Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary

Johan Ericson¹,², Stefan Norlin¹, Thomas M. Jessell² and Thomas Edlund¹,*

¹Department of Microbiology, Umeå University, S-901 87 Umeå, Sweden
²Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Center for Neurobiology and Behavior, Columbia University, New York, NY 10032, USA
*Author for correspondence (e-mail: Thomas.Edlund@micro.umu.s.e)

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SUMMARY

The mechanisms by which inductive signals control the identity, proliferation and timing of differentiation of progenitor cells in establishing spatial pattern in developing vertebrate tissues remain poorly understood. We have addressed this issue in the embryonic anterior pituitary, an organ in which distinct hormone cell types are generated in a precise temporal and spatial order from an apparently homogenous ectodermal primordium. We provide evidence that in this tissue the coordinate control of progenitor cell identity, proliferation and differentiation is imposed by spatial and temporal restrictions in FGF- and BMP-mediated signals. These signals derive from adjacent neural and mesenchymal signaling centers: the infundibulum and ventral juxtapituitary mesenchyme. The infundibulum appears to have a dual signaling function, serving initially as a source of BMP4 and subsequently of FGF8. The ventral juxtapituitary mesenchyme appears to serve as a later source of BMP2 and BMP7. In vitro, FGFs promote the proliferation of progenitor cells, prevent their exit from the cell cycle and contribute to the specification of progenitor cell identity. BMPs, in contrast, have no apparent effect on cell proliferation but instead appear to act with FGFs to control the initial selection of thyrotroph and corticotroph progenitor identity.

Key words: Progenitor cell, Corticotroph, Pituitary, BMP, FGF, Spatial patterning

INTRODUCTION

In vertebrate embryos, insight into the mechanisms by which secreted factors control tissue development has emerged from several different systems. Studies of mesodermal and neural tube patterning have provided information on the role of inductive factors in controlling the spatial pattern of progenitor cell identity and fates (Tanabe and Jessell, 1996; Bumcrot and McMahon, 1995; Smith, 1995) but have not resolved how the timing of terminal cell differentiation is regulated. In other embryonic tissues, notably the limb bud, the time at which cells leave the cell cycle appears to be an important determinant of their eventual identity (Tabin, 1995) but here it is less clear how environmental signaling is coordinated with temporal controls on progenitor cell identity. Thus, the mechanisms by which temporal and spatial controls on progenitor cell differentiation are coordinated to establish vertebrate tissue pattern remain poorly understood.

We have attempted to address this issue through an analysis of the development of a specialized endocrine organ, the pituitary gland. We selected the pituitary for analysis because each of its component hormone cell types have been defined (reviewed in Treier and Rosenfeld, 1996) and are known to be generated in a precise temporal and spatial order from an apparently homogeneous ectodermal primordium (Simmons et al., 1990; Japón et al., 1994; reviewed in Voss and Rosenfeld, 1992). The pituitary has a dual embryonic origin. The posterior pituitary derives from the infundibulum (INF), an evagination of the ventral diencephalon, whereas the anterior pituitary (AP) derives from Rathke’s pouch (RP), a specialized region of the oral roof ectoderm. In the mouse, AP development is initiated as the oral roof ectoderm involutes towards the ventral diencephalon to form RP. Subsequently, RP detaches from the adjacent ectoderm to form the AP. Soon after detachment of the AP, an early TSH-secreting population of thyrotrophs is generated in its ventral-most domain whereas ACTH secreting corticotrophs differentiate in an adjacent, intermediate, domain. At this stage, cells in the dorsal domain, close to the INF, continue to proliferate (Drolet et al., 1991; Ikeda and Yoshimoto 1991). These dorsal cells differentiate only at later stages, generating a second population of thyrotrophs, as well as somatotrophs, gonadotrophs and lactotrophs. The generation of most of these later-born hormone cell populations appears to be controlled by the activity of the POU-domain protein Pit1 (Li et al., 1990; Lin et al., 1994; Simmons et al., 1990; Sornson et al., 1996; Rhodes et al., 1994).
Several transcription factors in addition to Pit1 have been shown to regulate the commitment, proliferation and differentiation of pituitary cell types (Sheng et al., 1996; Sornson et al., 1996; Treier and Rosenfeld, 1996). Amongst these, members of the LIM homeobox gene family are expressed by cells in the AP prior to the onset of hormone gene expression. The LIM homeobox gene Lhx3 (Lim3/PLim) is expressed over a prolonged period of AP development (Bach et al., 1995; Zhadanov et al., 1995). Lhx3 null mice exhibit an arrest of AP development and most hormone-producing cell types are absent (Sheng et al., 1996). Another LIM homeobox gene, Isl1, is expressed in a variety of hormone secreting cell types, including cells in the AP (Thor et al., 1991). The early lethality of Isl1 null mice (Pfaff et al., 1996) has so far precluded an analysis of its function in pituitary development but Isl1 is known to be required for the generation of pancreatic endocrine cell types (Ahlgren et al., 1997).

