathke's pouch phenotypes from an enlarged, rostrally displaced pouch to induction of a second pouch and pituitary duplication (Davis and Camper, 2007).

With respect to its downstream targets, there is a suggestion that BMP4 signalling from the ventral diencephalon regulates expression of *Isl1* in the developing anterior pituitary; therefore, the regulation of *Isl1* expression could, at least in part, be responsible for the maintenance and survival effects of BMP4 on Rathke's pouch (Davis and Camper, 2007; Kelberman et al., 2009).

### **Fibroblast growth factor 8 (FGF8) and the FGF family**

Fibroblast growth factor 8 (FGF8) is a member of the FGF family of signalling molecules, that are involved in multiple processes during embryogenesis. FGFs activate their tyrosine kinase receptor (FGFR) and their extracellular association with heparan-sulfate proteoglycans is crucial for their activity (McCabe et al., 2011). *Fgf8*, along with *Fgf10* and *Fgf18*, are expressed in the infundibulum at E9.5, 24 hours later than the onset of *Bmp4*

(Treier et al., 2001; Treier et al., 1998). After the induction of Rathke's pouch, FGF signalling is necessary for cell proliferation within the pouch. FGF8 mediates the expansion of Rathke's pouch through induction of expression of *Lhx3* and *Lhx4* (Dasen and Rosenfeld, 2001; Zhu et al., 2007b), although at later stages (E16.5) expression of *Lhx3* appears to be independent of FGFs (Davis and Camper, 2007).

*Nkx2.1* null mutants, where expression of *Bmp4* initially occurs, do not express *Fgf8* in the ventral diencephalon and exhibit formation of a rudimentary pouch that fails to proliferate (Takuma et al., 1998). Treatment of pouch explants with *Fgf8* induces expression of *Lhx3*, whilst *Isl1* expression is restricted away from the factor source (Ericson et al., 1998). In contrast, treatment with a specific FGF receptor antagonist induces down-regulation of *Lhx3*, with significantly reduced proliferation and ectopic differentiation of prospective thyrotrophs (*Isl1*<sup>+</sup>; *Cga*<sup>+</sup>) and corticotrophs (ACTH<sup>+</sup>) in the dorsal region (Norlin et al., 2000). Moreover, early ectopic expression of *Fgf8* under the control of the *Cga* promoter within Rathke's pouch results in severe dysmorphogenesis and enlargement of the pituitary with an expansion of *Pomc* expressing cells (corticotrophs and melanotrophs) and loss of the other cell lineages (Treier et al., 1998; Kelberman et al., 2009).

Homozygous disruption of *Fgf8* in mice results in early embryonic lethality prior to gastrulation, whilst deletions of *Fgf10* or the gene encoding its receptor result in a poorly formed Rathke's pouch with widespread apoptosis resulting in absence of the pituitary. These observations confirm that FGFs from the ventral diencephalon have a proliferative effect on the pouch after its initial induction by BMP4 (Kelberman et al., 2009; Ohuchi et al., 2000).

*Fgf8* hypomorphic mice have a variable pituitary phenotype ranging from normal morphology to a markedly hypoplastic anterior pituitary gland, with or without an absent posterior pituitary and midline defects indicative of holoprosencephaly. In hypomorphic mutants the development of the hypothalamus is also compromised, with reduced numbers of hypothalamic neurons secreting arginine vasopressin and oxytocin (McCabe et al., 2011). Members of the FGF family are expressed in the diencephalon (FGF8, FGF10) and the embryonic pituitary transcriptome (FGF13, FGF14, FGF17), thus suggesting the potential for their functional redundancy at critical stages of development (Brinkmeier et al., 2009).

#### **Sonic hedgehog (SHH) signalling pathway**

Sonic Hedgehog (*Shh*) is expressed in the ventral diencephalon as well as throughout the oral ectoderm, with the exception of Rathke's pouch; *Shh* expression is subsequently lost within the oral epithelium at E12 and within the ventral diencephalon by E14 (Treier et al., 2001). Sonic hedgehog binds to its receptor Patched, leading to the activation or repression of target genes via the Gli-family of zinc finger transcription factors. In contrast to the expression pattern of *Shh*, *Patched* is highly expressed in Rathke's pouch (Treier et al., 2001) and members of the *Gli* gene family (*Gli1*, *Gli2* and *Gli3*) are expressed both in the ventral diencephalon and within the pouch (Zhu et al., 2007a; Treier et al., 2001), thus making the developing pituitary receptive to SHH signalling.

*Shh*-knockout mice have a severe phenotype with cyclopia and generalized loss of brain midline structures. Inhibition of SHH signalling through expression of its antagonist Hhip (Hedgehog-interacting protein) within the oral ectoderm and Rathke's pouch results in a

rudimentary pouch. In this case there is no disruption of BMP4 or FGF signalling from the ventral diencephalon, but the pouch appears severely hypoplastic with loss of expression of genes required for differentiation of the ventral cell types (*Bmp2*, *Gata2* and *Brn4/Pou3f4*) (Treier et al., 2001). On the other hand, ectopic overexpression of *Shh* in the developing Rathke's pouch under the control of *Cga* promoter results in up-regulation and maintenance of *Bmp2* expression within the developing pituitary at E17.5, over-expansion of the ventral thyrotrophs and gonadotrophs, and significant pituitary hyperplasia (Treier et al., 2001). These observations suggest that *SHH* signalling in the ventral diencephalon and the oral ectoderm is important for normal pituitary development, proliferation and, through induction of *Bmp2* expression, the specification and expansion of ventral cell types.

Mice with conditional loss-of-function of *Shh* in the ventral diencephalon (*Shh<sup>Ahyp</sup>*) exhibit severe hypothalamic, pituitary and ocular defects that are reminiscent of the phenotype of septo-optic dysplasia in humans. Hypomorphic *Shh<sup>Ahyp</sup>* animals have altered dorso-ventral patterning in the diencephalon, failure of the infundibulum to evaginate correctly with abnormal/duplicated invagination of Rathke's pouch, and significant reduction in the number of somatotrophs, thyrotrophs and corticotrophs in the developing anterior pituitary by E18.5 (Zhao et al., 2012).

Recent studies have shown that conditional deletion of *Shh* in the anterior hypothalamus, results in a fully penetrant phenotype characterised by a complete arrest of RP development. At 9.0dpc, there is a decrease in *Lhx3/Lhx4* expression in the epithelium of the RP, with a complete loss of the pituitary by 12.5dpc (Carreno G et al., 2017).

SHH signals are transduced through the Gli-transcription factors, with Gli2 primarily activating and Gli3 primarily repressing SHH transcriptional targets. Mice with homozygous deletion of the zinc finger domain of Gli2 ( $Gli2^{zfd}/Gli2^{zfd}$ ) have early perinatal lethality and severe skeletal and cartilage abnormalities (including cleft palate, incisor tooth anomalies, short limbs with poor ossification of the long bones). The Gli2 null embryos have hypomorphic pituitaries and reduced expression of Bmp4 and Fgf8, suggesting that there is a requirement for Gli2 at the very early stages of hypothalamo-pituitary axis formation (Wang et al., 2010).

Many members of the SHH pathway have been implicated in the aetiology of holoprosencephaly, a heterogeneous brain malformation resulting from incomplete cleavage of the prosencephalon, with variable failure of formation of the two hemispheres of the brain. Additionally, the condition affects both the forebrain and the face (Dubourg C et al., 2004). In rare severe cases, cyclopia may occur, mirroring features observed in the *Shh* knockout mouse. Haploinsufficient loss of function mutations in *SHH* itself are the most frequently occurring in these patients (Lami F et al., 2013). Furthermore, the production and activity of SHH has been affected by different mutations in the gene to varying degrees, giving rise to the variable phenotypic spectrum seen in HPE patients (Singh S et al., 2009). The transcription factor GLI2, a component of the SHH pathway, has also been described to be mutated in several HPE patients with variable penetrance (Roessler E et al., 2003). However a wider phenotypic variability is seen in patients with *GLI2* mutations, such as congenital hypopituitarism without midline defects (Gregory LC et al., 2015a) (Arnhold IJ et al., 2015).

PITX1 is a member of a family of homeodomain transcription factors related to the *Drosophila* protein Bicoid. In mice, expression of *Pitx1* is first detected in the anterior ectoderm at E8.0; by E9.5 it is expressed throughout the oral ectoderm and in Rathke's pouch and is maintained during pituitary development in all hormone-producing cell types. In the adult pituitary, *Pitx1* expression is highest in thyrotrophs expressing *Cga* and in gonadotrophs, with lower levels in other hormone-producing cell types (Lanctot et al., 1999; Goodyer et al., 2003). *Pitx1* null embryos have normal pituitary morphogenesis but the number of thyrotrophs and gonadotrophs are reduced at birth, with reductions in LH $\beta$  and TSH $\beta$  (Szeto et al., 1999). Although *Pitx1* is expressed throughout anterior pituitary development, the absence of early defects in *Pitx1* null mice may be explained by the redundant function of the closely related *Pitx2*.

Expression of *Pitx2* is detected widely in Rathke's pouch and in the developing pituitary, whilst in adults it is expressed predominantly in thyrotrophs and gonadotrophs (Gage et al., 1999; Suh et al., 2002; Charles et al., 2005). Homozygous loss of *Pitx2* results in early embryonic lethality with severe heart, craniofacial and eye defects and lung asymmetry (Gage et al., 1999; Lin et al., 1999; Liu et al., 2003). The development of the pituitary in these embryos is severely affected with initial specification of Rathke's pouch, but failure to maintain *Hesx1* expression and subsequent arrest of growth and differentiation by E12.5. This suggests that *Pitx2* is essential for pituitary gland development shortly after formation of the committed pouch (Gage et al., 1999). Expression of *Pitx2* is induced by activation of the Wnt signalling pathway or constitutive activation of  $\beta$ -catenin and, in turn, *Pitx2*



controls genes that regulate the cell cycle (*cyclin D1* and *cyclin D2*) (Kioussi et al., 2002; Ai et al., 2007). The undetectable *Lhx3* expression in *Pitx2* null pituitaries suggests that LHX3 is a potential target (Charles et al., 2005). It is therefore possible that the pituitary hypoplasia observed in *Pitx2* null mice may result from decreased cell proliferation (Kioussi et al., 2002) or increased apoptosis and reduced survival of pouch progenitors (Charles et al., 2005). Mice with targeted *Pitx2* deletion in thyrotroph cells have a significant increase in *Pitx1* transcripts in the pituitary, smaller thyroid glands and normal circulating TSH and T4 (Castinetti et al., 2011a).

