Developmental Biology 361 (2012) 208–219

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Modulation of Bmp4 signalling in the epithelial–mesenchymal interactions that take place in early thymus and parathyroid development in avian embryos

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ARTICLE INFO

Article history:
Received for publication 17 May 2011
Revised 2 October 2011
Accepted 3 October 2011
Available online 28 October 2011

Keywords:
Bmp4
Fgf10
Thymus
Parathyroid
Quail-chick
Foxn1
Gcm2

ABSTRACT

Epithelial–mesenchymal interactions are crucial for the development of the endoderm of the pharyngeal pouches into the epithelia of thymus and parathyroid glands. Here we investigated the dynamics of epithelial–mesenchymal interactions that take place at the earliest stages of thymic and parathyroid organogenesis using the quail-chick model together with a co-culture system capable of reproducing these early events *in vitro*. The presumptive territories of thymus and parathyroid epithelia were identified in three-dimensionally preserved pharyngeal endoderm of embryonic day 4.5 chick embryos on the basis of the expression of *Foxn1* and *Gcm2*, respectively: the thymic rudiment is located in the dorsal domain of the third and fourth pouches, while the parathyroid rudiment occupies a more medial/anterior pouch domain. Using *in vitro* quail-chick tissue associations combined with *in ovo* transplantations, we show that the somatopleural but not the limb bud mesenchyme, can mimic the role of neural crest-derived pharyngeal mesenchyme to sustain development of these glands up to terminal differentiation. Furthermore, mesenchymal-derived Bmp4 appears to be essential to promote early stages of endoderm development during a short window of time, irrespective of the mesenchymal source. *In vivo* studies using the quail-chick system and implantation of growth factor soaked-beads further showed that expression of Bmp4 by the mesenchyme is necessary during a 24 h-period of time. After this period however, Bmp4 is no longer required and another signalling factor produced by the mesenchyme, Fgf10, influences later differentiation of the pouch endoderm. These results show that morphological development and cell differentiation of thymus and parathyroid epithelia require a succession of signals emanating from the associated mesenchyme, among which Bmp4 plays a pivotal role for triggering thymic epithelium specification.

Introduction

The formation of thymus and parathyroid glands results from a series of complex epithelial–mesenchymal interactions taking place in the pharyngeal region of the early vertebrate embryo (reviewed by Grevellec and Tucker, 2010). The development of these glands is initiated with the budding off and outgrowth of rudiments from the foregut endoderm of the pharyngeal pouches (PP). The single, endodermal germ layer origin, of the thymic epithelium (TE) was demonstrated using the quail-chick chimera system (Le Douarin and Jotereau, 1975). In chicken, thymic and parathyroid organ rudiments derive from the third and fourth PP (PP3/4), which then separate from the pharynx at embryonic day-5 (E5) (HH-stage 26) (Hamburger and Hamilton, 1951). During this process, a thin mesenchymal capsule formed by neural crest-derived cells surrounds the thymic rudiment and, at E6.5 (HH-stage 29), the colonization of the TE by lymphoid progenitor cells begins (Le Douarin and Jotereau, 1975). Recently, it was shown in transgenic mice that the mesenchyme derived from cardiac neural crest cells forms the embryonic capsule and is associated with vasculature of both fetal and adult thymus (reviewed by Foster et al., 2008 and Rodewald, 2008).

The importance of establishing functional cellular interactions between the developing endoderm and the surrounding mesenchyme in order to initiate thymic development was well illustrated in studies using the quail-chick model. The PP3/4 endoderm isolated from early

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doi:10.1016/j.ydbio.2011.10.022

quail embryos was able to develop into TE with the cooperation of a heterologous mesenchyme derived from the somatopleure or splanchnopleure of E3-chick embryos, which thus could be considered "permissive" to endoderm development. Furthermore, the grafted endoderm was capable of inducing the heterologous mesenchyme to participate in the formation of a fully developed thymus (Le Douarin, 1967a; Le Douarin and Jotereau, 1975; Le Douarin et al., 1968). In contrast, mesenchyme from the somite and limb bud was "non-permissive" to PP3/4 endoderm development (Le Douarin, 1967a; Le Douarin et al., 1968). These data provided first evidence that epithelial–mesenchymal reciprocal interactions are essential for early thymic development; moreover, they revealed that some heterologous mesenchymal tissues are able to mimic the role played by neural crest-derived mesenchyme during normal development of the thymus in the pharyngeal region. Sonic Hedgehog (Grevellec et al., 2011; Moore-Scott and Manley, 2005) and signalling pathways belonging to families of the Bone morphogenetic proteins (Bmps) (Bleul and Boehm, 2005; Gordon et al., 2010; Soza-Ried et al., 2008) and Fibroblast growth factors (Fgfs) (Dooley et al., 2007; Erickson et al., 2002; Jenkinson et al., 2003; Revest et al., 2001) were reported to influence early thymic and parathyroid development. Moreover, Bmp and Fgf signalling pathways are mutually regulated at later stages of thymic development and in the adult thymus (Rossi et al., 2007; Tsai et al., 2003). It is however unknown how these signals crosstalk during endoderm–mesenchyme interactions in early thymic and parathyroid organogenesis.

In the mouse, expression of *Foxn1* transcription factor identifies the prospective TE (Gordon et al., 2001) and is required cell-autonomously for its differentiation and colonization by lymphoid progenitor cells (Blackburn et al., 1996; Bleul et al., 2006; Nehls et al., 1996). The parathyroid rudiment is defined by expression of *Gcm2* (*Glial Cell Missing 2*) transcription factor (Gordon et al., 2001); when *Gcm2* is deleted, no parathyroid glands are formed (Gunther et al., 2000; Liu et al., 2007). In chicken, *Gcm2* expression starts in PP3 from E2.5 (HH-stage 18) onwards and becomes evident in PP4 at E3.5 (HH-stage 22) (Okabe and Graham, 2004). The temporal and spatial identification of the presumptive TE in the chicken endoderm is still unknown.

In this work, we used the quail-chick model to define the molecular crosstalk between the endoderm of the PP3/4 and the mesenchyme, which controls the early stages of TE and parathyroid epithelium (PTE) development. We first identified the endodermal presumptive territories of the PTE and TE using probes for *Gcm2* and *Foxn1* genes and we determined the precise developmental time-window of thymus specification. We also analysed the dynamics of the interactions between PP endoderm and various mesenchymal tissues using *in vitro* tissue co-cultures and *in vivo* grafting combined with the quail-chick marker. The data show that development of the PP3/4 endoderm into PTE and TE requires sequential production of Bmp4 and Fgf10 factors by the local mesenchyme.

Materials and methods

Isolation of quail and chick embryonic tissues

Fertilised Japanese quail (*Coturnix coturnix japonica*) and chicken (*Gallus gallus*) eggs were incubated at 38 °C in a humidified incubator and embryos were dissected at specific times of development. Staging of embryos was according to Hamburger and Hamilton stages (HH, Hamburger and Hamilton, 1951) in the chick and to corresponding HH-stages in the quail. Isolation of PP3/4 endoderm was performed in E2.5 (25 to 30 somite-stage; HH-stage 16–17), E3 (HH-stage 21) and E3.5 (HH-stage 22) quail embryos and in E3 (HH-stage 20) to E4.5 (HH-stage 25) chick embryos as previously described (Le Douarin and Jotereau, 1975, and Suppl. Fig. 1A). Briefly, the wall of the embryonic pharynx was treated with a solution of pancreatin (8 mg/ml, Sigma) for 30–90 min on ice, which allowed separation of pure endoderm from the pharyngeal mesenchyme. Mesenchymal tissues of E2.5–E3 (HH-stages 18–19) chick embryos were dissociated from endodermal and ectodermal tissues by enzymatic digestion with pancreatin using the same procedure as above. Somatopleural and posterior limb bud mesenchymal tissues were obtained from the embryonic territories at the level of somites 19–24 and 25–30, respectively (Suppl. Fig. 1B).

In vitro tissue culture assay

PP3/4 endoderm isolated from E2.5 to E3 quail embryos was grown alone or in association with mesenchymal tissues isolated from E2.5 to E3 chick embryos (Fig. 5A). In brief, 2–3 endodermal explants were combined with 2–3 mesenchymal explants on Nucleopore membrane filters (Millipore) supported by fine meshed metal grids (Goodfellows). The grids were then placed in culture dishes and partly immersed in RPMI-1640 (Sigma) supplemented with 10% FBS and pen/step (control culture medium). In some experiments, the heterospecific associations were grown in culture medium supplemented with 100 ng/ml recombinant mouse Noggin (R&D Systems). Associated tissues were cultured for 48 h at 37 °C in a humidified incubator containing 5% CO2. Following the incubation period, cultured tissues were either used for RNA isolation or grafted on the chorioallantoic membrane (CAM) of E8-chick embryos that were left to develop for further 10 days in a humidified incubator at 38 °C as described (Le Douarin and Jotereau, 1975, and schematic representation in Fig. 5A). Triplicates were obtained for each culture condition analysed by RT-PCR.