The apposition of the INF and RP has led to suggestions that signaling between these two cell groups influences the growth of cells in the AP (Schwind, 1928; Diakoku, 1982). Consistent with this, mice in which Nkx2.1, a transcription factor expressed in the neural tube has been inactivated by gene targeting show defects in the development of the ventral diencephalon and the differentiation of the AP is also impaired (Kimura et al., 1996). However, the source, identity and mechanism of action of the inductive signals that control the early development of the AP remain to be defined. In this paper, we examine the origin and identity of secreted factors that control the differentiation of progenitor cells in the AP using LIM homeobox genes and polypeptide hormones as molecular markers in combination with in vitro assays of pituitary cell differentiation.

**MATERIALS AND METHODS**

**Animals**

Embryos were collected from CBA/B6 mice. The day of the appearance of the vaginal plug was considered embryonic day 0.5 (E0.5). Somite number was used to determine developmental stages up to E11.

**Isolation and culture of Rathke’s pouch**

RP and associated ventral diencephalic tissue was dissected at 4°C in L15 medium (GibcoBRL) and incubated with Dispase (Yamada et al., 1993) for 7 minutes. RP, the ventral hypothalamus and the INF were then separated from surrounding mesenchyme. The ventral juxtapituitary mesenchyme (VJM) was isolated from E11.5 embryos. RP explants were isolated from 28-31 somite embryos prior to Isl1 down-regulation. In experiments involving COS cells, RP tissue was derived from 35-37 somite (E10.5) and E11.5 embryos. The INF was isolated from E10.5-E12.5 embryos. Explants were cultured in collagen gels (Yamada et al., 1993) in OPTI-MEM (GibcoBRL) supplemented with N2 (GibcoBRL) for 24-96 hours. DEAE Affi-gel blue beads (Bio-Rad) or heparin beads (Pharmacia) were soaked in PBS or in PBS containing recombinant FGF8 or FGF2 (GibcoBRL).

**cDNA clones**

Lhx3 and FGF8 cDNAs were cloned from E11 AP and INF, respectively, using RT-PCR. The human BMP2 expression construct was provided by P. Brickell, human BMP7 cDNA by K. Liem and the mouse BMP4 expression construct by R. Derynck.

**Expression and purification of FGF8**

The FGF8 cDNA was used to isolate a cDNA corresponding to the FGF8(3) splice variant (Crossley and Martin, 1995). For bacterial expression, the pET32 expression system (Novagen) was used. A 6x His tag was fused to the N terminus of FGF8(3) and the fusion protein was purified on a Ni-NTA column (Qiagen). The eluted protein was refolded in 5 mM glutathione by successive removal of the urea by dialysis. The purity (90%) and concentration (approx. 500 μg/ml) of the FGF8 protein was estimated from a coomassie-stained protein gel using BSA as a reference standard.

**Expression in COS cells and preparation of conditioned media**

Transfection and aggregation of COS cells were performed as described by Roelink et al. (1994). Control vectors were without insert or contained Shh cDNA in antisense orientation. Conditioned medium from BMP2 or mock-transfected COS cells were concentrated 30 fold using Centricon spin filters. 10-25 μl of concentrated conditioned medium was added to 0.5 ml culture medium.