#### **Lim homeodomain transcription factors**

LIM homeodomain transcription factors are characterised by two tandemly repeated cysteine/histidine-rich, zinc-binding, LIM domains that are involved in protein-protein interactions, between the N-terminal end of the protein and the DNA binding homeodomain. Four LIM homeodomain transcription factors are currently known to be important for pituitary development: ISL1, LHX2, LHX3, and LHX4.

*Isl1* is the first to be detected in the oral ectoderm at E8.5 and becomes restricted to the pouch at E9.5; between E10.5 and E11.5, its expression is further restricted to the ventral portion of the pouch, in cells that will express *Cga* and subsequently *Tshb*, and that will become rostral tip thyrotrophs after E12.5 (Ericson et al., 1998). This changing expression pattern may be dictated by interactions with the surrounding tissues. Homozygous loss of *Isl1* in mice results in embryos with severe heart defects that die by E10.5. In these embryos, the rudimentary pouch fails to develop, suggesting that *Isl1* is necessary for pituitary progenitor cell proliferation and/or maintenance (Takuma et al., 1998); however, the early lethality of these animals made it difficult to assess the role of *Isl1* at later stages

of development. Recent detailed studies of the expression of *Isl1* in the developing and early postnatal murine pituitary demonstrated that *Isl1* expression is detected in Rathke's pouch from E11.5 to E13.5 in cells of the differentiating, but not of the proliferative, zone. At later stages, its expression is detected in scattered cells of the anterior lobe (E14.5, E16.5, P3), but not in the posterior lobe, whilst the ventral hypothalamus exhibits strong immunostaining for ISL1 through development. ISL1 immunostaining colocalised with many of the CGA (E16.5) or TSH $\beta$  positive cells (P7) as well as with NR5A1 positive cells (that will differentiate to gonadotrophs) at E16.5, but this was less pronounced in the postnatal pituitary (P7). These observations suggest that ISL1 might be involved in the development and function of thyrotrophs, as well as in the initiation of the differentiation of gonadotrophs, but not in their maintenance (Castinetti et al., 2015).

*Lhx3* and *Lhx4* are also characterised by the presence of the unique cysteine/histidine-rich zinc-binding LIM domain. After induction by *Fgf8*, *Lhx3* is expressed strongly and uniformly in Rathke's pouch from E9.5, in the ventral hindbrain and in spinal cord (Sheng et al., 1996). Early in pituitary development (E9.5-E10.5) there is overlap in the expression pattern of LHX3 and ISL1, but their expression becomes mutually exclusive at the later stages of development (Castinetti et al., 2015). By E16.5, *Lhx3* is expressed in the developing anterior and intermediate pituitary, but not in the posterior gland, and its expression persists into adulthood suggesting that *Lhx3* has a role in the establishment of hormone producing cell-types and in the maintenance of at least some cell types in the mature anterior pituitary (Sheng et al., 1996; Mullen et al., 2007).

*Lhx3* null mice (*Lhx3*<sup>-/-</sup>) show early lethality with lack of the anterior and intermediate pituitary lobes and, although Rathke's pouch is initially formed, development of the

pituitary gland is arrested with defects in the differentiation of all hormone-secreting cell types, as there is failure to maintain expression of *Hesx1* and induce *Pou1f1* (Ellsworth et al., 2008). The dorso-ventral cell specification appears impaired in *Lhx3*<sup>-/-</sup> mice and this may, in part, be due to disrupted Notch signalling, leading to ectopic gene activation dorsally and subsequent failure to activate *Pou1f1* with loss of lactotrophs, somatotrophs and thyrotrophs (Ellsworth et al., 2008). *Lhx3* null embryos have a transient lack of *Isl1* expression at E12.5, at a time that is critical for restricting expression of *Isl1* to the prospective anterior lobe, and its expression recovers at later stages in development. It is, therefore, suggested that this delay in the expression of *Isl1* may also contribute to the lack of thyrotrophs in the *Lhx3* null mice (Castinetti et al., 2015). The failure of gonadotroph differentiation is probably due to the downregulation of *Foxl2* and *Cga* (Ellsworth et al., 2006; Ellsworth et al., 2008). In addition, the down-regulation of *Tbx19* (*Tpit*) expression in the intermediate lobe is consistent with the observed absence of melanotrophs, whilst in the developing anterior lobe, the down regulation of *Tbx19* and *NeuroD1* results in the dramatic reduction of corticotrophs (Ellsworth et al., 2008).

*Lhx4* is also expressed in Rathke's pouch at E9.5, but its expression is restricted to the future anterior lobe and is down-regulated by E15.5. Expression of *Lhx4* is also detected in specific fields in the developing hindbrain, cerebral cortex and motor neurons of the spinal cord (Raetzman et al., 2002; Mullen et al., 2007). In *Lhx4* null mice, Rathke's pouch is formed and there is specification of all the anterior pituitary cell lines. However, their numbers are markedly reduced leading to anterior pituitary hypoplasia. Although there is a reduction in cell proliferation, the small size of the anterior pituitary in these animals is due to increased apoptosis which occurs by E14.5 (Raetzman et al., 2002). Double mutant

mice (*Lhx3*<sup>-/-</sup>, *Lhx4*<sup>-/-</sup>) exhibit a more severe phenotype than either single mutant, with an early arrest of pituitary development, suggesting that there is redundancy in their actions (Sheng and Westphal, 1999).

*Lhx2* is expressed in the diencephalon and posterior lobe, but not in Rathke's pouch. *Lhx2*<sup>-/-</sup> mutant embryos have complete failure of the evagination of the neuroectoderm and disorganised anterior and intermediate lobes of the pituitary, although all endocrine cell lineages are present (Zhao et al., 2010). The role of LHX2 in the developing hypothalamus is not yet clarified and may affect diverse processes from hypothalamic progenitors and development of tanycytes (Salvatierra et al., 2014) to neuronal connections and axon guidance (Marcos-Mondejar et al., 2012).

Patients with homozygous mutations in *LHX3* have hypopituitarism, with a short neck and limited neck rotation as a variable feature, considered to result from nervous system abnormalities (Netchine et al., 2000; Sloop KW et al., 2001; Bechtold-Dalla Pozza S et al., 2012). However, recent studies have described a heterozygous *LHX3* variant, p.L196P, associated with a milder form of hypopituitarism (Jullien N et al., 2018) compared to previous cases. Functional *in vitro* studies suggested haploinsufficiency due to the inability of p.L196P to bind DNA and in turn activate target promoters (Jullien N et al., 2018).

Heterozygous mutations in *LHX4* give rise to variably penetrant CPHD-related phenotypes usually through haploinsufficiency (Tajima T et al., 2007; Tajima T et al., 2010; Takagi et al., 2012). The first novel recessive lethal *LHX4* mutation was identified in a pedigree with severe panhypopituitarism, an ectopic posterior pituitary and mid-facial hypoplasia.

Affected patients died due to respiratory distress syndrome in combination with their severe hypopituitary phenotype (Gregory et al., 2015b).

## HESX1

The transcription factor *Hesx1* is a member of the paired-like class of homeobox genes and one of the earliest markers of the pituitary primordium. *Hesx1* is expressed early during gastrulation in a region that will form the forebrain and from E9.0-9.5, its expression is restricted to the ventral diencephalon and the developing Rathke's pouch (Dattani et al., 1998a; Andoniadou et al., 2007; Martinez-Barbera et al., 2000). *Lhx3* is important for maintaining expression of *Hesx1*; subsequently, expression of *Hesx1* gradually disappears from E12.5 in a spatiotemporal sequence that corresponds to progressive pituitary cell differentiation, and becomes undetectable by E15.5 (Dattani et al., 1998a; Dasen et al., 2001).

*Hesx1* is a transcriptional repressor and its down-regulation is important for the downstream activation of *Prop1*, and the emergence of the *Pou1f1*-cell lineage (Dasen et al., 2001). Prolonged expression of *Hesx1* can block *Prop1*-dependent activation, whilst premature expression of *Prop1* can repress *Hesx1* and block pituitary organogenesis (Dasen et al., 2001). This concomitant repression of *Hesx1* and activation of *Pou1f1* is mediated by the interaction between  $\beta$ -catenin and *Prop1* (Olson et al., 2006). This complex, along with co-factors, binds directly to *Hesx1* and *Pou1f1* cis-regulatory elements inducing the changes in repression and activation of these genes respectively.

*Hesx1* is essential for normal forebrain development and *Hesx1* null mice (*Hesx1*<sup>-/-</sup>) have a reduction in the prospective forebrain tissue, absence of the developing optic vesicles, optic

cups and olfactory placodes, markedly decreased head size, severe microphthalmia, hypothalamic abnormalities and abnormal morphogenesis of Rathke's pouch (Dattani et al., 1998a). Although a small percentage (5%) of the most severely affected *Hesx1*<sup>-/-</sup> mutants have complete lack of the pituitary, the majority show multiple oral ectodermal invaginations and abnormal branching resulting in the apparent formation of multiple pituitary glands (Dattani et al., 1998a; Dasen et al., 2001). In these animals, the expression domains of *Fgf8* and *Fgf10* in the infundibulum are expanded rostrally, implicating that *Hesx1* is important for the maintenance of their expression pattern (Dasen et al., 2001). The phenotype of *Hesx1*<sup>-/-</sup> mice is highly variable and reminiscent of patients with septo-optic dysplasia (Dattani et al., 1998a; Martinez-Barbera et al., 2000), with more recent studies suggesting that the pituitary may be more sensitive to changes in *Hesx1* dosage than the eyes or forebrain (Sajedi et al., 2008). The most severely affected *Hesx1* null embryos have significant reduction in the anterior forebrain structures, which is caused by posteriorisation of the anterior forebrain precursors at early somite stages and the ectopic activation of Wnt/ $\beta$ -catenin signalling within the *Hesx1* expression domain in the anterior forebrain (Andoniadou et al., 2007). In addition, mice with a homozygous null mutation in *Hesx1* (*Hesx1*<sup>R160C/R160C</sup>) may be severely affected with defects in the development of the telencephalon, eyes and craniofacial structures, and an ectopic anterior pituitary in the roof of the nasopharyngeal cavity (Sajedi et al., 2008). The less severely affected mutants had a eutopic but morphologically abnormal and bifurcated Rathke's pouch, resulting in the apparent formation of multiple pituitary glands, but the apparent overgrown gland becomes hypoplastic postnatally (Sajedi et al., 2008). In addition, embryos heterozygous for *Hesx1* and *Six3* null mutations had a phenotype similar to that of mildly affected *Hesx1* null

embryos. In these embryos, Rathke's pouch is initially expanded due to an increase in cell proliferation and the anterior pituitary gland appears bifurcated and occasionally ectopic in the nasopharyngeal cavity, but with unaffected cell differentiation (Gaston-Massuet et al., 2008).