RT-PCR analysis

Total RNA was extracted from quail endoderm isolated at different stages of development and from cultured tissues using TRIZOL reagent (Invitrogen). Reverse transcription was conducted with oligo-dT primers (Promega) and SuperScriptII reverse transcriptase (Invitrogen) according to the instructions from the manufacturer. PCR was carried out in 25 µl-reaction with a final concentration of 0.5 µM primers and using the Phusion Master Mix with HF buffer (Finnzymes) according to instructions from the manufacturer. Amplification was performed using the following chicken (c) and quail (q) primer sets and annealing conditions: *cFoxn1* (forward: 5′-ggctctgaaccctgccaaga-3′, reverse: 5′-ctgggaagacgttggggatg-3′, at 65 °C with a 707 bp product); *cGcm2* (forward: 5′-gccagcttccaagaggacca-3′, reverse: 5′-tgccggagtactcaccacca-3′, at 65 °C with a 349 bp product); *cGAPDH* (forward: 5′-caatgggcacgccatcacta-3′, reverse: 5′-ctccagacggcaggtcaggt-3′, at 65 °C with a 544 bp product); *qGAPDH* (forward: 5′-tgccaacccccaatgtctct-3′, reverse: 5′-tggcctctcacagcaggatg-3′, at 65 °C with a 412 bp product). PCR products were resolved on 1% agarose gels and visualised by GelRed (VWR) staining. Gels were loaded with 10 µl of each PCR product per lane. For the analysis of *GAPDH* expression, 1/10 of the PCR product was analysed by Typhoon-cell imaging system 9210 (GE Healthcare) with ImageQuant software and then used as a reference of cDNA amount in each sample. Samples of quail origin exhibited a second *cGAPDH* product of 172 bp used as a reference for the amount of quail cDNA in heterospecific co-cultures.

In vivo Noggin-bead implantation

Before implantation, Affi-Gel-Blue beads (Biorad) were soaked overnight at 4 °C in a solution of 10 µg/ml recombinant mouse Noggin (R&D Systems) in PBS (Noggin-beads) whereas control beads were soaked in PBS. Soaked beads (100–120 µm in diameter) were implanted unilaterally in E3-chicken embryos near the pharyngeal arches. Specifically, beads were primarily trapped in the pharyngeal clefts. In order to increase the chance of hitting the pharyngeal arches, in some cases, multiple beads were placed in the embryos.

Quail-chick chimera production by endoderm grafting

PP3/4 endoderm isolated from E2.5 (25–30 somite-stage) quail embryos was grafted into the body wall of E2.5–E3 chicken embryo. The endoderm was introduced into a slit made in the body wall of the embryo in the somatopleural region (between somites 20 and 25), as described previously (Le Douarin and Jotereau, 1975). Quail-chick chimeras were then incubated for further 24 h, 48 h, 72 h or 10 days of development.

Quail-chick chimera production by endoderm grafting combined FGF10-bead implantation

Before implantation, heparin acrylic beads (Sigma) were soaked overnight at 4 °C in 250 μg/ml recombinant mouse FGF10 (R&D Systems) reconstituted in PBS with 0.1% BSA (Fgf10-beads) whereas control beads were soaked in reconstitution solution.

Quail PP3/4 endoderm isolated at E3 (HH-stage 21) and E3.5 (HH-stage 22) was unilaterally grafted into E2.5–E3 chicken embryos by insertion into a slit made in the posterior limb bud. Chimeric embryos were incubated for 3–4 h and then, FGF10- or control beads (100–120 μm in diameter) were implanted near the grafted quail endoderm. Quail-chick chimeras with the implanted beads were analysed after further 36 h and 5 days of development.

Embryo processing and in situ hybridisation

Embryo and tissue explant cultures were fixed overnight with 4% paraformaldehyde at 4 °C and processed for whole-mount *in situ* hybridisation and immunohistochemistry. Whole-mount preparations and paraffin sections were hybridised with *Foxn1*, *Gcm2*, *Bmp4* (Francis et al., 1994), *Bmpr1b*, *BmprII*, and *Fgf10* (Ohuchi et al., 1997) probes as previously described (Etchevers et al., 2001; Henrique et al., 1995). The probes for avian *Foxn1* and *Gcm2* were produced using TOPO-cloning (Invitrogen). Templates were PCR-amplified for *Foxn1* and *Gcm2* products using primers described above and designed from GenBank database sequences ID: XM_415816 and ID: NM_001008480, respectively. Whole-mount preparations and paraffin sections were treated for immunocytochemistry with QCPN antibody (for labelling of quail cells) as described (Creuzet et al., 2002) and with HNK1 (Abo and Balch, 1981) to detect cells of the peripheral nervous system.

Results

Thymus and parathyroid gland rudiments can be identified in the avian embryo based on the expression of Foxn1 and Gcm2, respectively

To identify the presumptive territories of thymus and parathyroid glands in chicken and quail embryos, we devised new gene probes for chicken *Foxn1* and *Gcm2* transcription factors, which in mammals, are the first markers for TE and PTE, respectively (Gunther et al., 2000; Nehls et al., 1996). Using whole-mount *in situ* hybridisation, *Foxn1* was first detected bilaterally in the pharyngeal region of E4-quail embryos (qE4, Fig. 1A). At the same stage, *Gcm2* was strongly expressed in the PP3/4 endoderm in quail (not shown) and chicken (cE4, Fig. 1B). In E5-chicken (cE5), *Foxn1* and *Gcm2* transcripts occupied mutually exclusive and adjacent domains of PP3/4, with *Foxn1* expression located in the dorsal/anterior domain (Fig. 1C) and *Gcm2* in a more ventral one (Fig. 1D). In cE6, the expression domains of *Foxn1* and *Gcm2* marked thymus (Fig. 1E) and parathyroid (Fig. 1F) rudiments, respectively, and were later maintained in differentiated glands (Figs. 1G and H).

To precisely determine the spatial location/orientation of the TE and PTE territories, we isolated three-dimensionally-preserved PP3/4 endoderm in cE4.5. After *in situ* hybridisation, we observed strong signals of *Foxn1* in the dorsal domain (Figs. 2A–C) and of *Gcm2* in the median/anterior domain (Figs. 2D–F) of the epithelial pouches. These data show that both presumptive TE and PTE territories are established in cE4.5 PP3/4 endoderm; they occupy distinct and contiguous domains during chicken endoderm development (as schematically represented in Fig. 2G). We also determined the early stages of the expression of *Gcm2* and *Foxn1* by RT-PCR analysis of PP3/4 endoderm isolated from

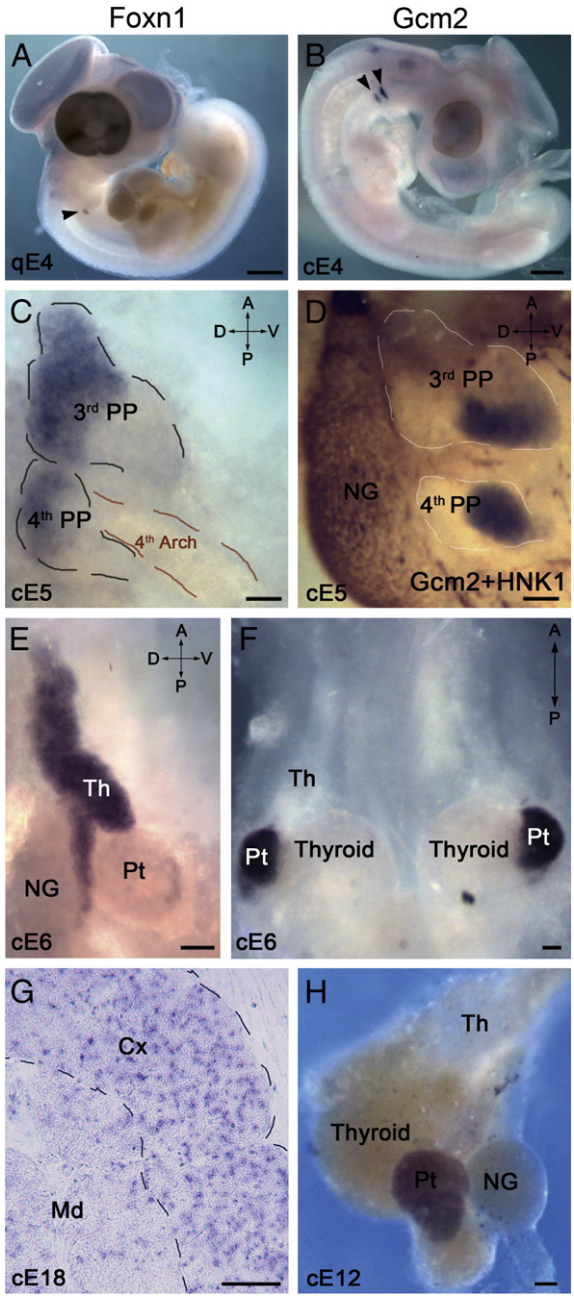

Fig. 1. Expression of *Foxn1* and *Gcm2* during thymic and parathyroid gland development in chick and quail embryos. *Foxn1* expression (A, C, E and G) detected by *in situ* hybridisation in the base of the neck of qE4 (arrowhead, A), in the dorsal/anterior domain of PP3 and PP4 endoderm of cE5 (C, lateral view), in the thymic rudiment of cE6 (E, lateral view) and in TE sections of cE18 showing a differentiated thymic lobe (G). *Gcm2* expression (B, D, F and H) was analysed by *in situ* hybridisation in PP3 and PP4 of cE4 (arrowheads, B) and cE5 (D, lateral view), in the bilateral parathyroid rudiments of cE6 (F, ventral view) and in parathyroid glands of cE12 (G). In D, *in situ* hybridisation of *Gcm2* (purple) was followed by immunocytochemistry using HNK1 (brown) for neural cell labelling. In C–F, hybridisation was performed after removing the ectodermal layer of the neck region. In H, neck glands and nodose sensory ganglion were isolated by microsurgery. In C, the arches and pouches are delimited using red and black lines, respectively; in D, pouches are delimited by white lines. (A, anterior; Cx, cortex; D, dorsal; Md, medulla; NG, nodose ganglion; P, posterior; PP, pharyngeal pouch; Pt, parathyroid gland; Th, thymus; V, ventral). Scale bars, 500 μm (A, B), 100 μm (G) and 50 μm (C–F and H).