**Immunohistochemistry and in situ hybridization**

Immunohistochemistry was performed as described by Yamada et al. (1991). Isl1 was detected by using rabbit anti-Isl1 antibodies (Thor et al., 1991; Ericson et al., 1992). Antisera to αGSU, ACTH and TSHβ were obtained from the National Hormone and Pituitary Program, NIDDK, USA. PCR fragments and cDNAs (see above) were used as templates to prepare digoxigenin-labeled RNA probes. In situ hybridization was performed essentially as described by Schaeren-Wiemers and Gerfin Moser (1993).

**BrdU labeling**

After 40 hours in culture, RP explants were incubated with BrdU (10 μM, Sigma) for 75 minutes and the fixed in 4% paraformaldehyde. BrdU+ cells were detected using an anti-BrdU monoclonal antibody (Becton-Dickinson).

**RESULTS**

**Progression of cell differentiation in Rathke’s pouch**

The pattern of cell differentiation in the embryonic mouse AP can be monitored by the spatially restricted expression of hormone genes (Rhodes et al., 1994). The pattern of cell types in the AP is first evident around E12.5. Thyrotrophs, defined by expression of the α-glycoprotein (αGSU) and thyroid stimulating hormone β (TSHβ) subunits, differentiate in a ventral domain (Fig. 1A' ,B' ) and corticotrophs, defined by expression of ACTH, differentiate in the intermediate domain (Fig. 1C'). At this stage cells in the dorsal domain, close to the INF, remain proliferative and do not express terminal markers of hormone cell differentiation (Fig. 1D'; see also Ikeda and Yoshimoto, 1991).

To investigate how this early pattern of cell types is generated from cells in RP we first examined the temporal expression, in situ, of the LIM homeobox genes Isl1 and Lhx3. At the 10 somite stage (E8.5), expression of Isl1 is detected in oral roof ectoderm cells that underlie the ventral diencephalon and give rise to RP (Fig. 1B). At E9.5, all RP cells express Isl1 (Fig. 1H and data not shown) but at later times (E10.5-E11), Isl1 expression is gradually extinguished from RP cells located closest to the INF (Fig. 1N). The expression of Isl1, however, persists in cells in the ventral domain of RP and by E11.5, these cells have begun to express αGSU (Fig. 1T and data not...
shown). We refer to these ventral Isl1+/αGSU+ cells (Fig. 1X,A’,B’) as prospective thyrotrusts since only at E12.5, do they initiate expression of TSHβ, a definitive thyrotrust marker (Fig. 1B’ and data not shown). Expression of Lhx3 (Bach et al., 1995; Zhadanov et al., 1995) is initiated at the 20 somite stage (E9.5) in the dorsal domain of RP (Fig. 1A,G) and from E10.5-E11.5 Lhx3 appears to be expressed at high levels by all cells in RP (Fig. 1M,S; Bach et al., 1995; Zhadanov et al., 1995). By E12.5, however, expression of Lhx3 is no longer uniform: there is a high level in the dorsal domain of the AP, a moderate level in the intermediate domain and a very low level in the ventral domain (Fig. 1Y). Thus, Isl1 and Lhx3 appear initially to be coexpressed by cells in RP but their expression subsequently segregates into distinct domains within the AP (Fig. 1Y,Z).

The early potential of Rathke’s pouch cells
To test if the regulation of transcript factor expression and the early pattern of hormone cell differentiation within RP is controlled intrinsically or by signals from surrounding tissues, we isolated RP explants from E10 embryos. At this stage virtually all cells still express Isl1 and Lhx3 (Fig. 1G,H and data not shown). Explants were cultured in vitro for 65 hours and the expression of Lhx3, Isl1, αGSU and TSHβ was monitored. In these explants, ~90% of cells maintained Isl1 expression (Fig. 2B) and cells expressed only low levels of Lhx3 (Fig. 2A). Most Isl1+ cells expressed αGSU (Fig. 2C), but fewer than 1% of cells expressed TSHβ (data not shown). The down-regulation of Lhx3 and the maintenance of Isl1 expression in RP explants suggests that signals provided by surrounding cell types are required to establish the normal pattern of LIM homeobox gene expression and the ventral restriction of thyrotrust differentiation in RP. In addition, the maintenance of Isl1 expression and the onset of αGSU expression in vitro shows that most RP progenitor cells acquire molecular markers characteristic of prospective thyrotrusts in the absence of additional signals. The absence of expression of TSHβ in vitro, however, indicates that these cells are not able to progress to a definitive thyrotrust state.