In humans, the first homozygous *HESX1* mutation was described in two siblings from a consanguineous pedigree who presented with hypopituitarism in association with SOD and an ectopic/undescended posterior pituitary (Dattani et al., 1998a). Homozygous and heterozygous *HESX1* mutations are an uncommon cause of hypopituitarism and SOD (less than 1% of cases) (McNay et al., 2007) and have since been described in patients with highly variable phenotypes without obvious genotype-phenotype correlation. Homozygous *HESX1* mutations have also been reported in patients with panhypopituitarism who had pituitary aplasia with a normally sited posterior pituitary, with (Sobrier et al., 2005) or without (Sobrier et al., 2006b) ocular defects. For instance, two siblings from a consanguineous family were homozygous for an Alu-element insertion in exon 3 of *HESX1*, which contains the homeobox, yet they had distinct ocular phenotypes. Both had panhypopituitarism with aplasia of the anterior pituitary and a normal posterior pituitary and infundibulum. However, one had unilateral blindness and retinal coloboma, whilst the other sibling had no ocular abnormalities, but displayed a left-sided diaphragmatic hernia and aortic coarctation and died shortly after birth (Sobrier et al., 2005). In addition, homozygous frameshift (c.449\_450delAC) and splice site (c.357+2T>C) *HESX1* mutations have been reported in patients with life-threatening neonatal panhypopituitarism, who had aplasia of the anterior pituitary but absence of an ectopic posterior pituitary and no optic nerve abnormalities; in vitro studies suggested that the mutant HESX1 proteins had lost

their ability to inhibit PROP1 activity (Sobrier et al., 2006b).

Rarely, heterozygous *HESX1* mutations (p.E149K, p.S170L or p.T181A) may be associated with isolated growth hormone deficiency in patients exhibiting a relatively milder phenotype compared with the severe manifestations of SOD, who may or not have optic nerve hypoplasia, and with or without an ectopic/undescended posterior pituitary and anterior pituitary hypoplasia (Thomas et al., 2001a).

## **OTX2**

*Otx2* (Orthodenticle homeobox 2) is a transcription factor homologous to the *Drosophila* orthodenticle protein. In mice, expression of *Otx2* is localised to the developing neural and sensory structures, such as brain, eye, nose and ear. Homozygous knockout mice die at mid-gestation with mutant embryos lacking anterior structures corresponding to the future head (Acampora et al., 1995); targeted deletion of *Otx2* in the anterior neuroectoderm revealed that it is required for maintenance of the forebrain (Kurokawa et al., 2004). *Otx2* is expressed in the ventral diencephalon and in Rathke's pouch from E10.5, but by E12.5 its expression persists only in the ventral diencephalon and it becomes undetectable at both sites from E16.5 (Mortensen et al., 2011). This spatial and temporal pattern of *Otx2* expression suggests its potentially important role for the development of the ventral diencephalon and posterior pituitary.

More recently, conditional deletion of *Otx2* in the neural and oral ectoderm suggests that *Otx2* plays a minor role in the organogenesis of Rathke's pouch but it is necessary for normal development of the infundibulum and for induction of FGF signalling in the ventral diencephalon, thus affecting indirectly the development of the anterior pituitary (Mortensen et al., 2015). Mice with targeted *Otx2* deletion in the oral ectoderm (*Otx2<sup>FX</sup>*;



*Pitx2-Cre*) had normal pituitary morphology at E14.5 with normal immunostaining for  $\alpha$ GSU and POU1F1 and the postnatal pituitaries (P10) had normal terminal cell differentiation with immunostaining for GH, TSH $\beta$ , ACTH and LH $\beta$ . On the contrary, mice with *Otx2* deletion in the ventral diencephalon (*Otx2<sup>FX</sup>;Nkx2.1-Cre*) showed lack of invagination of the infundibulum and a smaller anterior lobe compared to wild type embryos at E12.5-16.5. Cell specification, however, was not affected as immunohistochemistry of postnatal pituitaries (P10) for TSH $\beta$ , ACTH, LH $\beta$ , FSH $\beta$ , GH and  $\alpha$ MSH indicated that all cell types were present with no differences between *Otx2<sup>FX</sup>;Nkx2.1-Cre* mutants and controls, apart from the smaller pituitaries (Mortensen et al., 2015).

In humans, heterozygous *OTX2* mutations or gene deletions have been implicated in the etiology of 2-3% of anophthalmia/microphthalmia syndromes (Ragge et al., 2005; Hever et al., 2006; Wyatt et al., 2008). The pituitary phenotype of patients with heterozygous *OTX2* mutations ranges from partial (Dateki et al., 2008) to complete GHD (Ashkenazi-Hoffnung et al., 2010a) or hypopituitarism (Diaczok et al., 2008a) with (Tajima et al., 2009a) or without an ectopic posterior pituitary on MRI (Dateki et al., 2008). There is no clear genotype-phenotype correlation, even among patients with the same mutation and in rare cases, such as the p.N233S mutation, patients may not even exhibit an ocular phenotype (Diaczok et al., 2008a). However, due to the relative short term follow-up data in these reports, the possibility of developing other pituitary hormone deficiencies over time cannot be excluded.

Previous *in vivo* studies have shown that *OTX2* loss of function mutations alter otocephaly and/or dysgnathia phenotypes in humans when in the presence of a second mutation in a

known causative otocephaly gene (Chassaing N et al., 2012). These data indicate that *OTX2* mutations contribute to craniofacial defects such as micrognathia, thus increasing the severity of the phenotype.

### **Wnt/ $\beta$ -catenin pathway**

The Wingless (Wnt) signalling pathway is an early developmental pathway implicated in diverse processes including cell proliferation, cell fate, differentiation and adhesion, as well as morphogenesis, patterning and axis formation (Clevers, 2006; Bienz, 2005). Wnts are a family of evolutionary conserved, cysteine rich, secreted glycoproteins (Cadigan and Peifer, 2009) and in the developing pituitary members of the Wnt-family and components of the downstream signalling pathway (such as  $\beta$ -catenin and TCFs) are expressed within Rathke's pouch, in the diencephalon or in the surrounding tissues (Douglas et al., 2001) (Potok et al., 2008).

In summary, the canonical pathway is activated by binding of Wnts to Frizzled receptor complex (Fzd/LRP) and its effects are mediated by  $\beta$ -catenin. The cytoplasmic pool of  $\beta$ -catenin is tightly regulated by a multi-protein destruction complex that contains adenomatous polyposis coli protein (APC), the scaffolding proteins Axin-1 and Axin-2, and the kinases GSK3 $\beta$  (glycogen synthase kinase-3 $\beta$ ) and CK1 (casein kinase-1) (MacDonald et al., 2009). In absence of Wnt signalling, CK1 phosphorylates  $\beta$ -catenin at a serine residue (S45), which triggers further phosphorylation of the N-terminal region by GSK3 $\beta$  at conserved serine and threonine residues (T41, S37 and S33 in sequence). Phosphorylation is enhanced by the action of Axin that acts as a scaffolding protein binding to  $\beta$ -catenin and the two kinases, CK1 and GSK3 $\beta$ . In addition, APC binds  $\beta$ -catenin and

“protects” it from dephosphorylation. The result of this co-ordinate action is that the phosphorylated  $\beta$ -catenin is recognised by the E3 ubiquitin-ligase ( $\beta$ -Trcp) leading to  $\beta$ -catenin ubiquitination and subsequent proteasomal degradation, thus limiting the pool of soluble cytoplasmic  $\beta$ -catenin (Cadigan and Peifer, 2009).

In the nucleus, in the absence of Wnt signalling, TCF/LEF (T-cell factor/Lymphoid enhancer factor) proteins are bound to target genes, along with co-repressors, and act as transcriptional repressors silencing gene transcription. The TCF/LEF transcription factors are members of the HMG group of proteins and bind to target DNA sequences via their HMG (High mobility group) domain (Clevers and van de Wetering, 1997; Schilham and Clevers, 1998).

Binding of Wnt ligands to the cell surface receptor inhibits GSK3 $\beta$  kinase, which leads to the inactivation of the destruction complex and stabilisation of  $\beta$ -catenin. As a result  $\beta$ -catenin accumulates and translocates to the nucleus, where it interacts with TCF/LEF transcription factors, displaces co-repressors such as Groucho (GPRK2), releases histone deacetylase (HDAC) and recruits co-activators (such as the histone acetyl-transferase CBP-p300) and chromatin remodelling proteins (BRG1) to stimulate transcription (Daniels and Weis, 2005; Clevers, 2006) (Cadigan and Peifer, 2009).

Study of the Rathke’s pouch at E12.5 by semi-quantitative RT-PCR, confirmed the expression of 10 of the 19 known members of the Wnt family (Wnt 3, 4, 5a, 5b, 6, 7a, 7b, 10a, 11 and 15) (Olson et al., 2006). Although subsequent studies did not confirm these results for three of the Wnt members (Wnt 6, 7a and 7b) (Potok et al., 2008), the Wnt/ $\beta$ -catenin pathway has been shown to be transcriptionally active during pituitary development

and in Rathke's pouch, at E13.5,  $\beta$ -catenin is associated with a Wnt-responsive element in the LEF1 promoter (Olson et al., 2006).