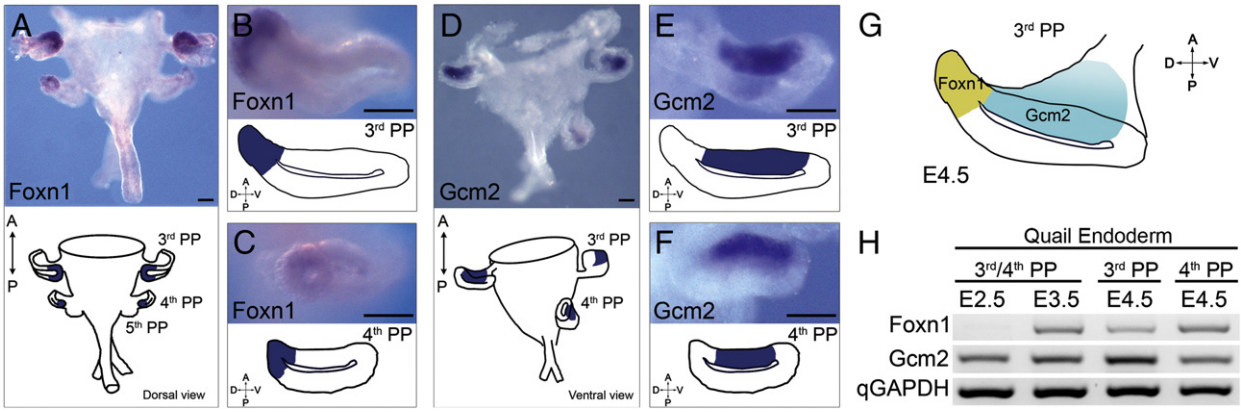

Fig. 2. Early gene expression pattern of *Foxn1* and *Gcm2* in isolated PP3/4 endoderm. *In situ* hybridisation and corresponding schemes showing *Foxn1* (A–C) and *Gcm2* (D–F) expression in PP3/4 endoderm isolated by microsurgery in cE4.5. Magnified images and corresponding schemes of the endoderm of the PP3 (B and E) and PP4 (C and F). Schematic 3D-representation of *Foxn1* and *Gcm2* expression domains in PP3 endoderm of cE4.5 (G). RT-PCR analysis of *Foxn1*, *Gcm2* and *GAPDH* transcripts in quail PP3/4 endoderm isolated at different stages of development from E2.5 to E4 (H). (A, anterior; D, dorsal; P, posterior; PP, pharyngeal pouch; V, ventral). Scale bars, 100 μm.

E2.5 to E4.5-quail embryos. *Gcm2* expression was found from E2.5 (25–30 somite-stage) while *Foxn1* was detected only from E3.5 onwards (Fig. 2H), indicating that endoderm specification into PTE occurs earlier than into TE.

Expression patterns of Bmp4 and Bmp-signalling genes in the PP3/4 endoderm and surrounding mesenchyme during chicken development

In this work we investigated the role of Bmp4 signalling in the epithelial–mesenchymal interactions that take place during early chicken thymic and parathyroid gland development. We first characterized the three-dimensional expression pattern of Bmp-signalling genes in the endodermal and mesenchymal compartments during PP3/4 morphogenesis in the chicken embryo.

We analysed PP3/4 development in cE3 and cE3.5, *i.e.*, before the establishment of thymic and parathyroid presumptive endoderm territories (that is, before the onset of *Foxn1* and *Gcm2* expression *in situ*; Figs. 1 and 2). When the endoderm was isolated in cE3 at the level of PP2 to PP4, *Bmp4* was expressed in the dorsal tip of all the pouches (Fig. 3A) whereas *Noggin* was faintly detected throughout the pouch territory (Fig. 3B). At the same stage, *BmpRIb* transcripts were present in the anterior/dorsal domain of PP2 and PP3 (Fig. 3C) while *BmpRII* showed ubiquitous expression in the pouches and pharynx as well as a strong hybridisation signal restricted to the most posterior and recently formed PP3/4 (Fig. 3D). In whole-mount preparations of cE3.5 embryos, *Bmp4* expression was found at the dorsal tip of the pouches and extended to the local ectoderm (Fig. 3E). The distribution of *Noggin*, *BmpRIb* and *BmpRII* transcripts identified in cE3 was maintained in cE3.5 (Figs. 3F–H), with the exception of a strong hybridisation signal of *BmpRIb* in the anterior/dorsal domain of the most recently formed PP4 (Fig. 3G), not observed at E3 (Fig. 3C). Sections showed strong hybridisation signals of *Bmp4* in mesenchymal cells in the vicinity of the recently formed PP3 (Fig. 3I) and PP4 (Fig. 3J) in cE2.5 and cE3.5, respectively. *Noggin* was faintly detected in the pharyngeal region during PP3/4 formation (data not shown) and strongly expressed in the sensory nodose ganglion located close to PP3/4 in cE4 (Fig. 3K).

We next studied the expression of Bmp-signalling genes in PP3/4 endoderm isolated in cE4.5 (Fig. 4), *i.e.*, a stage in which we have identified both *Foxn1*$^+$ thymic and *Gcm2*$^+$ parathyroid territories (see Fig. 2). *Bmp4* was strongly expressed in the dorsal domain of PP3 (Figs. 4A and B) and in dorsal and posterior domains of PP4 (Figs. 4A and C). Low expression of *Noggin* was detected in dorsal PP3 and posterior PP4 (Figs. 4D–F). In addition, strong *Bmp4* (Fig. 4A) and *Noggin* (Fig. 4D) hybridisation signals marked the prospective territory of PP5, known to participate in ultimobranchial body formation (Le Douarin and Le Lièvre, 1970; Le Douarin et al., 1974). *Noggin* was also detected in a narrow line of cells located in ventral/lateral (Fig. 4D) and dorsal/lateral (not shown) regions of the pharynx. *BmpRIb* was expressed strongly in the most anterior and dorsal region of PP3/4 and, more weakly in the posterior domain of PP3 (Figs. 4G–I). In contrast, *BmpRII* exhibited dim and ubiquitous expression in PP3/4 and PP5, as well as in the pharynx down to the branch point between respiratory and digestive tubes (Fig. 4J).

Therefore, in the PP endoderm, the thymic rudiment displayed higher *Bmp4* expression than the parathyroid territory, while both rudiments exhibited high levels of *BmpRIb* transcripts. Noticeably, *BmpRIb* expression in the endoderm coincided with a strong *Bmp4* expression in the adjacent arch mesenchymal cells, suggesting that Bmp4 produced by the neighbouring mesenchyme may act on PP3/4 endoderm to promote its development into TE and PTE.

In vitro association of endoderm and mesenchymal tissues can reproduce early epithelial–mesenchymal interactions leading to thymic and parathyroid gland development

To overcome the complexity of tissue interactions and the highly dynamic spatial modification of gene expression in the developing pharyngeal region *in vivo*, we set up an *in vitro* experimental system to analyse epithelial–mesenchymal interactions and the influence of Bmp4 during the early formation of thymic and parathyroid glands. We performed co-cultures of early PP endoderm and mesenchymal tissues isolated from quail and chick embryos, respectively, to examine subsequent gene expression and endoderm-derived tissue fate (Fig. 5A). The quail pharyngeal endoderm was isolated between E2 and E3 (from 15- to 30-somite-stage) (Suppl. Fig. 1A) and associated *in vitro* for 48 h with E2.5-chicken mesenchyme taken from the somatopleure or the posterior limb bud (Suppl. Fig. 1B). Both types of mesenchyme expressed *Bmp4* (Suppl. Figs. 1C–D); however, as opposed to the "non-permissive" limb bud, only the "permissive" somatopleural mesenchyme was capable to sustain the development of the PP endoderm into a fully differentiated thymus, as previously shown in grafting experiments (Le Douarin, 1967a; Le Douarin and Jotereau, 1975; Le Douarin et al., 1968).