Patterning of Rathke’s pouch by the infundibulum
The INF abuts the dorsal region of Rathke’s pouch and constitutes one potential source of factors that might regulate the early expression of LIM homeobox genes and the pattern of hormone cell differentiation. To test this possibility, E10 RP explants were grown in vitro, together with INF tissue isolated from E10.5 embryos. After 65 hours in coculture, Isl1 and αGSU expression was absent from most (80-90%) cells and the residual Isl1+ and αGSU+ cells were confined to the region of the explant distant from the junction with the INF (Fig. 2E,F). Lhx3 expression was maintained by most cells within these explants, with those cells located close to INF tissue expressing Lhx3 at a higher level than more distant cells (Fig. 2D). Complete extinction of Isl1 and αGSU expression was achieved when two INF explants were placed at opposite sides of RP (Fig. 2H,I) and now Lhx3 expression was maintained at a high level by most cells (Fig. 2G). Similar results were observed when RP explants were grown with E11.5-E12.5 INF (data not shown). Basal hypothalamic tissue isolated at E11.5 did not mimic the ability of the INF to restrict the expression of Isl1 in RP explants (data not shown). Thus, within the relevant region of the embryonic ventral diencephalon, the ability to pattern RP and control the position of prospective thyrotrust differentiation appears to be restricted to the INF.

FGFs mimic the patterning activity of the infundibulum
Two secreted factors, BMP4 and FGF8, are expressed in the embryonic INF (Jones et al., 1991; Heikinheimo et al., 1994; Crossley and Martin, 1995; MacArthur et al., 1995), raising the possibility that one or both contribute to the patterning activity of the INF. To begin to assess the potential contributions of these two factors, we analyzed their profiles of expression in the INF over the period that the early pattern of RP cells is established. The expression of BMP4 in the presumptive INF is initiated at E8.5 and a high level of BMP4 is detected by E9.5 (Fig. 1C,I; Jones et al., 1991), a stage when Isl1 is expressed by all RP cells (Fig. 1H). By E11.5, however, expression of BMP4 has been extinguished (Fig. 1U; Jones et al.,1991). FGF8 expression is initiated in the presumptive INF at E9.25 (Fig. 1D,J) and persists until at least E14.5 (Fig. 1P and data not shown; Crossley and Martin, 1995). The onset of FGF8 expression in the INF coincides with that of Lhx3 expression (Fig. 1A,G) and precedes the extinction of Isl1 in RP cells (Fig. 1N). The ability of the INF over the period E10.5 to E12.5 to extinguish Isl1 and promote Lhx3 expression, thus, corresponds more closely to the temporal expression of FGF8 than of BMP4.

To investigate whether FGF8 can mimic the ability of the INF to repress Isl1 and maintain Lhx3 expression, E10 RP explants were grown for 65 hours in contact with beads adsorbed with FGF8 protein. In these explants Lhx3 expression was maintained at a high level and Isl1+ αGSU+ cells were restricted to the region of the explant distant from the beads (Fig. 3D-F). In contrast, with control beads most cells ceased to express Lhx3 (Fig. 3A) and Isl1 and αGSU expression were detected in many cells (Fig. 3B,C). Complete repression of Isl1 was not observed in presence of FGF8 beads, possibly because of the low activity of our bacterially expressed FGF8 protein (Fig. 3E). Since FGF8 and FGF2 show similar inductive activities in many tissues (Fallon et al., 1994; Cohn et al., 1995; Crossley et al., 1996b; Mahmood et al., 1995; Neubuser et al., 1997) we tested whether FGF2 also repressed Isl1 expression in RP explants. Explants grown with FGF2 beads did not express Isl1 and αGSU (Fig. 3H,I) and Lhx3 was expressed uniformly at a high level (Fig. 3G). Thus, both FGF2 and FGF8 mimic the ability of the INF to repress Isl1, maintain Lhx3 and control the pattern of prospective thyrotrust differentiation. Since the INF does not express FGF2, its signaling activity is likely to be mediated by FGF8.