*Wnt4* is expressed in the pouch from E9.5 and its deletion (*Wnt4*<sup>-/-</sup>) results in pituitary hypoplasia and reduced expression of the transcription factor POU1F1, which is important for the differentiation of cells of the somatotroph lineage (Potok et al., 2008). By E17.5, *Wnt4*<sup>-/-</sup> mice show a decrease in the population of somatotrophs and thyrotrophs compared to the wild type littermates (Potok et al., 2008). In addition, *Wnt5a* is expressed in the ventral diencephalon and its conditional deletion (*Wnt5a*<sup>-/-</sup>) leads to an abnormal and bifurcated pouch (Cha et al., 2004), probably due to the disruption and extension of the expression domains of *Bmp4* and *Fgf*. This results in the recruitment of a wider region of the oral ectoderm leading to bifurcations within the developing pouch (Brinkmeier et al., 2007). There is evidence that members of the Wnt/ $\beta$ -catenin signalling pathway interact with transcription factors SOX2 (Mansukhani et al., 2005) and SOX3 (Zorn et al., 1999) and this interaction drives developmental processes such as neurogenesis and gliogenesis (Meyers et al., 2012). Disruption of the interaction between SOX2 and  $\beta$ -catenin in humans may be associated with the development of slow progressing hypothalamo-pituitary tumours (Alatzoglou et al., 2011a).

### **SIX Homeodomain Proteins**

At least four members of the SIX (sine oculis-related homeobox) family are expressed during pituitary development but the exact role of these proteins was at first difficult to determine due to their functional redundancy and the severity of the phenotype affecting

anterior forebrain structures in mouse mutants. *Six1*, *Six2*, *Six4*, and *Six5* have a broad expression profile during embryogenesis, but expression of *Six3* and *Six6* is restricted to the developing eye and brain (Jean et al., 1999; Conte et al., 2005).

*Six6* is first expressed in the invaginating pouch and becomes restricted to its dorsal region, distant from the periluminal region which is involved in cell differentiation and where proliferation is maintained (Jean et al., 1999). *Six6* is also expressed in the developing eye and hypothalamus and is maintained in the post-natal anterior pituitary (Jean et al., 1999). Although there is initial overlap in the expression domains of *Six6* and *Six3* in the anterior-most neural plate, they exhibit distinct expression patterns during late embryonic and postnatal development and their expression coincides only in a few hypothalamic and thalamic nuclei (Conte et al., 2005).

Initial studies on *Six6*<sup>-/-</sup> mice showed that these animals exhibited a hypoplastic pituitary, likely due to an early impairment of progenitor proliferation, and variable retinal hypoplasia with or without optic nerve hypoplasia (Li et al., 2002). Despite the anterior pituitary hypoplasia, immunohistochemistry confirmed the presence of all anterior pituitary hormone-producing cells (Li et al., 2002). *Six6*<sup>-/-</sup> female mice had significant reduction in fertility with a decrease in the total number of GnRH neurons (Larder et al., 2011).

*Six6* and *Six3* seem to have distinct but compensatory roles in the differentiation of gonadotrophs, as *Six3* is expressed early in gonadotroph cells and represses transcription of GnRH receptor (*GnRHR*) and *Cga*, whilst *Six6* is expressed in mature gonadotrophs and represses the expression of *LHβ* and *FSHβ* that occurs later in development. This action of

SIX6 involves competition for DNA-binding sites with the transcriptional activator PITX1 and the interaction with co-repressor proteins (TLE) (Xie et al., 2015).

Six3 is required for normal forebrain development and its targeted disruption in *Six3*<sup>-/-</sup> embryos results in forebrain defects that are comparable, although more severe, to those observed in *Hesx1*<sup>-/-</sup> mutants (Lagutin et al., 2003). *Six3*<sup>-/-</sup> mice have a severely truncated prosencephalon with rostral expansion of the expression domain of Wnt1. Further insight into the function of Six3 during pituitary development was obtained by the generation of *Six3*<sup>+/-</sup>; *Hesx1*<sup>Cre/+</sup> double heterozygous mice (Gaston-Massuet et al., 2008). These animals have marked growth failure, which is first detectable at weaning with severe gonadal and thyroid gland defects, and they die by week 5-6 of age. The Rathke's pouch of mutant embryos is initially expanded due to an increase in cell proliferation at E12.5 and up-regulation of Wnt/β-catenin signalling, leading to hypertrophy and dysmorphogenesis. The anterior pituitary gland appears bifurcated and occasionally ectopic in the nasopharyngeal cavity, but cell differentiation is not affected (Gaston-Massuet et al., 2008).

### **SOX family of transcription factors**

Transcription factors SOX2 and SOX3 are members of the SRY-related high mobility group (HMG) box (SOX) family; they are early markers of progenitor cells and their expression is down-regulated as cells differentiate (Pevny and Nicolis, 2009; Wegner, 2011; Pevny and Lovell-Badge, 1997). SOX2 and SOX3 are members of the SOXB1 subgroup, which has the highest degree of similarity to SRY, are expressed throughout the developing neural system, and have overlapping expression patterns, suggesting that there is a degree of redundancy in their functions (Collignon et al., 1996; Uwanogho et al., 1995)

## SOX2

SOX2 is expressed at the earliest stages of murine development, before gastrulation, in cells at the morula stage (E2.5) and in the inner cell mass of the blastocyst (E3.5) (Avilion et al., 2003). After implantation, SOX2 expression becomes restricted to the presumptive anterior ectoderm and by E9.5 it is expressed throughout the developing central nervous system (Collignon et al., 1996; Avilion et al., 2003; Episkopou, 2005; Wood and Episkopou, 1999), as well as in sensory placodes, inner ear, cochlea and in the developing lens, retina and optic nerve (Wood and Episkopou, 1999; Hume et al., 2007; Kiernan et al., 2005; Collignon et al., 1996; Avilion et al., 2003; Episkopou, 2005; Kamachi et al., 1998; Taranova et al., 2006; Dabdoub et al., 2008). In the developing CNS, expression of SOX2 persists in areas of proliferation in the ventricular zone, marking neural progenitors, whilst its expression is reduced in layers where cells are differentiated (Ferri et al., 2004). However, in areas such as the thalamus and septum, its expression is maintained postnatally. In the adult murine brain, SOX2-expressing cells are detected within the cortex and thalamus and within the adult neurogenic regions (such as the periventricular ependyma, subependymal areas and hippocampus) (Ferri et al., 2004).

SOX2 is expressed uniformly in Rathke's pouch and the infundibulum (Kelberman et al., 2006a) at E11.5. By E18.5, its expression is detected in proliferating cells of the dorsal zone and in scattered cells of the anterior pituitary (Kelberman et al., 2006a). SOX2 expression persists in a small population of cells of the adult murine pituitary, that line the pituitary cleft and maintain their potential to proliferate and differentiate into all pituitary cell types, therefore representing a progenitor/stem cell pool (Fauquier et al., 2008). This persistence of SOX2-expressing cells in the adult pituitary may be important for the

plasticity and dynamic response of the gland to fluctuating endocrine demands, its capacity to regenerate after trauma, or even account for the potential for tumour formation (Fauquier et al., 2008) (Vankelecom, 2007; Andoniadou et al., 2013).

*Sox2*<sup>-/-</sup> null embryos die shortly after implantation, however, heterozygous animals (*Sox2*<sup>*βgeo*/+</sup>) have a reduction in size and fertility (Avilion et al., 2003). Mutant mice exhibit an abnormal morphogenesis of the gland and at E12.5 almost one third have a bifurcated pouch, with subsequent extra clefts in some of the adult pituitaries. In comparison to wild type littermates the embryonic pituitaries at E18.5 are smaller and have significantly reduced numbers of somatotrophs and gonadotrophs, with reduced GH and LH content, although this reduction may also reflect the reduction in the size of the gland, or result from hypothalamic dysregulation associated with the disruption of SOX2 (Kelberman et al., 2006a).

Evaluation of the hormonal content postnatally, in two month old heterozygotes, showed that there was also reduction in GH and LH, the latter being significant in males, whilst the other pituitary hormones were variably affected. In some adult pituitaries there was reduction in TSH and PRL, whilst a significant reduction in ACTH was observed in SOX2 heterozygotes up to early postnatal life (P7) but not in adult pituitaries (Kelberman et al., 2006a). Transgenic animals with selective absence of SOX2 in the developing pituitary gland (*Hesx1*<sup>Cre/+</sup>;*Sox2*<sup>fl/fl</sup>) survive to birth but die perinatally (Jayakody et al., 2012). They exhibit severe anterior pituitary hypoplasia, detectable from E12.5-14.5, and by E18.5 there are remnants of the pituitary tissue embedded within the oropharyngeal ectoderm, whilst the intermediate and posterior lobes are normal (Jayakody et al., 2012). This suggests that the early induction of Rathke's pouch is not affected, but there is a failure of the developing



pituitary to expand. Mutant mice have a marked reduction in the expression of POU1F1 resulting in reduction in the differentiation of somatotrophs and thyrotrophs, whilst gonadotrophs are present, although in smaller numbers compared to the wild type pituitary (Jayakody *et al.* 2012). In addition, they exhibit a significant reduction in the number of hypothalamic GnRH neurons suggesting that the hypogonadal phenotype may be due to hypothalamic dysregulation (Jayakody *et al.*, 2012).