To validate our *in vitro* approach, we first verified if the tissue interactions in endodermal–mesenchymal co-cultures could mimic the early developmental events needed to pursue thymus and parathyroid gland formation. For this purpose, 48 h-cultures of quail endoderm and chick somatopleural mesenchyme were explanted onto the chorioallantoic membrane (CAM) of cE8, which provides the conditions for long-term growth of grafted tissues *in ovo* (Fig. 5A). After 10 days on

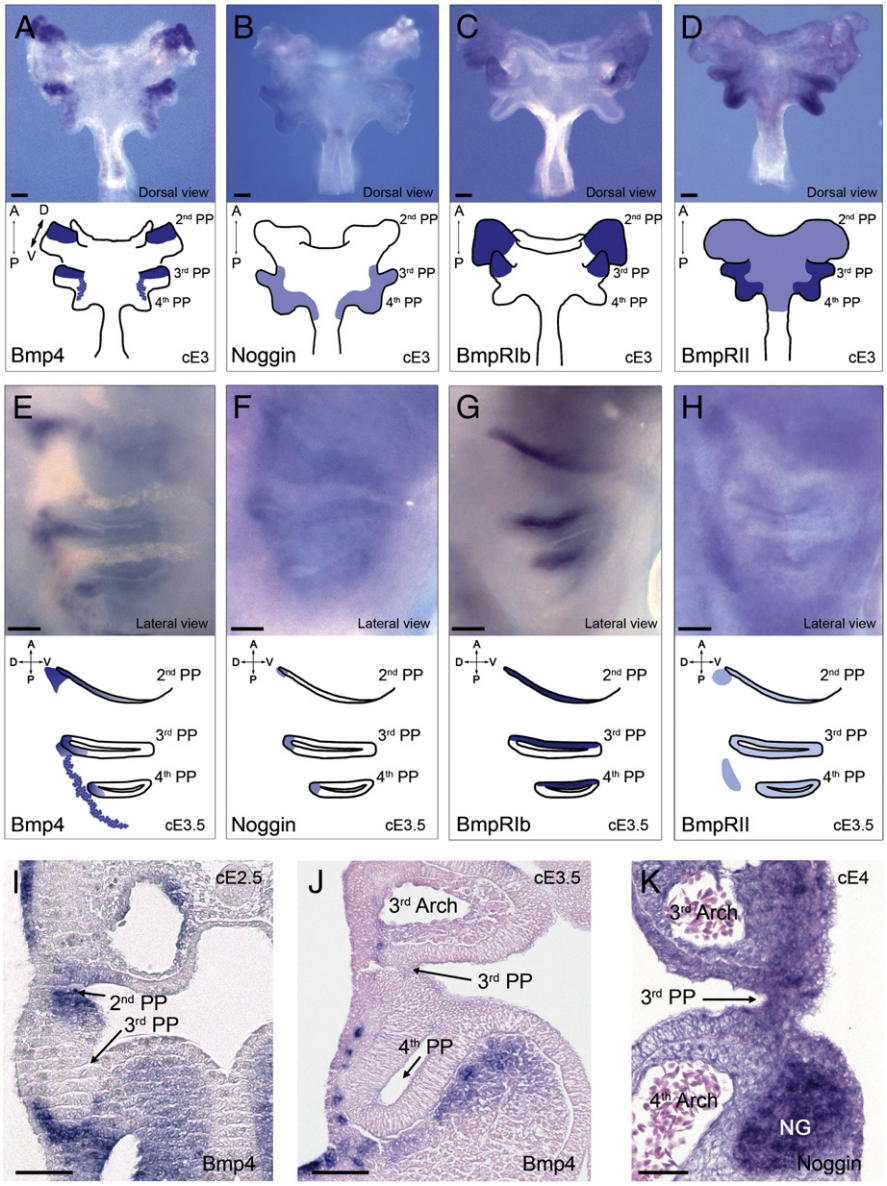

Fig. 3. Spatio-temporal expression of Bmp-signalling genes in PP3/4 before the onset of *Foxn1* expression. *In situ* hybridisation of *Bmp4* (A, E), *Noggin* (B, F), *BmpRIb* (C, G) and *BmpRII* (D, H) in PP3/4 endoderm isolated from cE3 (and corresponding schemes, A–D) and in whole-mount preparations of cE3.5 (magnified arch region and corresponding schemes, E–H). Frontal sections of the pharyngeal region in cE2.5 (I) and cE3.5 (J) after hybridisation with *Bmp4*. Frontal section of cE4 PP3/4 region hybridised with *Noggin* (K). (A, anterior; D, dorsal; NG, nodose ganglion; P, posterior; PP, pharyngeal pouch; V, ventral). Scale bars, 100 μm (A–H) and 50 μm (I–K).

host CAM, the grafted co-cultures gave rise to fully developed thymus and parathyroid glands (Fig. 5B). The yield of gland formation increased with endoderm stage of the donor embryos (Table 1). Even the pharyngeal endoderm isolated as early as 15-somite-stage (approximately 60 h before *Foxn1* starts to be expressed *in situ*) was able to form a full thymus when associated with the somatopleural mesenchyme. Immunostaining with quail-specific marker showed that the gland structures formed in the graft comprised PTE and TE derived from the quail endoderm, whereas hematopoietic cells in the ectopic thymus were of chick host origin (Fig. 5B). In agreement with previous chimera experiments, no gland formation but necrosis was observed after implantation on CAM of the PP endoderm associated with non-permissive limb bud mesenchyme (not shown).

Therefore, the interactions between the PP endoderm and the permissive mesenchymal tissue during this *in vitro* co-culture provide appropriate early signals for long-term development of thymus and parathyroid glands.

Effects of Noggin treatment on Foxn1 activation during in vitro interactions between quail PP endoderm and chick mesenchyme

We used the *in vitro* 48 h-tissue culture assay described above to study early endodermal–mesenchymal interactions at two distinct stages of endoderm development (qE2.5 and qE3), before the detection of *Foxn1* transcripts (see Fig. 2H). The presence of TE and PTE was evaluated by RT-PCR analysis of *Foxn1* and *Gcm2*, respectively.

In the associations of qE2.5 (25–30 somite-stage) PP3/4 endoderm with somatopleural or posterior limb bud mesenchyme (cE2.5–cE3), *Foxn1* transcripts were upregulated after 48 h in control medium (Fig. 5C). To study the role of Bmp4 signalling in TE specification and PTE development, the co-cultures were treated with Noggin (100 ng/ml). In contrast to control medium, when the co-cultures were treated with Noggin, the induction of *Foxn1* expression in the presence of the mesenchymes was abolished (Fig. 5C). *Gcm2* transcripts, as opposed to those of *Foxn1*, were present when the endoderm

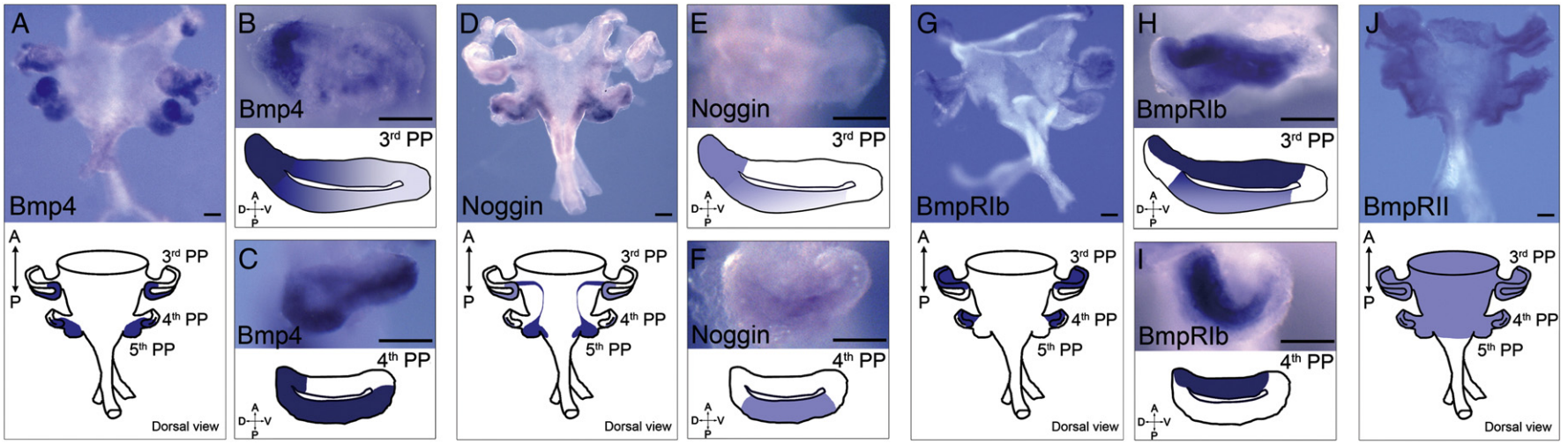

Fig. 4. Expression of Bmp-signalling genes in early thymic and parathyroid rudiments. *In situ* hybridisation and corresponding schemes showing transcripts of *Bmp4* (A–C), *Noggin* (D–F), *BmpRIb* (G–I) and *BmpRII* (J) in the PP endoderm isolated from cE4.5. Magnified images and corresponding schemes of PP3 (B, E and H) and PP4 (C, F and I) endoderm. (A, anterior; D, dorsal; P, posterior; PP, pharyngeal pouch; V, ventral). Scale bars, 100 μm.