We next examined whether cells in the ventral domain of AP that have progressed to an Isl1+αGSU+ state remain sensitive to signals from the INF and to FGFs. Explants isolated from the ventral domain of E11.5 AP were cultured for 40 hours either alone, with E11.5 INF or with FGF beads and the expression of Isl1, αGSU and TSHβ examined. Under all three conditions, ~90% of cells within these explants maintained Isl1 (Fig. 3K,N and data not shown) and αGSU (data not shown) expression despite exposure to INF or FGF2. These cells also expressed the definitive thyrotrust marker TSHβ (Fig. 3L,O). Conversely, few if any cells maintained expression of Lhx3 (Fig. 3J,M). These results show that by E11.5, Isl1+/αGSU+
cells are no longer sensitive to inhibition by FGFs and thus appear to be committed to a definitive thyrotroph fate.

**Late FGF8 signaling controls corticotroph differentiation**

The activity of the INF and the expression of FGF8 are maintained at stages after the commitment of thyrotrophs (Fig. 1V), raising the possibility that the differentiation of other hormone cell types may be regulated by FGF8-mediated signals from the INF. To test this possibility we first analyzed the fate of the Isl1− progenitor cells present in the intermediate and dorsal domains of the AP. By E12.5-E13, Isl1− progenitor cells in the intermediate domain have begun to differentiate into ACTH+ corticotrophs (Fig. 1C; Fig. 4A), whereas Isl1− cells in the dorsal domain continue to proliferate and fail to express hormone markers. We examined whether the restriction of corticotroph differentiation to the intermediate domain observed in vivo is recapitulated when E11.5 AP is
isolated and grown in vitro. After 40 hours in culture, corticotrophs were generated throughout both the intermediate and dorsal domains of the AP and were excluded only from the ventral thyrotroph domain (Fig. 4B; data not shown). This result raises the possibility that the domain of corticotroph differentiation is normally constrained by signals from the INF.

To test if signals from the INF are able to restrict corticotroph differentiation to the intermediate domain, the entire E11.5 AP was grown with INF for 40 hours. In the presence of INF the generation of ACTH+ cells was largely restricted to the intermediate domain (Fig. 4C). To exclude the possibility that the appearance of corticotrophs in dorsal E11.5 explants results from the migration of a ventral population of corticotroph progenitors or the action of inductive signals from thyrotrhps we isolated explants that comprised solely the dorsal or intermediate regions of E11.5 AP and cultured them with INF, with FGF2 beads or with control beads. In explants cultured alone or with control beads 15-20% of cells expressed ACTH (Fig. 4D and data not shown) and Lhx3 was expressed at a low level (Fig. 4G and data not shown). ACTH expression was almost completely inhibited when dorsal or intermediate explants were grown with INF or FGF2 beads (Fig. 4E,F and data not shown) and conversely, the expression of Lhx3 was maintained at a high level (Fig. 4H). These results suggest that FGF-mediated signals from the INF are sufficient to

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**Fig. 2.** Patterning of Rathke’s pouch by the infundibulum and FGF8. (A-C) E10 RP explants grown alone for 65 hours express low levels of Lhx3 (A) whereas most cells maintain expression of Isl1 (B) (88% of cells, n=25 explants) and many of these initiate expression of αGSU (C). (D-F) Lhx3 expression is maintained in RP cells when cocultured with E10.5 INF tissue (D). Lhx3 expression is higher in RP cells adjacent to INF compared to more distant cells. Isl1 is downregulated in RP by INF tissue and cells that maintain Isl1 expression (20% of cells, n=10 explants) are located at a distance from the INF (E). αGSU expression is detected in RP cells located at a distance from the INF tissue (F). (G-I) Most cells within RP express high levels of Lhx3 (G) when cocultured with two INF whereas Isl1 (H) and αGSU (I) are not expressed in RP when cultured with two INF (<1% Isl1+ cells, n=8 explants).