De novo *SOX2* mutations have been identified in a number of patients with eye defects, such as anophthalmia or microphthalmia, in association with hypogonadotropic hypogonadism, variable GH deficiency, with or without associated features including agenesis of the corpus callosum, hypothalamic hamartoma, slow progressing hypothalamo-pituitary tumours, esophageal atresia, spastic diplegia or sensorineural hearing loss (Schneider *et al.*, 2009; Sato *et al.*, 2007a; Kelberman *et al.*, 2006a; Kelberman *et al.*, 2008a; Alatzoglou *et al.*, 2011a; Williamson *et al.*, 2006; Bakrania *et al.*, 2007). Hypogonadotropic hypogonadism is a constant feature of the phenotype in humans, whereas growth hormone deficiency is rarer and other cell types do not seem to be affected

### **SOX3**

*Sox3* expression in mouse is first detected by E6.5 before the appearance of the primitive streak, throughout the epiblast, and in a band of extraembryonic ectoderm at the boundary of the embryonic and extraembryonic tissues (Wood and Episkopou, 1999). Subsequently it is expressed along the full length of the developing central nervous system, including the brain and spinal cord, in dividing undifferentiated neural progenitor cells (Bylund *et al.*, 2003). At E12.5, *Sox3* expression is detected in most cells of the neuroepithelium and its expression domain overlaps with *Sox2* and *Pax6* (Rogers *et al.*, 2013). High levels of

expression are noted in the ventral diencephalon, including the infundibulum and presumptive hypothalamus, where it exhibits a dynamic pattern of expression (Rizzoti et al., 2004; Rogers et al., 2013). At the earliest stages (E10.5-11.5) *Sox3* is expressed in periluminal progenitor/stem cells and the infundibulum, whilst by E14.5, expression is detected across the presumptive hypothalamus (Rogers et al., 2013). *Sox3* expression is also detected in the primordium of the subcommissural organ (E11.5), a small secretory organ at the dorsal midline of the caudal diencephalon, and its expression is maintained into adulthood (Lee et al., 2012). As neurogenesis progresses and during neuronal differentiation, *Sox3* expression is expected to be downregulated (Bergsland et al., 2011; Bylund et al., 2003). However, SOX3 expression in the hypothalamus is detected in subpopulations of immature neurons that will give rise to hypothalamic nuclei and even in some magnocellular neurons of the median eminence, suggesting that its expression is not restricted to progenitor/stem cells (Rogers et al., 2013).

Deletion of *Sox3* in mice results in animals with a complex phenotype including craniofacial abnormalities, hypopituitarism, midline defects, and a reduction in size and fertility (Weiss et al., 2003; Rizzoti et al., 2004). *Sox3* mutant mice of both sexes are born with expected frequency, with no evidence for embryonic lethality, and approximately one-third are viable and fertile with no gross abnormalities (Weiss et al., 2003; Rizzoti et al., 2004). Heterozygous females (*Sox3*<sup>-X</sup>) appear normal, although some display a mild craniofacial phenotype. However, approximately 40% of *Sox3* null mice (*Sox3*<sup>-/-</sup>) did not survive to weaning, and the most severely affected exhibited profound growth insufficiency and general weakness with craniofacial defects, including overgrowth and misalignment of the front teeth and an abnormal or absent pinna (Rizzoti et al., 2004; Rizzoti and Lovell-

Badge, 2007).

Study of the hypothalamo-pituitary axis of *Sox3* mutant mice revealed that these animals have a variable endocrine deficit, the extent of which correlates with body weight and, at two months of age, pituitary levels of GH, LH, FSH and TSH were lower compared to wild-type animals. The anterior pituitary lobe was smaller with the presence of an additional abnormal cleft disrupting the boundary between the anterior and intermediate lobes. Severely affected mice had additional midline abnormalities including dysgenesis of corpus callosum or failure of the hippocampal commissure to cross the midline. The embryonic pituitaries of *Sox3* mutants had an abnormally expanded and bifurcated Rathke's pouch (E11.5), which possibly resulted in the additional cleft observed in adult pituitaries. The evagination of the infundibulum was less pronounced and the presumptive hypothalamus was thinner and shorter with a marked reduction in cell proliferation (Rizzoti et al., 2004). At an earlier stage (E10.5) there was expansion of the expression domain of *Fgf8* and *Bmp4*, suggesting that *Sox3* may pattern the development of the pituitary indirectly, by restricting signalling domains and controlling the development of the infundibulum and hypothalamus and, in the absence of *Sox3*, the hypopituitary phenotype may be the result of the hypothalamic defect (Rizzoti et al., 2004).

The hypothalamic defects of *Sox3* null embryos are similar to the patterning abnormalities observed in mouse embryos lacking Shh in the prospective hypothalamus (*Shh*<sup>Δhyp</sup>). The *Shh*<sup>Δhyp</sup> mutants have anterior expansion of the expression of *Fgf10* and *Bmp4*, reduction of proliferation in the ventral diencephalon and failure of infundibular development (Zhao et al., 2012). In addition, embryos lacking *Sox3* and heterozygous for *Sox2* (*Sox3*<sup>Y/-</sup>;

Sox2<sup>+/-</sup>), have no detected expression of Shh or Six6 in the ventral midline of the anterior hypothalamus with expansion of the expression domain of *Fgf10*. The hypothalamic defect in Sox3<sup>Y/-</sup>; Sox2<sup>+/-</sup> embryos is consistent with the notion that members of the SoxB1 family function in a dose dependent manner to regulate the expression of Shh in the ventral midline of the anterior hypothalamus (Zhao et al., 2012).

In humans *SOX3* is implicated in the etiology of X-linked hypopituitarism with a highly variable phenotype in patients with gene dosage abnormalities (Laumonnier et al., 2002; Woods et al., 2005). Over- and under-dosage of *SOX3* results in isolated growth hormone deficiency or combined pituitary hormone deficiencies, with or without variable mental retardation and learning difficulties. The anterior pituitary may be hypoplastic with an ectopic/undescended posterior pituitary or other abnormalities including persistence of the craniopharyngeal canal (Woods et al., 2005; Laumonnier et al., 2002; Alatzoglou et al., 2011b; Burkitt Wright et al., 2009; Alatzoglou et al., 2014; Takagi et al., 2013).

### **Genes regulating cellular differentiation**

A number of transcription factors are implicated in the specification and expansion of the hormone-producing cells of the anterior pituitary and, for many of them, genetic changes result in the development of hypopituitarism in humans (Table 1). However, the pathway to cell specification is not strictly a linear one, with one factor being solely and directly responsible, but additional factors and modulators have an effect on cell specification and network formation. For example, in the case of somatotrophs, in addition to the role of the cell-type specific factors (*PROPI*, *POU1F1*), other extracellular factors (hormones, neuropeptides, signalling molecules), diverse molecular pathways and the developing

pituitary capillary network are also required for their establishment, proliferation, maintenance and organisation. There is now increasing evidence that oestrogens, glucocorticoids, chemokines and the dopaminergic system are important for the differentiation, function and maintenance of somatotrophs (Vakili and Cattini, 2012; Nogami and Hisano, 2008; Schaeffer et al., 2011; Mazziotti and Giustina, 2013; Deneff, 2008). In addition, changes in chromatin structure may affect the subsequent binding of transcription factors and direct cellular specification. The detailed study of PAX7 has revealed a remarkable example of a transcription factor that binds gene enhancers either opening the chromatin to permit binding of TPIT (TBX19), or suppressing binding, therefore acting as selector for the differentiation into corticotrophs rather than melanotrophs (Budry et al., 2012).

## **PROP1**

PROP1 (Prophet of PIT-1) is the earliest expressed pituitary-specific transcription factor. This paired-like homeodomain transcription factor is initially detected in the dorsal portion of Rathke's pouch at E10-10.5, its expression peaks at E12.0 and becomes undetectable by E15.5 (Ward et al., 2005; Kelberman et al., 2009; Olson et al., 2006). Depending on the associated co-factors, PROP1 is required for both activation and silencing of genes implicated in pituitary development. The onset of *PROP1* expression is required for the emergence of the *POU1F1* lineage (somatotrophs, lactotrophs and thyrotrophs), activation of *NOTCH2* with emergence of the gonadotroph lineage, and repression of *HESX1* and *OTX2* (Raetzman et al., 2006) (Dasen et al., 2001) (Mortensen et al., 2011). The temporal regulation of PROP1 expression is important as its premature expression in Rathke's pouch

leads to agenesis of the anterior pituitary, probably by early repression of *Hesx1* (Olson et al., 2006), whilst its persistent expression delays the differentiation of gonadotrophs (Cushman et al., 2001). By expressing a constitutively active form of  $\beta$ -catenin throughout the pouch, Olson et al, showed that the interaction between  $\beta$ -catenin and PROP1 mediates this dual role of concomitant repression of *Hesx1* and activation of *Pou1f1*. This complex, along with different co-factors, binds directly to cis-regulatory elements of *Hesx1* and *Pou1f1* inducing repression and activation of these genes respectively (Olson et al., 2006). The Ames dwarf mouse has a naturally occurring mutation within the homeodomain of *Prop1* that results in an eight-fold reduction in DNA-binding activity (Sornson et al., 1996). These animals exhibit severe proportional dwarfism and infertility, with GH, TSH and PRL deficiency and reduced gonadotropin expression correlating with low plasma LH and FSH. The anterior pituitary gland is reduced in size by about 50%, displaying an abnormal looping appearance (Ward et al., 2005; Ward et al., 2006). In *Prop1* deficient mice (*Prop1*<sup>-/-</sup>) progenitor cells of the periluminal area fail to migrate from the proliferative zone and differentiate, resulting in a dysmorphic embryonic pituitary, whilst postnatally, a late wave of apoptosis may explain the subsequent pituitary hypoplasia (Ward et al., 2005). This failure of normal cell differentiation occurs in parallel with a reduction in the normal vascularisation of the pituitary and an abnormal pattern of expression of vascular endothelial factors (Ward et al., 2006).

In humans, mutations in *PROPI* are the commonest genetic cause of combined pituitary hormone deficiency. Recessive mutations are associated with GH, prolactin and TSH deficiencies, in addition to ACTH and gonadotropin deficiencies that may be present at a young age, or develop over time. The anterior pituitary may be hypoplastic or enlarged and

the size can wax and wane over time (Bottner et al., 2004; Pfaffle and Klammt, 2011; Vallette-Kasic et al., 2001; Voutetakis et al., 2004; Turton et al., 2005a).