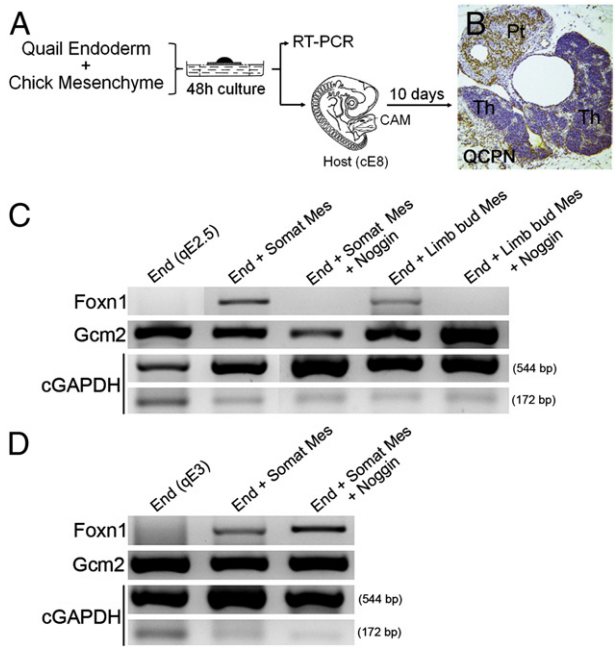

Fig. 5. Effects of mesenchymal tissues and modulating Bmp4 signalling levels on early stages of thymic and parathyroid development in endoderm tissue culture. Schematic representation of the *in vitro* experiments wherein quail PP3/4 endoderm was associated with chicken mesenchyme and cultured for 48 h (A). The co-cultured tissues were then either explanted and grown *in ovo* for further 10 days onto the CAM of cE8, before immunostaining with QCPN (B) or they were analysed by RT-PCR for *Foxn1*, *Gcm2* and *GAPDH* transcripts (C, D). (B) Section of 48 h-associated tissues explanted on CAM for 10 days, which gave rise to fully developed parathyroid glands and thymus comprising epithelia of quail origin (QCPN, brown staining) and vascular endothelium and connective tissues of chick origin (Gill's hematoxylin, blue staining); in the thymus, hematopoietic (basophilic) cells are also of chick origin. (C) RT-PCR analysis of qE2.5 PP3/4 endoderm, alone or associated with the somatopleure or the limb bud mesenchyme of cE2.5–E3, after 48 h-culture in the presence or absence of Noggin (100 ng/ml). (D) RT-PCR analysis of qE3 PP3/4 endoderm, grown alone or associated with the somatopleural mesenchyme for 48 h. An additional and specific quail-derived *GAPDH* transcript (172 bp) was amplified when using the chicken primer sets. No *Foxn1* or *Gcm2* expression was detected in cultures of mesenchymal tissues in the absence of endoderm (data not show). (CAM, chorioallantoic membrane; End, endoderm; Mes, mesenchyme; Pt, parathyroid gland; Somat Mes, somatopleural mesenchyme; Th, thymus). Scale bars, 100 μm (C–F) and 50 μm (E, H).

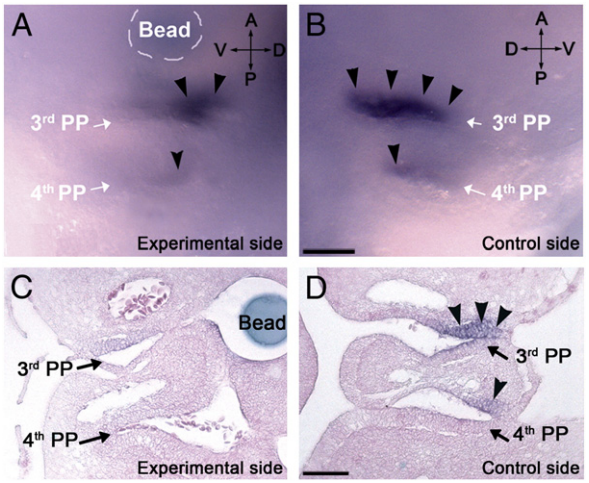

Fig. 6. *In vivo* effect of an ectopic source of Noggin on the expression of *Gcm2* in the developing PP3/4 endoderm. A Noggin-soaked blue agarose bead was unilaterally placed in the vicinity of PP3/4 endoderm of cE3 (A and C). After 24 h, the embryos were processed for *in situ* hybridisation with *Gcm2*. Magnified images of the PP3/4 region in whole-mount embryos (A, B) and corresponding post-hybridisation sections (C, D). (A, anterior; D, dorsal; P, posterior; PP, pharyngeal pouch; V, ventral). Scale bars, 100 μm (B) and 50 μm (D).

was grown in the absence of mesenchyme. Moreover, *Gcm2* expression was maintained when the endoderm was cultured with somatopleural or limb bud mesenchyme, in both control and Noggin-supplemented media (Fig. 5C). However, a decrease of *Gcm2* expression was observed in Noggin-treated associations of endoderm and somatopleural mesenchyme, suggesting that reducing the levels of Bpm4 may interfere with early PTE development *in vitro* (Fig. 5C). Therefore, during the 48 h-*in vitro* period, the E2.5-endoderm was capable of sustaining *Gcm2* expression in the absence of mesenchymal influence; however, it required Bmp4 signals provided by association with mesenchymal tissues, in order to become specified into *Foxn1*-expressing TE.

Similar to qE2.5, qE3 PP3/4 endoderm cultured for 48 h without mesenchyme did not show *Foxn1* expression by RT-PCR, while expressing both *Gcm2* and *Foxn1* transcripts after 48 h-association with the somatopleural mesenchyme (Fig. 5D). Surprisingly, addition of Noggin (100 ng/ml) to the cultures of associated tissues did not change *Foxn1* and *Gcm2* expression, when compared to co-cultures grown in control medium (Fig. 5D). These results suggest that signals produced by the mesenchyme (or the combined endoderm and mesenchyme) are essential for TE specification in both E2.5 and E3-PP endoderm. However, in contrast to early (E2.5) endoderm, in which the inhibition of Bmp signalling by Noggin impaired the induction of *Foxn1*, the older (E3) PP3/4 endoderm no longer required Bmp4 signals to upregulate *Foxn1*. Therefore, the effect of Bmp4 signalling in regulating the specification of the PP endoderm into TE is temporally restricted to a short period between qE2 and qE3.

Table 1
Differentiation of parathyroid glands and thymus from associated PP endoderm and mesenchyme after explantation onto the CAM.

Tissues implanted on CAM[a]		PP-derived differentiated glands (%)[b]	
Isolated endoderm	Isolated mesenchyme	Parathyroid glands	Thymus
PP3/4 (25–30ss)	–	0/4	0/4
–	Somatopleure	0/4	0/4
PE (15–20ss)	Somatopleure	5/8 (62%)	4/8 (50%)
PE (20–25ss)	Somatopleure	12/15 (84%)	7/15 (52%)
PP3/4 (25–30ss)	Somatopleure	14/16 (88%)	10/16 (62%)
PP3/4 (25–30ss)	Limb	0/4	0/4

[a] Quail PP endoderm isolated between 15- and 30-somite-stage (ss) was cultured *in vitro* for 48 h, either alone or associated with chick mesenchyme of the somatopleure or limb bud and then grafted onto the CAM of cE8.

[b] After 10 days, the formation of endoderm-derived parathyroid and thymic glands was analysed by histochemistry and immunochemistry of CAM sections. Data are expressed as the number (and percent) of the tissue cultures that formed parathyroid glands and thymus on CAM. (PE, pharyngeal endoderm).

Local application of Noggin interferes with early in vivo development of PP3 endoderm into PTE

Our *in vitro* analysis suggested that inhibition of Bmp signalling in co-cultures of qE2.5 endoderm with somatopleural mesenchyme could affect the maintenance of *Gcm2* expression during early development of the endoderm into PTE (see Fig. 5C). To further clarify this issue, we used an *in vivo* approach that consisted in implanting an exogenous source of Noggin (Noggin-soaked bead) unilaterally, in the vicinity of the PP3/4 endoderm in cE3 (n = 3), when *Gcm2* expression is faintly detected by *in situ* hybridisation (not shown). Twenty-four hours after bead implantation, we observed expression of *Gcm2* in the anterior domain of PP3 in the control (non-implanted) side of the embryos (Figs. 6B and D). As compared with the control side, *Gcm2* expression was strongly decreased in the contralateral pouch implanted with Noggin-soaked bead (Figs. 6A and C). Control beads (without Noggin) similarly placed in the arch region did not perturb normal development of PP3/4 (not shown). These results therefore show that the reduction of local Bmp4 levels by Noggin interferes with the *in vivo* expression of *Gcm2* and the early development of PP3/4 endoderm into PTE.