**Fig. 3.** Patterning of Rathke’s pouch by FGFs. (A-C) E10 RP explants grown with PBS-soaked beads express low levels of Lhx3 (A). Most cells maintain expression of Isl1 (B) (85% of cells, n=6 explants) and many cells initiate expression of αGSU (C). (D-F) Expression of Lhx3 (D) is maintained in RP when cultured in contact with FGF8-soaked beads. Isl1 expression is downregulated and cells that maintain Isl1 (E) (32% of cells, n=8 explants) and αGSU (F) expression are located at a distance from the bead. (G-I) Most cells within RP express high levels of Lhx3 (G) when cultured in contact with FGF2-soaked beads. Isl1 (H) and αGSU (I) expression are not detected in this condition. Soaking concentrations of FGF2 below 25 ng/ml resulted in the maintained expression of Isl1 by some cells distant from the bead. (J-O) Lhx3 expression is not maintained by INF (J) or FGF2 beads (M) in explants of the ventral anterior pituitary (APv) isolated from E11.5 embryos. Under both conditions most or all cells maintain expression Isl1 (KN) and initiate expression of TSHβ (L,O), a definitive thyrotrhph marker.

b, beads.
restrict corticotroph differentiation to the intermediate domain of the AP.

**BMPs promote prospective thyrotoph and suppress corticotroph differentiation**

We next considered whether the early patterning of cells in the AP can be accounted for exclusively by FGF-mediated signaling from the INF. The ventral domain of RP contains IsI1+/αGSU+ presumptive thyrotophs and is flanked by VJM cells that express BMP2 and BMP7 (Fig. 1Q,W and data not shown). Moreover, the presumptive INF tissue initiates expression of ACTH. The ventral domain of RP contains Isl1+ cells detected in the intermediate and dorsal domains of E11.5 AP explants cultured alone for 40 hours. No ACTH+ cells are detected in the ventral domain of AP (region adjacent to dotted line) that expresses TSHb (data not shown). (C) E11.5 AP explant grown in contact with INF. Only cells at a distance from the INF tissue initiate expression of ACTH. (D) Numerous ACTH+ cells (17%, n=8) are detected in E11.5 dorsal AP (APd) explants grown alone or with PBS-soaked beads (data not shown) for 40 hours. (E) Expression of ACTH is suppressed at high levels in APd explants grown in contact with E11.5 INF. (F) Expression of ACTH is suppressed at high levels in APd explants grown in contact with FGF2-soaked beads. (G) Very few Lhx3+ cells are detected in E11.5 dorsal AP (APd) explants grown alone or with PBS-soaked beads for 40 hours. (H) Lhx3 expression is maintained at high levels in APd explants grown in contact with FGF2-soaked beads.

To test this possibility, RP and dorsal AP explants were isolated at E10.5 and E11.5 respectively and were grown for 60 hours in contact with COS cells expressing BMP2 or with mock-transfected COS cells. At E10.5, Isl1 expression had ceased in approx. 50% of cells (Fig. 1N) and in dorsal AP at E11.5 no cells expressed Isl1 (Fig. 1T). When E10.5 RP explants were grown on control COS cells, <15% of RP cells expressed Isl1 and αGSU (Fig. 5A,D) and many ACTH+ cells were present (Fig. 5G). In contrast, addition of BMP2 resulted in the expression of Isl1 in E10.5 RP explants (n=6 explants). (D) Few cells in E10.5 RP cells grown with mock-transfected COS cells express αGSU. (E) Most E10.5 RP cells grown with BMP2 expressing COS cells express αGSU. (F) BMP2 fails to induce αGSU expression in E11.5 APd explants (n=6 explants). (G) ACTH+ cells are detected in E11.5 APD explants grown with mock-transfected COS cells, (n=8 explants). (H) ACTH expression is suppressed by exposure of E10.5 RP explants to BMP2-expressing COS cells (n=8 explants). (I) ACTH+ cells are detected in E11.5 APD explants exposed to BMP2 (n=6 explants).

**Integration of FGF and BMP signaling by anterior pituitary cells**

The opponent activities of FGFs and BMPs on the establishment of progenitor cell identity raised the issue of the
fate of cells exposed simultaneously to both factors. Cells in the ventral domain of RP coexpress Isil and Lhx3, which suggests that these cells are subject to concurrent FGF and BMP signaling. We therefore considered whether the maintenance of the ventral thyrotroph progenitor cell population might involve a signal that opposes the actions of FGF8. One source of such a signal might be VJM cells.