## **POU1F1**

POU1F1 (previously referred to as PIT1) is a pituitary specific transcription factor that belongs to the POU-homeodomain family. POU1F1 controls the terminal differentiation and expansion of the POU1F1-dependent cell lineages (somatotrophs, lactotrophs and thyrotrophs) and is required for the transcriptional regulation of *GHI*, *PRL*, *TSH $\beta$* , *GHRHR* and the repression of gonadotroph cell fate. POU1F1 is expressed late during pituitary development (E13.5), reaches its peak in the differentiated somatotrophs (E16) and its expression persists throughout adulthood. Once its expression has reached a critical threshold, autoregulation of *POU1F1* is required in order to sustain its expression (Andersen and Rosenfeld, 1994; Dasen and Rosenfeld, 2001; Kelberman et al., 2009).

Two dwarf mice strains (Snell and Jackson) show naturally occurring disruption of *POU1F1*. The Snell dwarf mouse has a recessive mutation affecting DNA binding, whilst the Jackson mouse has a chromosomal rearrangement affecting the expression of *POU1F1*. They both exhibit similar phenotypes with postnatal, but not embryonic, anterior pituitary hypoplasia and GH, TSH and PRL deficiencies (Kelberman et al., 2009; Prince et al., 2011). This suggests that POU1F1 is necessary for the emergence but not the expansion of somatotrophs, thyrotrophs and lactotrophs; postnatally, hypothalamic and other factors stimulate the expansion of these lineages (Nogami and Hisano, 2008; Gahete et al., 2009; Vakili and Cattini, 2012; Ansell et al., 2007).

Independent of its DNA binding properties, POU1F1 inhibits transcription factor GATA2 in gonadotrophs, thus preventing their differentiation. However, in thyrotroph cells

POU1F1 and GATA2 act synergistically to promote thyrotroph fate (Dasen et al., 1999). In somatotrophs, the POU1F1 target gene *Math3* is required for the terminal differentiation and maturation of growth hormone producing cells (Zhu et al., 2006).

Autosomal recessive and dominant *POU1F1* mutations are associated with growth hormone deficiency, manifesting as a severe growth deficit in the first years of life, prolactin and variable TSH deficiencies, and a hypoplastic anterior pituitary (Turton et al., 2005b; Bas et al., 2015; Kelberman and Dattani, 2009; Pfaffle and Klammt, 2011). However, there are reports of adult patients with isolated growth hormone deficiency and a hypoplastic anterior pituitary who, although homozygous for a *POU1F1* mutation (p.E230K), did not develop other pituitary hormone deficiencies (Turton et al., 2005b).

## **IGSF1**

Murine immunoglobulin Superfamily Member 1 (*Igsf1*) is expressed in thyrotrophs, lactotrophs, and somatotrophs. In addition expression is seen in the Leydig and germ cells, with trace levels in Sertoli cells in both murine and human testes (Sun Y et al., 2012; García M et al., 2017). *Igsf1*-deficient male mice (*Igsf1<sup>-/-</sup>*) have increased body mass and display diminished pituitary and serum TSH, as well as triiodothyronine, concentrations, with lower levels of pituitary TRH receptor expression (Sun Y et al., 2012). Recent studies have shown that *Igsf1* mouse models with a loss-of-function mutation in the C-terminal domain have diminished TSH subunit transcripts, as well as reduced TSH and TRH protein expression (Turgeon MO et al., 2017). IGSF1 indirectly reduces *FSHB* expression in gonadotrophs, via down-regulation of the activin-Smad pathway. Conversely, IGSF1 stimulates transcription of *TRHR* by negative modulation of the TGFβ1-Smad signalling pathway, thereby enhancing TSH synthesis and biopotency (Garcia M et al., 2017). Patients



with *IGSF1* mutations manifest an X-linked form of central hypothyroidism, which mimics the murine model of *Igsf1* deficiency, with macroorchidism usually being a prominent feature of the phenotype (Sun Y et al., 2012; Garcia M et al., 2017), albeit not in every case (Hughes JN et al., 2016). Intriguingly, mild hypothyroidism may occur in heterozygous *IGSF1* female carriers (Joustra SD et al., 2013).

## GATA2

GATA2 is member of a family of transcription factors that are characterised by the presence of at least one N-terminal transactivation domain and a zinc finger DNA-binding domain. Depending on the tissue, GATA2 functions as a stem cell maintenance factor or as a promoter of cellular differentiation (DePater E. et al., 2013). In the developing pituitary, expression of GATA2 is first detected in the ventral Rathke's pouch (E10.5), where it is induced by BMP2 along with  $\alpha$ -GSU, marking the prospective and definitive thyrotrophs and gonadotrophs, and its expression is maintained in the adult pituitary (Gordon et al., 1997). In the cascade of pituitary development, PITX2 activates the transcription of *GATA2* (Suh et al., 2002). GATA2 activates the *Cga* promoter, regulating expression of  $\alpha$ -GSU which is the common  $\alpha$ -glycoprotein subunit of TSH, LH and FSH (Gordon et al., 2002) and acts synergistically with POU1F1 and SF1 (*Nr5a1*) to activate the transcription of *Tsh $\beta$*  and *Lh $\beta$*  respectively (Ohba et al., 2011).

Ectopic expression of *GATA2* under the control of the *POU1F1* promoter results in dorsal expansion of gonadotrophs at the expense of cells of the POU1F1 lineage (Dasen et al., 1999). On the other hand, expression of a dominant-negative form of GATA2 in which the

N-terminal transcriptional activation domain is replaced with the engrailed repressor domain, causes loss of gonadotrophs and reduction of thyrotrophs, with expansion of the *POU1F1* expression domain (Dasen et al., 1999). Further evidence for the role of GATA2 in the determination of gonadotrophs and thyrotrophs came from the study of mice with targeted loss of *GATA2* in the anterior pituitary (Charles et al., 2006). These animals have a reduced number of thyrotrophs at birth and reduced function of gonadotrophs and thyrotrophs in adult life. The pituitary defect results in a transient reduction in body weight, which is only apparent in male animals that are both viable and fertile as adults. The recovery of the number of thyrotrophs and of the function of gonadotrophs in adult mice suggests that GATA2 may be important, but not critical, for the function of the adult pituitary (Charles et al., 2006).

#### **SF1**

In the most ventral aspect of the anterior pituitary, high levels of GATA2 restrict expression of *POU1F1* and, in its absence, GATA2 induces transcription factors that will determine gonadotroph differentiation, including Steroidogenic factor-1 (*SF1*).

Steroidogenic factor-1 (encoded by *Nr5a1*), is a zinc-finger nuclear receptor that regulates a number of genes involved in sex determination, steroidogenesis and reproduction, including *CGA*, *LH $\beta$* , *FSH $\beta$*  and *GnRHR* (Zhao et al., 2001b; Achermann et al., 2001; Achermann et al., 1999). It is expressed in gonadotroph cells (E13.5), the ventromedial hypothalamus as well as in the developing gonads and adrenal glands (Zhao et al., 2001a). In the developing pituitary GATA2 induces expression of *SF1* after the initiation of *CGA* expression but prior to that of *LH $\beta$*  and *FSH $\beta$*  (Dasen et al., 1999).

Deletion of *Sfl* in mice causes agenesis or apoptosis of the adrenal glands and gonads early in development, resulting in a postnatal phenotype associated with gonadal dysgenesis/agenesis, retained Müllerian structures and impaired androgenisation in males, with abnormal gonadotropin release and late-onset obesity (Luo et al., 1994; Majdic et al., 2002). Mice with a conditional *Sfl* deletion within the pituitary have gonadal hypoplasia with a dramatic decrease in pituitary gonadotropin expression and fail to develop normal secondary sexual characteristics, whilst the adrenal glands and hypothalamus are unaffected. In these animals, supraphysiological doses of GnRH can result in expression of LH $\beta$  suggesting that, in the absence of *Sfl*, another co-factor (*Egfr*) can activate LH $\beta$  (Zhao et al., 2001a). In this context, *Sfl* is necessary for maturation of gonadotrophs but not for the specification of their cell fate. In humans, heterozygous or rare homozygous *SF1* mutations have been reported in 46,XY patients with a wide spectrum of phenotypes ranging from adrenal and gonadal failure, through to mild gonadal dysgenesis and impaired androgenisation with normal adrenal function associated with SF1 haploinsufficiency (Lin L et al., 2006; Lin L et al., 2007; Philibert et al., 2007).

#### **TIPT (TBX19)**

TPIT (more recently referred to as TBX19) is a member of the T-Box family of transcription factors. It is exclusively expressed in the developing pituitary at E12.5 in the most ventral part of Rathke's pouch in proopiomelanocortin-positive cells (POMC-positive) and then in corticotrophs and melanotrophs, where expression is maintained in the adult gland (Lamolet et al., 2001). TBX19 acts synergistically with PITX1 to activate the *POMC* promoter by binding to contiguous sites within the same regulatory element

(Pulichino et al., 2003b; Liu et al., 2001), while it actively suppresses differentiation of gonadotrophs by antagonising SF1 (*Nr5a1*) (Pulichino et al., 2003b; Davis et al., 2010).

*TBX19* knockout mice almost completely lack *POMC*-expressing cells, resulting in severe ACTH and glucocorticoid deficiencies, adrenal hypoplasia and defects in pigmentation, whilst the intermediate lobe is hypoplastic (Lamolet et al., 2001; Pulichino et al., 2003b). Most of the cells “destined” to express *POMC* instead remain blocked in a non-cycling precursor state (Bilodeau et al., 2009), although a few appear to differentiate to *POMC*-expressing, and about 10% adopt an alternative fate and differentiate to gonadotrophs, probably due to the lack of the antagonistic action of *TBX19* on SF1 (Pulichino et al., 2003b). In these embryos the transient expression of *NeuroDI* is not affected, pre-corticotrophs and pre-melanotrophs are present, and both cell lineages are initially specified in normal numbers, suggesting that *TBX19* is required for the maturation and maintenance of both populations (Pulichino et al., 2004; Pulichino et al., 2003a). Premature stop codons, aberrant splicing and chromosomal deletions have been identified in patients with isolated ACTH deficiency (Couture C et al., 2012), with complete or severe loss of function in DNA-binding and/or transactivation in over two thirds of patients (Lamolet B et al., 2001; Unal E et al., 2018). Compound heterozygosity in *TBX19*, with a novel frameshift in combination with a previously described mutation, has been described in a patient with isolated ACTH deficiency combined with recurrent respiratory tract infections. The authors concluded that patients with unexplained recurrent infections should be tested for adrenal insufficiency to prevent a delay in diagnosis and possible fatal consequences (Akcan N et al., 2017).