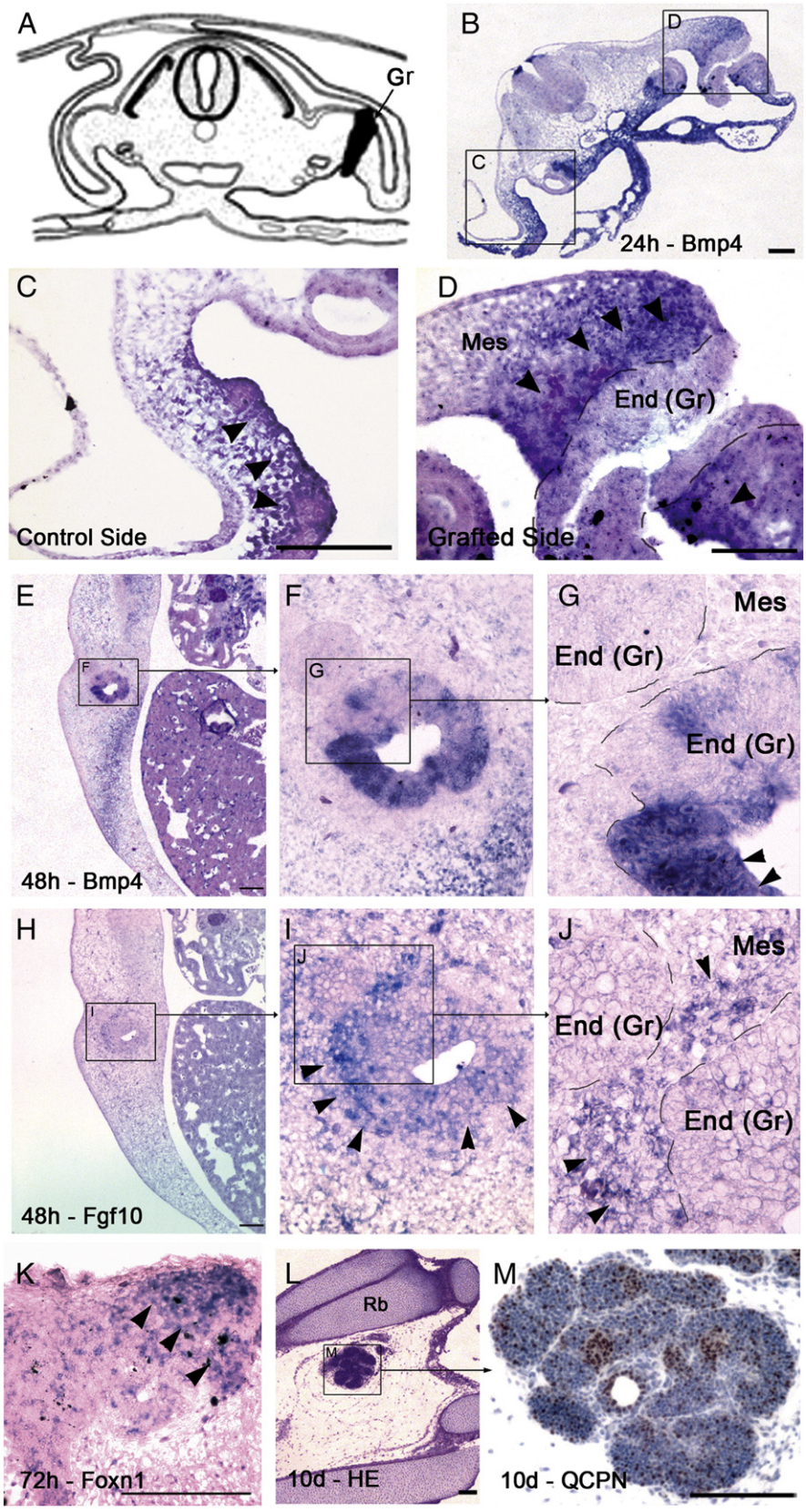

Fig. 7. Ectopic thymus development *in vivo* after grafting of quail PP3/4 endoderm into chick somatopleural mesenchyme. Schematic representation showing the grafting of isolated quail PP3/4 endoderm (qE2.5) into the somatopleural region of cE2.5–E3 host (A). Transverse sections of chick host embryos 24 h (B–D), 48 h (E–J) and 72 h (K) after endoderm grafting, were processed for *in situ* hybridisation with *Bmp4* (B–G), *Fgf10* (H–J) and *Foxn1* (K) probes. Serial sections are shown in (E–G) and (H–J). Arrowheads indicate strong hybridisation signals. Transverse sections of chick host embryos 10 days post–grafting showing ectopically developed thymus (with clear discrimination between cortical and medullary compartments) detected between the ribs (L, M). Only TE cells are quail endoderm-derived and positive for QCPN staining (brown); lymphoid and mesenchymal cells of host origin are evidenced by Gill's hematoxylin contrast (blue staining) (M). (End, endoderm; Gr, graft; HE, hematoxylin–eosin; Mes, somatopleural mesenchyme; Rb, ribs). Scale bars, 100 μm.

Heterospecific grafting experiments show that endoderm–mesenchyme interactions prior to TE specification involve sequential production of Bmp4 and Fgf10 by the mesenchyme

In order to further understand the role of Bmp4 signalling and the respective contribution of endodermal and mesenchymal signals in early TE development *in vivo*, we performed heterospecific graft experiments, as previously described (Le Douarin, 1967a; Le Douarin and Jotereau, 1975; Le Douarin et al., 1968). As depicted in Fig. 7A, we implanted the PP endoderm isolated from qE2.5 into the permissive somatopleural mesenchyme of cE2.5–E3 and subsequently examined *Bmp4* expression in the developing grafted region. Host embryos 24 h after endoderm implantation (n = 3) showed high levels of *Bmp4* expression and increased size of the somatopleural mesenchyme in contact with the grafted endoderm (Figs. 7B and D), whereas mesenchymal cells in the control side exhibited low *Bmp4* expression and loose cellular arrangement (Fig. 7C). In contrast, when the qE2.5 PP endoderm was implanted into the chick somite for 24 h, this non-permissive mesenchyme was unable to upregulate *Bmp4* activity in response to the presence of the implanted endoderm (n = 2) (not shown). In recipient embryos 48 h after grafting of the qE2.5 PP endoderm into the permissive somatopleural mesenchyme (n = 4), we found down-regulation of *Bmp4* expression in the mesenchyme surrounding the graft, accompanied by an increase of *Bmp4* transcripts in the grafted endodermal epithelium (Figs. 7E–G).

Therefore, *Bmp4* expression by the host somatopleural mesenchyme is temporally modulated in response to the presence of the implanted endoderm, being first increased after 24 h-interaction and decreased

