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# Notch and Hedgehog in the thymus/parathyroid common primordium: Crosstalk in organ formation

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ABSTRACT

The avian thymus and parathyroids (T/PT) common primordium derives from the endoderm of the third and fourth pharyngeal pouches (3/4PP). The molecular mechanisms that govern T/PT development are not fully understood. Here we study the effects of Notch and Hedgehog (Hh) signalling modulation during common primordium development using *in vitro*, *in vivo* and *in ovo* approaches. The impairment of Notch activity reduced Foxn1/thymus-fated and Gcm2/Pth/parathyroid-fated domains in the 3/4PP and further compromised the development of the parathyroid glands. When Hh signalling was abolished, we observed a reduction in the *Gata3*/*Gcm2*- and *Lfng*-expression domains at the median/anterior and median/posterior territories of the pouches, respectively. In contrast, the *Foxn1* expression-domain at the dorsal tip of the pouches expanded ventrally into the *Lfng*-expression domain. This study offers novel evidence on the role of Notch signalling in T/PT common primordium development, in an Hh-dependent manner.



## 1. Introduction

The parathyroid glands and the thymus are organs with distinct functions, carried out mainly by epithelial cells which have a common embryological origin, that is, the endoderm of the pharyngeal pouches (PP). The epithelia of these organs in the avian model originate from the third and fourth PP (3/4PP) endoderm. It is worth noting, that in mammals the thymic epithelium derives from the 3PP endoderm (Farley et al., 2013; Gordon et al., 2001) and in mouse and human the epithelium of parathyroids derives from the 3PP and 3/4PP, respectively. The main function of the parathyroid endocrine epithelium is to secrete a peptidic hormone, the parathyroid hormone (Pth), essential for the regulation of calcium and phosphate homeostasis (Potts, 2005). In the thymus, the epithelial cells establish complex interactions with the developing lymphocytes to produce self-restricted and self-tolerant T-cells, which generate central immune tolerance.

Parathyroid and thymic organogenesis starts with the budding off and outgrowth of rudiments from pouches of the foregut endoderm (Manley and Condie, 2010), accompanied by the lining of neural crest-derived connective tissues (Grevellec and Tucker, 2010). These early steps involve pouch patterning and the establishment of a common primordium (Manley and Condie, 2010) in which the distinct parathyroid and thymic prospective domains, can be distinguished by the expression of the organ-specific genes, Gcm2 (Glial cells missing 2) and Foxn1, respectively.

In avian embryos, *Gcm2* transcripts were first detected by RT-PCR in isolated quail (q) endoderm at embryonic day (E) 2.5 (25–30 somite-stage) (Neves et al., 2012). However, *in situ* expression of *Gcm2* has only been observed in the anterior domain of the 3PP and 4PP at Hamburger and Hamilton Stage 18 (HH18) and HH22, respectively (Okabe and Graham, 2004). This temporal sequence of *Gcm2* expression follows the chronological formation of the pouches. As development proceeds, Pth is upregulated in the developing glands. In avian, *Pth* expression was first observed *in situ* at chicken (c) E5.5 (HH28) (Grevellec et al., 2011). In Gcm2

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homozygous null mutant mice, the expression of *Pth* is not initiated and no parathyroid glands are formed (Günther et al., 2000; Liu et al., 2007).

The transcription factor of the winged helix/forkhead class, Foxn1, is the earliest known marker of the thymic rudiment. *Foxn1* transcripts were detected in isolated quail endoderm 24 h after *Gcm2* expression. At cE4.5, *Foxn1* expression was observed *in situ* in the dorsal tip of the 3/4PP and transcription endures until birth (Neves et al., 2012). The gene is mutated in the nude mouse strain, which displays abnormal hair growth and failure of thymus development, leading to immunodeficiency (Nehls et al., 1996; Blackburn et al., 1996; Bleul et al., 2006).

As in other developmental processes, the activation of the correct transcriptional programs during parathyroid (Neves and Zilhão, 2014) and thymic (Manley and Condie, 2010) organogenesis depends on the crosstalk of several signalling pathways which respond to extracellular signals.

Notch signalling is a major pathway during development that acts in a juxtacrine fashion and is responsible for cell-fate decisions (Lewis, 1998; Lai, 2004). In the last fifteen years, several reports have shown that Notch is fundamental during epithelial-lymphoid cell interactions at late-stages of thymus formation (Rodewald, 2008). Notably, perinatal mutant mice with loss of Notch ligand Jag2 exhibit aberrant thymic morphology with smaller medullar compartments (Jiang et al., 1998). Notch activity is also required for the commitment of lymphoid progenitor cells to the T-cell lineage (Pui et al., 1999; Radtke et al., 1999), in a ligand dependent manner (Jaleco et al., 2001; Dorsch et al., 2002). Whilst largely unknown, there is some evidence for the role of Notch signalling in the early-development of these organs. In mice, the loss of Notch-target Hes1 promotes a spectrum of malformations of pharyngeal endoderm-derived organs, including parathyroid glands aplasia/hypoplasia (Kameda et al., 2013) and abnormal thymic formation (Tomita et al., 1999; van Bueren et al., 2010; Kameda et al., 2013).