## PAX7

Pituitary corticotrophs are the first cell type to reach terminal differentiation; however, relatively little is known about the factors that direct cell specification towards the development of anterior lobe corticotrophs rather than the intermediate lobe melanotrophs. Although both cell lineages express *POMC*, expression is regulated by different signalling pathways and they process differentially the *POMC*-protein precursor: corticotrophs express only prohormone convertase-1 (*PC1*) and process *POMC* into ACTH, whilst melanotrophs express both *PC1* and *PC2* and further process ACTH into  $\alpha$ MSH (Dores et al., 2014; Cortes et al., 2014; Kim et al., 2014).

More recently, *PAX7* has been identified as a critical factor that determines the melanotroph cell fate (Budry et al., 2012). *PAX7* belongs to a family of nine paired-domain DNA-binding transcription factors that exhibit complex and variable patterns of DNA recognition, exert their action through recruitment of chromatin remodelling complexes, and act as transcriptional activators or repressors (Mayran et al., 2015). In murine pituitary, *PAX7* has a highly restricted expression only in the intermediate lobe of the embryonic pituitary, persisting in the adult pituitary.

At E14.5 in murine embryogenesis, *TBX19* expression is only detected in the corticotrophs. At E15.5, most cells in the intermediate lobe express *PAX7*, with a subset expressing *TBX19*, suggesting that *PAX7* expression precedes *TBX19* in the intermediate lobe. By E16.5, most cells of the intermediate lobe co-express *PAX7* and *TBX19* correlating with expression of *POMC* in both the anterior and intermediate lobes. *PC2* expression in the intermediate lobe starts at E15.5; therefore, the onset of *PAX7* expression in the

intermediate lobe melanotrophs precedes their terminal differentiation (defined by *TBX19*, *POMC*, and *PC2* expression) (Budry et al., 2012). Interestingly, depending on the genetic background, *PAX7*<sup>-/-</sup> knock out mice die perinatally or can survive to a month of age (Kuang et al., 2006). In these animals *TBX19* expression is unaffected in the intermediate lobe cells but there is loss of melanotroph-specific gene expression (*POMC*, *PC2*, and *DRD2*) and, melanotrophs switch to a corticotroph cell fate (Budry et al., 2012). *PAX7* is not solely responsible for the differentiation of melanotrophs, as it requires *TBX19*; however, it is critical for the activation of melanotroph-specific genes and the repression of corticotroph-specific genes, thus acting as a selector switch between the two POMC-lineages.

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#### **KCNQ1**

The *KCNQ1* paternally imprinted gene, encodes a voltage-gated ion channel Kv7.1 subunit, and is associated with heart defects such as cardiac arrhythmia syndromes (Wang et al., 1996). Phenotypically variable patients with maternally inherited gingival fibromatosis, hypopituitarism, and accompanying mild craniofacial dysmorphic features have been reported to have missense mutations in *KCNQ1* (Tommiska J, et al., 2017). GHD was identified in all mutation-positive patients, whereas gonadotrophin, TSH and ACTH deficiencies were more variable. Transcripts of the gene are expressed in postnatal mouse and human somatotroph and gonadotroph cells, in hypothalamic GHRH neurons during murine development, and in the human hypothalamus (Tommiska J, et al., 2017). These data, together with previous reports of voltage-gated potassium channel currents in

pituitary cells, suggest that ion channels may be imperative regulators of pituitary function in humans (Stojilkovic SS et al., 2010), (Stojilkovic SS et al., 2017) and (Xu R et al., 1999).

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## **Conclusion**

Over the last few years there has been an explosion in the knowledge of the genetic factors that orchestrate the development of the pituitary gland. Transgenic animal models and detailed study of human phenotypes have provided an insight in the genes involved in hypothalamo-pituitary development, whilst technological advances and next generation sequencing have vastly improved our understanding and quest for mutations. What is becoming clearer, however, is that pituitary development is far more complex than a linear sequential expression of transcription factors and signalling molecules that lead to cell specification and organ commitment. The developed anterior pituitary is a dynamic organ, with hormone-producing cells organised in homotypic networks that confer the plasticity of the gland. Study of factors involved in diverse processes such as the vascularity of the gland, cell cycle regulation, the role of pituitary stem/progenitor cells and the identification of novel genes involved in pituitary development, will further our knowledge in the years to come.

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**Table 1: Genetic factors implicated in syndromic hypopituitarism and combined hormone deficiencies**

Gene	Mutation	Hormone Deficits	MRI	Additional Features	References
<b><i>Syndromic Hypopituitarism</i></b>					
<i>HESX1</i>	AD	Panhypopituitarism; GHD; GHD with evolving ACTH and TSH deficiency	APH, EPP, ONH, ACC; Reported normal AP in association with EPP and ONH (p.S170L)	Septo-optic dysplasia and its variants	(Thomas et al., 2001b) (Coya et al., 2007; Thomas et al., 2001b; Corneli et al., 2008)
	AR	Panhypopituitarism; GH, LH, FSH, evolving ACTH and TSH deficiencies	APH, EPP, ONH, ACC; Reported normal ON in association with APH and EPP (p.I26T); pituitary aplasia with normal PP and normal ON; pituitary aplasia with normal PP and optic nerve coloboma		(Dattani et al., 1998b; Carvalho et al., 2003; Sobrier et al., 2006a; Sobrier et al., 2005)
<i>LHX3</i>	AR	GH, TSH, PRL, LH, FSH deficiencies; reported ACTH deficiency	APH, enlarged/cystic AP Normal PP and stalk	Limited neck rotation, short cervical spine, sensorineural deafness.	(Netchine et al., 2000; Bhangoo et al., 2006; Pfaeffle et al., 2007; Rajab et al., 2008)
<i>LHX4</i>	AD	Panhypopituitarism; GHD with variable TSH, ACTH, gonadotropin deficiencies	APH, PP normal or EPP, Chiari malformation	Cerebellar abnormalities	(Machinis et al., 2001; Tajima et al., 2007; Pfaeffle et al., 2008)
	AR	ACTH, TSH, PRL and probable GH deficiencies	Aplastic AP, EPP		Lethality in the first weeks of life with severe sepsis, poor tone, lung atelectasis, mid facial hypoplasia, low set ears.
<i>SOX2</i>	AD	LH and FSH deficiency, rare GHD	APH, thin corpus callosum; hippocampal abnormalities; hypothalamic hamartoma; slow progressing hypothalamo-pituitary tumour	Anophthalmia bilateral/unilateral; spastic diplegia, developmental delay; esophageal atresia; sensorineural deafness	(Kelberman et al., 2006b; Kelberman et al., 2008b; Sato et al., 2007b; Alatzoglou et al., 2011a; Schneider et al., 2009)

<i>SOX3</i>	XL	Panhypopituitarism; GH, TSH, ACTH, LH, FSH deficiencies; isolated GHD	APH, EPP; persistent craniopharyngeal canal	Variable mental retardation	(Woods et al., 2005; Laumonnier et al., 2002; Alatzoglou et al., 2011b; Alatzoglou et al., 2014; Burkitt Wright et al., 2009)
<i>OTX2</i>	AD	Isolated or partial GHD; GH, TSH, ACTH, LH, FSH deficiencies	Normal pituitary; APH, EPP, Chiari malformation	Anophthalmia bilateral/unilateral retinal dystrophy; normal eye phenotype	(Diaczok et al., 2008b) (Tajima et al., 2009b; Dateki et al., 2010) (Ashkenazi-Hoffnung et al., 2010b)

***Rarer causes of syndromic hypopituitarism***

<i>ARNT2</i>	AR	DI, ACTH, GH, TSH deficiencies	APH, absent PP, thin corpus callosum; frontal and temporal lobe hypoplasia, large Sylvian fissure	Microcephaly, fronto-temporal hypoplasia, seizures, visual impairment, renal tract abnormalities	(Webb et al., 2013)
<i>GLI2</i>	AD	Panhypopituitarism, GH, TSH, ACTH, LH, FSH deficiencies; isolated GHD	APH, EPP or normal PP, hypoplastic CC, cavum septum pellucidum.	Holoprosencephaly; midfacial defects, cleft lip/palate, single central incisor; postaxial polydactyly, optic nerve hypoplasia	(Roessler et al., 2003; Gregory et al., 2015a; Franca et al., 2010; Cohen, 2012) Arnold et al 2015
<i>PITX2</i>	AD	Reduced GH concentration	Hypoplasia of sella turcica	Axenfeld-Rieger syndrome (malformation of the anterior segment of the eye, dental hypoplasia, a protuberant umbilicus, and brain abnormalities)	(Tumer and Bach-Holm, 2009; Wang et al., 2003)
<i>FGF8</i> *	AD	Borderline peak GH concentration	Absent corpus callosum, ONH	Moebius syndrome, spastic diplegia, developmental delay	(McCabe et al., 2011; Raivio et al., 2012)
	AR	DI, TSH, ACTH deficiencies.	Semilobar HPE; bulky AP, normal PP	Microcephaly, micrognathia, high arched palate	(McCabe et al., 2011)
<i>FGFR1</i> *	AD	GH, TSH, ACTH, LH, FSH deficiencies, DI	APH, EPP, stalk thin or normal, agenesis of corpus callosum	Cleft lip/palate, brachydactyly, central incisor	(Correa et al., 2015; Raivio et al., 2012)