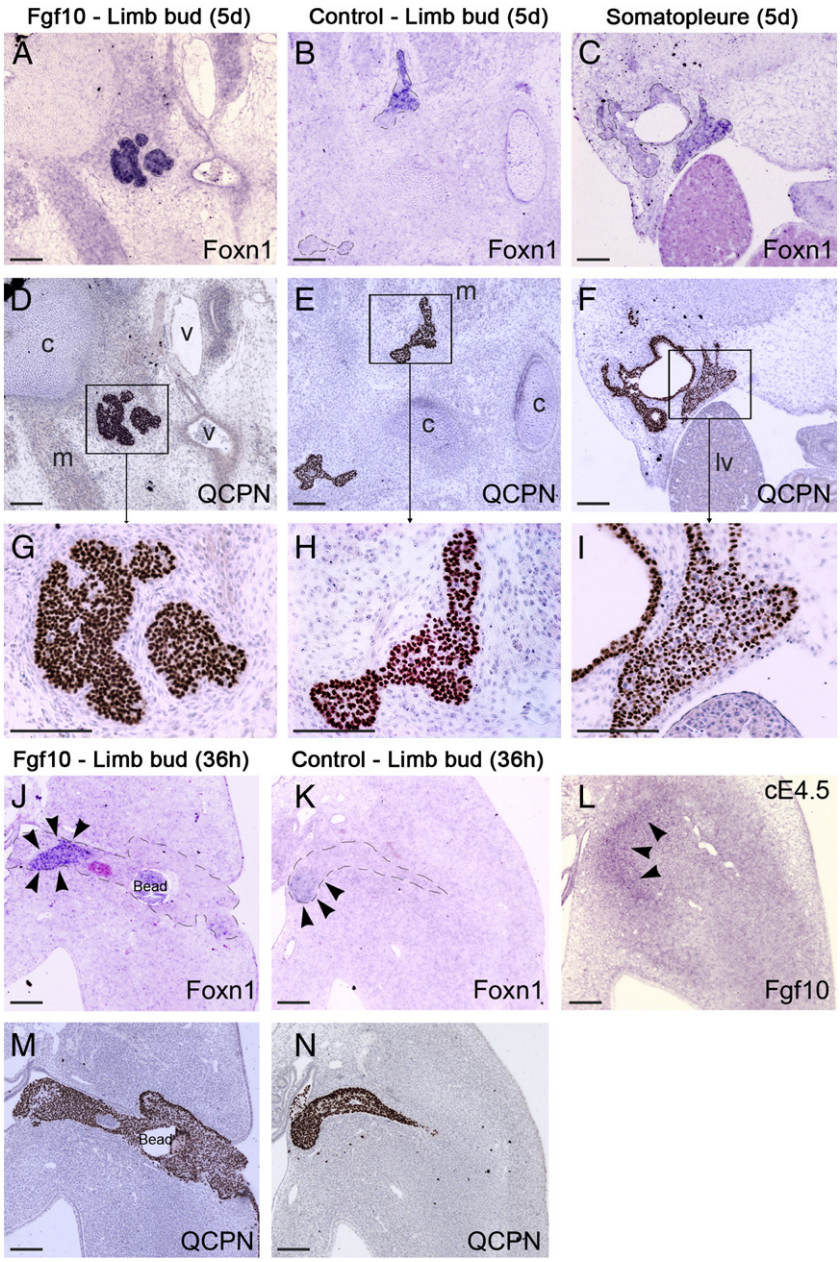

Fig. 8. *In vivo* effect of an ectopic source of Fgf10 on the expression of *Foxn1* by PP3/4 endoderm grown in the limb bud territory. Transverse sections of chick host embryos 5 days (A–I) and 36 h (J, K, M and N) after grafting quail E3 and E3.5 endoderm, respectively. Fgf10-beads (A, D, G, J and M) or control-beads (B, E, H, K and N) were implanted together with the endoderm into the posterior limb bud (A, B, D, E, G, H, J, K, M and N) and somatopleural (C, F and I) regions of recipient embryos. Transverse serial sections were processed for *in situ* hybridisation with *Foxn1* probe (A–C, J and K) and staining with QCPN antibody and Gill's hematoxylin (D–I, M and N). Transverse section of the limb bud in cE4.5 hybridised with *Fgf10* (L). Arrowheads indicate hybridisation signals (J–L). (c, cartilage; lv, liver; m, muscle; v, vessel; 5d, 5 days). Scale bars, 100 μm.

one day later, as the grafted PP3/4 endoderm develops. These data raised the question of the identity of the mesenchymal signals able to support further TE development, at stages when the mesenchyme shows low *Bmp4* activity. In the mouse, *Fgf10* is expressed by mesenchymal cells of the pharyngeal arches (Revest et al., 2001) while Fgf and Bmp signalling pathways are mutually regulated in late thymic organogenesis (Rossi et al., 2007; Tsai et al., 2003). Hence, we investigated whether Fgf10 could be one of the mesenchymal factors able to promote thymic development of the PP endoderm in our grafting assays. In chicken host embryos 24 h after grafting the qE2.5 PP endoderm (n = 3), *Fgf10* transcripts were not detected in the graft area (data not shown); however, 48 h post-operation, *Fgf10* expression was present in the implanted endoderm and the surrounding mesenchyme (Figs. 7H–J, n = 3). The permissive somatopleural mesenchyme therefore responded to endodermal instructive signals by sequentially producing Bmp4 and Fgf10, suggesting that, after initial involvement of mesenchymal Bmp4, Fgf10 might substitute for Bmp4 to sustain later development of the endoderm into a *Foxn1*$^+$ TE rudiment. Moreover, modulation of *Bmp4* and *Fgf10* expression in the mesenchymal compartment occurred one day prior to endoderm specification into TE, since *Foxn1* started to be expressed by the quail endoderm only 72 h after grafting (Fig. 7K). Further incubation of operated embryos until E13 (10 days post-grafting) allowed development of a well-differentiated ectopic thymus (Fig. 7L) with TE cells derived from the (quail) endoderm and hematopoietic cells of mesenchymal (chick) origin (Fig. 7M), as previously described in similar grafting experiments (Le Douarin, 1967a; Le Douarin and Jotereau, 1975; Le Douarin et al., 1968).

Heterospecific endoderm grafting combined with bead implantation show that Fgf10 favours TE specification

Our data suggested that, following the early influence of Bmp4, mesenchymal-derived Fgf10 might sustain later development of the endoderm into a *Foxn1*$^+$ TE rudiment. To further address this question, we investigated whether an exogenous source of Fgf10 could influence the fate of the endoderm (older than E2.5) when grafted in a non-permissive environment. For this purpose, qE3–E3.5 PP endoderm and Fgf10-soaked beads were implanted together into the E3-chick posterior limb bud mesenchyme and *Foxn1* expression was examined 36 h and 5 days after the operation.

E3-quail endoderm grafts analysed after 36 h, showed faint expression of *Foxn1* in only one of the host embryos treated with Fgf10-beads (n = 5) and no expression in those implanted with control beads (n = 4) (data not shown). However, 5 days post-grafting, the quail endoderm showed strong *Foxn1* expression in 3 of 4 host embryos implanted with Fgf10-beads (Fig. 8A), as compared to 2 of 4 control host embryos (Fig. 8B). Additionally, although expressing *Foxn1* in this non-permissive environment, the quail epithelium in both conditions showed no colonization by chick lymphoid progenitor cells (Figs. 8D, E, G and H). Conversely, colonization of *Foxn1*$^+$ thymic epithelium occurred when the endoderm was grafted in the permissive mesenchyme of the somatopleural region (Figs. 8C, F and I).

When the quail endoderm was isolated at later stage (E3.5), *Foxn1* expression could be identified 36 h after grafting into the limb bud mesenchyme of E3-chick host embryos. In the presence of Fgf10-beads, 5 of 6 recipient embryos exhibited strong *Foxn1* hybridization signal in the endoderm (Figs. 8J and M); in contrast, weaker *Foxn1* expression occurred in only 2 of 6 embryos implanted with control beads (Figs. 8K and N). In fact we detected endogenous *Fgf10* expression in the limb bud mesenchyme of E4.5 chick embryos (Fig. 8L), which suggests that Fgf10 produced locally by the non-permissive mesenchymal cells can support, at least in part, the maintenance of *Foxn1* expression by the endoderm.

These results show that Fgf10 acts on E3.5 quail PP3/4 endoderm to promote its development into TE.

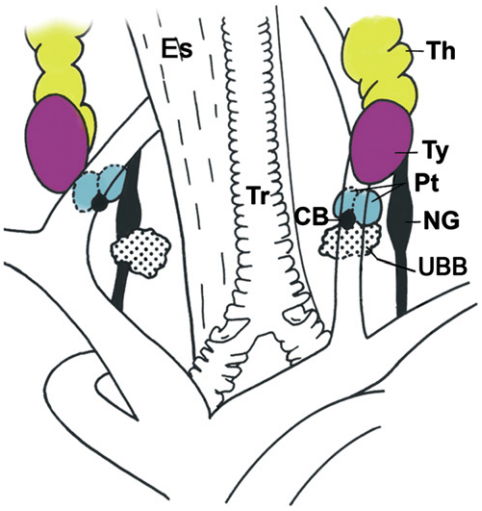

Fig. 9. Schematic representation of the anatomic location of foregut endoderm-derived glands in adult chicken. (CB, carotid body; Es, esophagus; NG, nodose ganglion; Pt, parathyroid glands; Th, thymus; Tr, trachea; Ty, thyroid; UBB, ultimobranchial body).

Discussion

Pharyngeal organs such as the thymus and parathyroid glands develop from endoderm territories in cooperation with the surrounding mesenchyme, implying dynamic reciprocal signalling between the two tissues. Here we investigated the role of mesenchymal tissues in PP3/4 endoderm development in the avian embryo, taking advantage of the capacity to isolate three-dimensionally-preserved endoderm at early developmental stages. Quail-chick chimeras were constructed *in vivo* and *in vitro* by associating isolated quail endoderm with chick mesenchyme from different sources, such as that of the somatopleure and posterior limb bud. We present evidence for the role of Bmp4 produced by the mesenchyme in the epithelial–mesenchymal interactions taking place at early stages of thymic and parathyroid gland development. Bmp4 produced by the mesenchyme was able to sustain *Gcm2* expression in the presumptive parathyroid epithelium. Moreover, mesenchymal derived Bmp4 was critical for further development of the thymic rudiment during a short period of time, between E2 and E3 in the quail (corresponding to E2.5–E3.5 in the chick). Later on, the endoderm itself took over the production of Bmp4. This later step coincided with the onset of *Fgf10* expression in the mesenchyme. Sequential production of Bmp4 and Fgf10 by the mesenchyme thus results in *Foxn1* activity in the endoderm. In the non-permissive limb bud environment, the grafted endoderm expressed *Foxn1* but exhibited a block or delay in lymphoid cell colonization.

Identification of the presumptive endodermal territories of thymus and parathyroid gland epithelia in the chicken embryo

In chicken, the rudiments of the thymus and parathyroid glands develop from the PP3/4 endoderm. In this work, we determined the spatial distribution and position of these rudiments in the three-dimensionally-preserved PP endoderm, using combined techniques of whole-mount *in situ* hybridisation and enzymatic isolation of embryonic tissues. We first observed the expression of *Gcm2* followed by that of *Foxn1*, which identify parathyroid and thymic rudiments, respectively. Moreover, *Foxn1* expression in the prospective thymus rudiment was preceded by *Bmp4* expression in the developing PP3/4. Although these data are similar to those reported in the mouse (Gordon et al., 2001; Patel et al., 2006), the domain of *Foxn1* expression is inverted along the dorsal–ventral axis of the pouches in chicken and quail, when compared to mouse embryos. These distinct positions during embryogenesis might contribute to the different anatomical locations of the adult

thymus between mammals and birds. Accordingly, in mammals, the thymus (in a ventral early position in the pouch; Gordon et al., 2001) is located in upper anterior position in the chest cavity just above the heart, while in the chicken and quail, it initially forms in a dorsal position in the pouch and, later, becomes bilaterally located near the jugular vein and along the neck (Fig. 9).