Paracrine Hedgehog (Hh) signalling is also involved in craniofacial and neck morphogenesis (Grevellec and Tucker, 2010), and regulates T/PT common primordium development (Moore-Scott and Manley, 2005). In Sonic Hh (Shh) homozygous null mutants the rudiment boundaries are compromised, displaying an expanded domain of the prospective thymic territory at the expense of the Gcm2/parathyroid-fated domain (Moore-Scott and Manley, 2005). This mutant fails to form parathyroid glands (Moore-Scott and Manley, 2005) and displays functional defects in the thymus (Shah et al., 2004). At later stages of development, Shh and Indian Hh, other Hh signalling molecule, are known to regulate thymocyte differentiation after thymic epithelium colonization by lymphoid progenitor cells (Outram et al., 2009).

Hh and Notch pathways interact in multiple biological scenarios (McGlinn et al., 2005; Lawson et al., 2002; Stasiulewicz et al., 2015). In distinct developmental contexts, Notch signalling is known to control morphological boundary formation by the mechanism of lateral inhibition (Lewis, 1998; Lai, 2004; Kiernan, 2013). In light of this evidence, we hypothesized that similar mechanisms could operate in the development of T/PT common primordium. In order to test this hypothesis, Notch and Hh signals were inhibited *in vitro* and *in vivo* in the presumptive territories of thymus and parathyroids by ectopic administration of the respective pharmacological inhibitors. Briefly, our results show a positive regulatory effect of Notch signalling in T/PT common primordium development and parathyroid gland formation. Hh positively regulates the Gata3/Gcm2/parathyroid-fated domain. Furthermore, Hh establishes the dorsal/posterior boundary of Foxn1/thymic rudiment by positively regulating Lfng/Notch signals at the posterior/median territory of the developing 3/4PP endoderm.

## 2. Materials and methods

### 2.1. Embryo preparation

Fertilised Japanese quail (*Coturnix coturnix japonica*) and chicken (*Gallus gallus*) eggs were incubated at 38 °C in a humidified incubator. Chicken Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Quail (q) embryos were dissected at embryonic day (E) 3 and E4 for *in vitro* development studies and whole-mount *in situ* hybridisation (WM-ISH) procedures. Chicken (c) embryos were used at E2.5 (HH17) and E3.5 (HH21) for *in vivo* assays and at E8 for *in ovo* organ formation assays. Chicken pharyngeal endoderm was isolated at E3.5 and E4.5 (HH24-25) and used for WM-ISH, as previously described (Neves et al., 2012).

### 2.2. In vitro organotypic assay

The third and fourth pharyngeal arches region (3/4PAR) was dissected from qE3 on PBS (3/4PAR-0 h), and kept on ice until culture. The 3/4PAR included the 3/4PP and foregut endoderm and the ventral mesenchymal- and ectodermal-neighbouring cells. The dorsal structures like notochord, somites and neural tube were removed (Fig. 1A–F). Explants were then placed on a 24 mm Transwell® with 0.4 μm Pore Polycarbonate Membrane Insert (Corning Product #3412). Seven explants per well were placed with the ventral side up and the dorsal side in contact with the membrane (Fig. 1G). The tissues were grown partially immersed in culture medium, RPMI-1640 Medium (Sigma) supplemented with 10% FBS (Invitrogen), 1 × Pen/Strep (Invitrogen) in a humidified incubator at 37 °C with 5% $CO_2$, for 48 h (3/4PAR-48 h).

For Notch signalling inhibition assays, culture medium was supplemented with LY-411.575 (Ly, Stemgent - Stemolecule™) at 50 nM (Ly-50), 100 nM (Ly-100) or 200 nM (Ly-200) or with Dibenzazepine (DBZ, Selleckchem) at 5 μM (DBZ-5), 10 μM (DBZ-10) or 15 μM (DBZ-15) (experimental conditions). For Hh signalling inhibition assays, culture medium was supplemented with 20 μM of Cyclopamine (Cyc, Sigma) or with 10 μM of Vismodegib (Vis, Selleckchem) (experimental conditions). In parallel, explants were grown with culture medium supplemented with the drug solvent, DMSO, at similar concentrations as the ones present in the medium of experimental conditions [Control-50 (Ly) – 0.0005% DMSO; Control-100 (Ly) – 0.001% DMSO; Control-200 (Ly) – 0.002% DMSO; Control-5 (DBZ) – 0.05%; Control-10 (DBZ) – 0.10%; Control-15 (DBZ) – 0.15%; Control (Cyc) – 0.16%; Control (Vis) – 0.2%] (control conditions).

Following the incubation period, cultured explants were either used for RNA isolation (see Quantitative real time RT-PCR section) or grafted onto chorioallantoic membrane (CAM) at cE8.

### 2.3. In ovo organ formation assay

The 3/4PAR explants grown *in vitro* for 48 h were grafted onto CAM of chicken embryos at cE8 (Fig. 1G). Transplanted tissues were allowed to further develop *in ovo* for 10 days in a humidified incubator at 38 °C, as previously described (Neves et al., 2012). For Notch inhibition assays, the 3/4PAR-48 h explants derived from Ly-200 (3/4PAR Ly-200) were grafted and developed in CAM (Graft-Ly) (experimental condition). For the control conditions, 3/4PAR-48 h explants derived from Control-200 (3/4PAR Control-200) were grafted and developed in CAM (Graft-Control). For both conditions, transplanted tissues were allowed to further develop *in ovo* for 3 and 10 days in a humidified incubator at 38 °C. Survival and organ formation were evaluated in CAM-derived explants grown *in ovo* for 10 days.