<i>PROKR2*</i>	AD	GH, ACTH, TSH, LH, FSH deficiencies; GHD	APH or normal AP, EPP, absent stalk, dysgenesis of corpus callosum	Facial asymmetry, schizencephaly, cerebellar hypoplasia, Hypoplastic optic discs and optic nerves	(McCabe et al., 2013; Correa et al., 2015; Raivio et al., 2012)
<b><i>Combined Pituitary hormone deficiencies</i></b>					
<i>POU1F1</i>	AD, AR	GH, PRL and TSH deficiencies; TSH deficiency may present early or develop much later	APH or normal AP, normal PP and infundibulum, but with no extrapituitary abnormalities	-	(Tenenbaum-Rakover et al., 2011; Turton et al., 2005b; Pfaffle et al., 1992; Carlomagno et al., 2009; Inoue et al., 2012)
<i>PROPI</i>	AR	GH, TSH, PRL LH, FSH, evolving ACTH deficiencies; variable time of onset and severity of pituitary deficiencies	APH, normal or enlarged AP that may change over time; normal PP and stalk	-	(Deladoey et al., 1999; Fluck et al., 1998; Turton et al., 2005a; Voutetakis et al., 2004; Vallette-Kasic et al., 2001; Bas et al., 2015)

ACC, Agenesis of corpus callosum; ACTH, Adrenocorticotrophic hormone; AD, Autosomal Dominant; APH, Anterior pituitary hypoplasia; AR, Autosomal recessive; DI, Diabetes insipidus; EPP, Ectopic posterior pituitary; FSH, follicle-stimulating hormone; GH, growth hormone; GHD, growth hormone deficiency; LH, luteinizing hormone; ONH, Optic nerve hypoplasia; PP, posterior pituitary; TSH, thyroid-stimulating hormone; XL, X-linked

\* Mutations in these genes are more common in patients with Kallman syndrome (hypogonadotropic hypogonadism in association with anosmia). These phenotypes denote genetic overlap between hypopituitarism/SOD and Kallman syndrome

## Figure Legends

### Figure 1

Schematic presentation of the stages of pituitary development in rodents: (a) Oral ectoderm (b) Rudimentary pouch (c) Definitive pouch (d) Adult pituitary gland.

The close contact between the developing Rathke's pouch (red) and the infundibulum (yellow) is maintained throughout and is important for the normal morphogenesis of the gland.

I infundibulum; NP neural plate; N notochord; PP pituitary placode; OM oral membrane; H heart; F forebrain; MB midbrain; HB hindbrain; RP Rathke's pouch; AN anterior neural pore; O oral cavity; PL posterior lobe; OC optic chiasm; P pontine flexure; PO pons; IL intermediate lobe; AL anterior lobe; DI diencephalon; SC sphenoid cartilage.

*Figure from Sheng and Westphal, Trends in Genetics 1999;15:236-240 with permission (193).*

## **Figure 2**

Pituitary organogenesis during human embryonic development

A: Midline sagittal hematoxylin and eosin-stained section of a Carnegie stage (CS) 13 embryo, at approximately 5 weeks of development, showing the invagination of the oral ectoderm to form Rathke's pouch (*arrow*). B: Sagittal section of CS14 embryo showing the developing Rathke's pouch coming into contact with the overlying neuroectoderm.

C: Sagittal section of CS15 embryo showing the definitive Rathke's pouch becoming separated from the oral ectoderm.

D: at CS 17 the definitive Rathke's pouch is fully separated from the oral ectoderm and maintains contact with the neural ectoderm of the diencephalon.

Rp, Rathke's pouch; oe, Oral ectoderm; Di, Diencephalon. Scale bars: A and D, 300  $\mu\text{m}$ ; B and C, 100  $\mu\text{m}$ .

*Figure taken from Kelberman et al, Endocr Rev 2009;30:790-829 with permission (118).*



### Figure 3

Schematic cascade of transcription factors and signalling molecules during pituitary development. The terminal differentiation of the anterior pituitary cell types is the result of complex interactions between extrinsic signalling molecules and transcription factors (*HESX1*, *SOX2*, *SOX3*, *OTX2*, *LHX3*, *LHX4*, *GATA2*, *ISL1*, *PROP1*, *POU1F1*). Mutations in the early developmental transcription factors result in pituitary hormone deficiencies in association with structural pituitary abnormalities and/or extra-pituitary defects (ie ocular or skeletal abnormalities, midline and other central nervous system defects, sensorineural deafness, developmental delay). Mutations in the later transcription factors result in combined or isolated pituitary hormone deficiencies, depending on the factor affected.

*Figure taken from Kelberman et al, Endocr Rev 2009;30:790-829 with permission (118).*

### Figure 4

*Sox2* expression and abnormal morphogenesis of the pituitary gland in *Sox2* heterozygote mice. (A) Sagittal section of an 11.5-dpc wild-type embryo hybridized to *Sox2*, shows expression in both the CNS and Rathke's pouch. (B and C) Sagittal sections of 12.5 dpc wild-type (B) and *Sox2* heterozygous (C) embryos demonstrate bifurcation of the pouch in the mutant embryo. (D and E) Pituitary transverse sections of 5-week-old wild-type (D) and *Sox2* heterozygous mice (E). There is presence of an extra cleft in the *Sox2* heterozygous pituitary (arrow).

The bifurcated Rathke's pouch is reminiscent of the abnormal shape in mouse mutants with disruption of *Hesx1* (Dattani et al., 1998), *Sox3*, *Wnt5a* (Cha et al., 2004) or *Shh* (Zhao et al., 2012).

HYP, presumptive hypothalamus; RP, Rathke's pouch. Scale bars: 0.1 mm.

*Figure taken from Kelberman et al JCI 2006;116(9):2442-2445 with permission (117).*

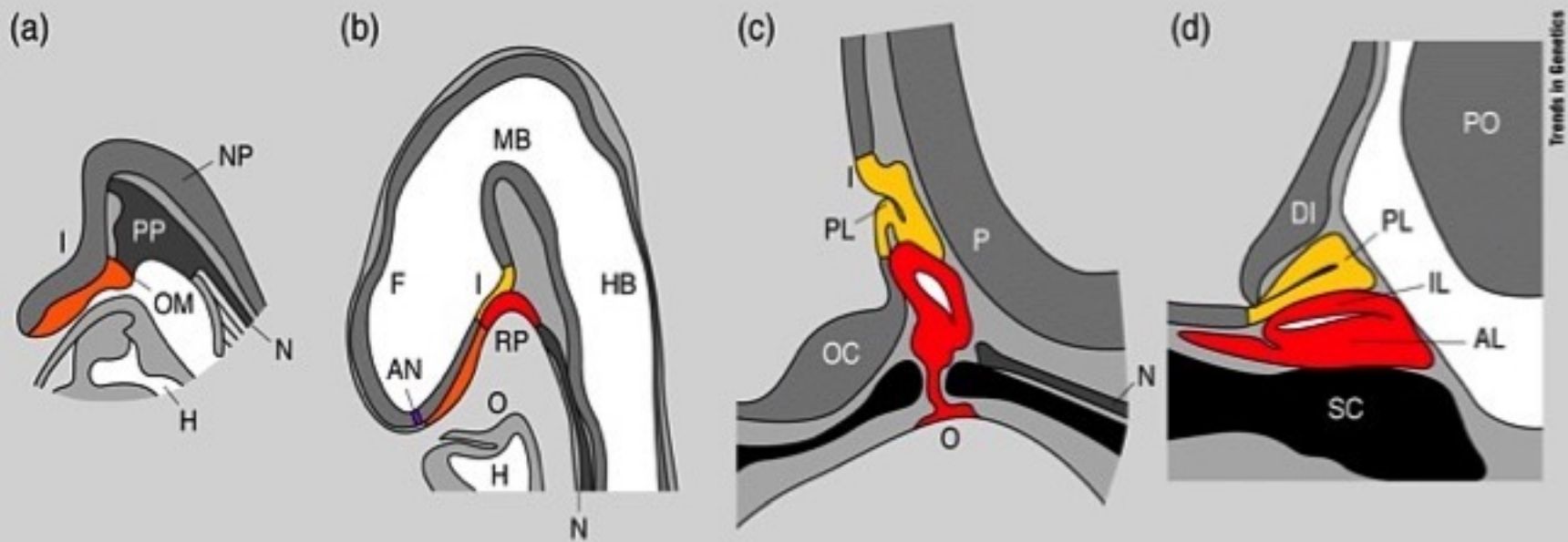
## Figure 5

Pituitary MRI of patients with congenital hypopituitarism (B-F) compared to normal MRI appearance (A)

- A. Midsagittal MRI scan of a normal child, showing a well-formed corpus callosum (CC), normal optic chiasm (OC), and the posterior pituitary (PP), which appears as a bright spot within the sella turcica.
- B. Sagittal MRI scan of two siblings with a homozygous p.R160C mutation in *HESX1*. In the first sibling (i) the splenium of the corpus callosum is more hypoplastic than the rest of the structure and the posterior pituitary is partially descended as compared with the other sibling (ii) who has a severely hypoplastic corpus callosum, ectopic posterior pituitary, and lack of visible pituitary stalk (PS).
- C. Coronal and sagittal MRI scans from one patient [panels (i) and (ii)] and sagittal scan from a second patient (iii) with *SOX3* duplication showing anterior pituitary (AP) hypoplasia, partial hypoplasia of the infundibulum (I) in the first patient and complete absence in the second, and an ectopic posterior pituitary which is more severe in the second patient.
- D. MRI scans from patient with *SOX2* mutations. Sagittal section from patient with c60insG mutation showing anterior pituitary (ap) hypoplasia with normal posterior pituitary (pp) and infundibulum (i) and a hypothalamic hamartoma (h).
- E. Sagittal MRI scan in patient with compound heterozygosity for p.E230K and p.R172Q mutations in *POU1F1*, showing hypoplasia of the anterior pituitary gland with a normal posterior pituitary and infundibulum.

- F. Sequential MRI scanning of a patient with a 13-bp deletion (c.112\_124del13) in *PROP1* reveals waxing and waning of a pituitary mass (*arrow*); (i) on initial presentation, (ii) after 4 months, (iii) after 12 months, and (iv) 21 months after initial MRI.

*Figure taken from Kelberman et al, Endocr Rev 2009;30:790-829 with permission (118).*



Oral ectoderm → Rudimentary pouch → Definitive pouch → Pituitary gland

Rat	8.5	11	14.5	19.5
Mouse	8.0–8.5	9.5	12	17

Embryonic  
days