Role of the mesenchyme during cellular interactions with PP3/4 endoderm to promote thymic and parathyroid gland development

Our tissue co-culture experiments have shown that ectopic mesenchymal tissues of the somatopleure or limb bud can provide the early (qE2.5) PP endoderm with signals required for setting up the expression of TE and PTE early markers. However, in contrast to the limb bud, only the somatopleural mesenchyme was able to promote full differentiation of TE and PTE leading to gland formation after transplantation on CAM. Furthermore, *Foxn1*$^{+}$ epithelium grown in the non-permissive mesenchymal environment showed no colonization by lymphoid progenitors, suggesting that specification of TE and its colonization by hematopoietic cells can occur independently during thymus organogenesis.

Our data showing that only the somatopleural mesenchyme is capable of coordinated long-term development of endoderm indicate the need of continuous molecular crosstalk between these tissues during pharyngeal gland organogenesis. In the embryo, the PP3/4 endoderm develops in close contact with Bmp4-expressing mesenchyme of the arches, suggesting that, in the graft paradigm, the capacity of ectopic mesenchymal tissues to sustain early development of these glands may depend on their ability to produce Bmp4. Indeed, *Bmp4* is expressed by both somatopleural and limb bud mesenchymal tissues when they are isolated and associated *in vitro* with the endoderm. Furthermore, *Bmp4* expression is upregulated in the somatopleural mesenchyme after *in vivo* grafting of the PP3/4 endoderm into the body wall. Considering that the presumptive territories of the glands express *BmpR1b* and *BmpRII* and, that Bmp2/4/7 preferentially signal through heterodimeric complexes of BmpR1a (or 1b) and BmpRII (Feng and Derynck, 2005), altogether our data strongly suggest that the establishment of the prospective domains of TE and PTE initially depends on mesenchymal-derived Bmp4 signals.

Reciprocal signalling between PP endoderm and surrounding mesenchyme in driving specification of TE and PTE: involvement of mesenchymal-derived Bmp4 and Fgf10

Our analysis of early endoderm–mesenchyme interactions *in vitro* revealed that Bmp4 produced by mesenchymal cells is capable of sustaining early development of PP3/4 endoderm into TE (*Foxn1* expression) and PTE (*Gcm2* expression). The requirement for Bmp signalling varied with endoderm developmental stages: the Bmp signalling inhibitor Noggin thus interfered with activation of *Foxn1* in qE2.5, but not in qE3-endoderm grown *in vitro* in association with mesenchymal tissues. Although some tissue-autonomous effect of Bmp signalling in the endoderm itself could not be excluded, the levels of Bmp4 signalling within the PP endoderm seemed insufficient or not properly maintained above-threshold levels, to induce *Foxn1* expression. In agreement, *Bmp4*-conditional deletion driven by *Foxg1-Cre* activity in mice clearly indicated that Bmp4 produced by the pharyngeal endoderm is dispensable for TE and PTE specification (Gordon et al., 2010). Therefore our results argue that Bmp4 signalling of mesenchymal origin acts during an early and narrow window of time in order to control TE specification. In addition, we have shown that inhibition of Bmp signals also interfered with early development of PTE: administration of exogenous Noggin caused a decrease of *Gcm2* expression in the PP3/4 endoderm (qE2.5) both *in vivo* (after Noggin-bead implantation) and *in vitro* (after treatment of endoderm-somatopleural mesenchyme co-cultures).

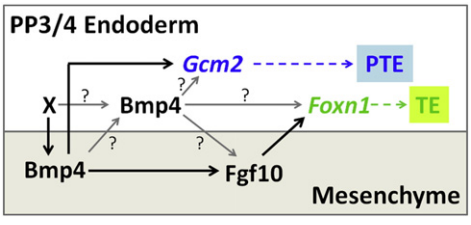

Fig. 10. Schematic model for Bmp4 and Fgf10 signalling crosstalk during epithelial–mesenchymal interactions in early thymic and parathyroid development. The temporal sequence of molecular changes is depicted for both endodermal and mesenchymal compartments. Arrows indicate putative signalling crosstalk involved in the epithelial–mesenchymal dialogue (see Discussion for details).

Together, our results provide evidence that mesenchymal-derived Bmp4 signalling controls endoderm expression of *Foxn1* and *Gcm2*, two master genes of thymus and parathyroid development, respectively. Our results are thus in apparent discrepancy with recent data showing a normal pattern of expression of *Foxn1* and *Gcm2* after targeted mutation of *Bpm4* in the early PP endoderm and adjacent mesenchyme in *Foxg1-Cre;Bmp4* mice (Gordon et al., 2010). However, these mice exhibited incomplete and variable deletion of *Bmp4* in the pharyngeal mesenchyme prior to the onset of *Foxn1* expression in the endoderm (Gordon et al., 2010), which raises the possibility that residual Bmp signalling in the mesenchyme could have been sufficient to trigger normal specification of the endoderm.

Using heterospecific grafting of the PP endoderm into permissive mesenchyme we were, for the first time, able to follow the *in vivo* dynamics of the reciprocal signals between the two tissues. We first noticed a temporal regulation of *Bmp4* expression in the mesenchymal compartment, in which *Bmp4* activity increased 24 h after endoderm grafting, then decreased one day later. This suggests that the Bmp4 levels need to be tightly regulated in the developing pouches. In agreement, *Bmp4* gene deletion in the mouse pharyngeal epithelia induced apoptosis in mandibular mesenchyme (Liu et al., 2005). Additionally, the impairment of Bmp signalling inhibition led to defects in pharyngeal arch development and hypoplasia of thymus and parathyroid glands (Bachiller et al., 2003; Stottmann et al., 2001).

We further showed that cellular interactions between the endoderm and adjacent mesenchyme involved a sequential expression of *Bmp4* and *Fgf10* in the mesenchymal compartment. In mouse, it was previously shown that the survival/expansion of TE at late stages of development and in adulthood requires Bmp4-regulated Fgf10 signalling (Tsai et al., 2003). Our data reveal that the successive action of Bmp4 and Fgf10 signalling in thymic development occurs earlier than previously reported.

Here, we propose a model for crosstalk between Bmp and Fgf signalling molecules during tissue interactions in thymic and parathyroid development, which emphasizes the highly dynamic temporal and spatial dialogue between the PP endoderm and mesenchyme (Fig. 10). The signals from the early PP3/4 endoderm, which can induce the mesenchyme to participate in thymus and parathyroid gland formation, remain to be identified. Our experiments clearly show that the first signal for the onset of thymic and parathyroid development arises from the endoderm, as the grafted pharyngeal endoderm is able to recruit the somatopleural mesenchyme in order to trigger its maturation into a fully developed glandular tissue. Similarly, previous experiments using the quail-chick model have shown that any type of somatosplanchnopleural mesenchyme is able to develop into dense connective tissues, capsule or multilayer smooth muscle, according to the endodermal-derived organ formed following the grafting of early quail pharyngeal endoderm into the body wall of a chick embryo (Le Douarin, 1967a, 1967b; Le Douarin et al., 1968). One molecular candidate for the first instructive signal derived from the endoderm is Fgf8, which is expressed in the posterior domain of the pouches and excluded from TE and PTE presumptive territories in chicken (not shown). Moreover, Fgf8 is known to be required for pharyngeal

arch development and to regulate *Fgf10* expression in pharyngeal arch mesenchyme in the mouse embryo (Abu-Issa et al., 2002; Frank et al., 2002). Another candidate is Noggin, which is expressed, albeit faintly, in the posterior domain of chicken PP3/4 endoderm and may synergize with Fgf8, as described in mouse mandible development (Liu et al., 2005; Stottmann et al., 2001). Further experiments aimed at modifying gene activity in the endoderm will help in defining the early instructive signals emanating from the PP endoderm.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.10.022.

Acknowledgments

The authors are grateful to Dr. Nohno (Department of Molecular and Developmental Biology, Kawasaki Medical School, Japan) for the *Bmpr1b* and *BmprII* chicken probes, to Interaves Portugal for contributing with quail fertilised eggs, to S. Morgado and V. Proa for technical assistance and to G. Calloni, R. Garcez and A. Cidadão for helpful discussions. This work was supported by Centre National de la Recherche Scientifique (CNRS), Association pour la Recherche contre le Cancer (ARC), Fondation Bettencourt-Schueller and Fundação para a Ciência e Tecnologia (FCT), and partially conducted at the facilities of the Instituto de Medicina Molecular, Lisbon. H.N. received research fellowships from the Institut Curie and the Fondation pour la Recherche Médicale (FRM).

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