To test this possibility, RP explants were grown in contact with FGF2 beads but in the presence of VJM. Many Isil+ cells were detected in the presence, but not in the absence of VJM (Fig. 6B,C). To test if this activity is mimicked by BMPs, RP explants were cultured in contact with FGF2 beads but also with BMP2 or control medium. In the presence of BMP2, but not with control medium, many Isil+ cells were detected (Fig. 6B,D). However, no αGSU+ cells were detected in this condition (Fig. 6G,H). Expression of αGSU was detected in explants grown for an additional 24 hours after removal of FGF2 beads (data not shown), indicating that the FGF-induced blockade of thyrotroph progenitor progression to an αGSU+ state is reversible. These findings support the hypothesis that VJM cells, and specifically the BMPs they produce, function to oppose the actions of FGF8 and maintain an early ventral population of thyrotroph progenitors. Nevertheless, BMP signaling is not able to overcome the FGF-mediated block in the conversion of thyrotroph progenitors to prospective thyrotrhops.

Hormone cell differentiation and cell cycle exit

The ability of the INF and FGFs to maintain a high level of Lhx3 expression and to prevent the terminal differentiation of RP cells prompted us to examine whether the INF and FGF might maintain RP cells in a proliferative state. To test this, RP explants from E10 embryos were cultured for 40 hours in contact with INF, FGF8 or FGF2 beads and the incorporation of BrdU (75 minute pulse) was measured. An approx. 6-fold increase in the number of BrdU+ cells over controls was detected in the presence of INF or FGF2 and an approx. 4-fold increase was detected with FGF8 (Fig. 6J; Table 1). In contrast, BMPs had no effect on BrdU incorporation by cells in E10 RP explants (Fig. 6I; Table 1). Moreover, the number of BrdU+ cells in E10 explants grown with either VJM or BMP2 and FGF2 was similar to that in explants exposed to FGF2 alone (Fig. 6J-L; Table1). Thus, BMP signaling does not prevent FGFs maintaining RP cells in a proliferative state.

Finally, we made use of the ability of FGF and BMP signaling to maintain cells at specific stages of hormone cell differentiation to investigate the point at which cells leave the cell cycle. To assay cells at the prospective thyrotrhoph stage, E10 RP explants were grown in the absence of FGF2 or BMP2 and BrdU incorporation measured at 40 hours. These explants contained many Isil+/αGSU+ cells (Fig. 2B,C), but few if any of these had incorporated BrdU (Table 1). To analyze cells at the thyrotrhoph progenitor stage, E10 RP explants were exposed jointly to FGF2 and either BMP2 or VJM. Such explants contained many Isil+/GSU- cells, many of which incorporated BrdU (Fig. 6K,L; Table 1). To assay cells at the corticotroph progenitor stage, E10 explants were grown in the presence of FGF2 alone. These explants contained many Isil+/corticotroph progenitors, many of which incorporated BrdU (Fig. 6J; Table 1). These results suggest that the progression from progenitor to prospective or definitive hormone cell types is accompanied by exit from the cell cycle. They show also that FGF signaling appears dominant over that of BMPs in the control of cell cycle exit and in the conversion to hormone producing cell types.

DISCUSSION

This study provides evidence that the identity, proliferation and timing of progenitor cell differentiation in the early embryonic AP are controlled in large part by the coordination of FGF and BMP signaling. These factors are provided by two cell groups that act as local signaling centers: the INF and the VJM. The

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<th>Table 1. Infundibulum and FGFs but not BMP2 promote the proliferation of Rathke’s pouch cells</th>
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Percentage of BrdU+ cells in E10RP explants cultured alone or with BMP2, E11.5 INF, FGF2 beads, FGF8 beads or FGF2 beads and BMP2. After 40 hours in culture explants were pulsed with BrdU for 75 minutes and the percentage of BrdU+ cell determined. Number in parenthesis indicates number of explants analyzed. Values indicate mean ± s.d.
INF appears to have a dual signaling function, serving initially as a source of BMP4 and subsequently of FGF8. The VJM appears to serve as a later source of BMP2 and BMP7. In vitro, FGFs promote the proliferation of progenitor cells, prevent their exit from the cell cycle and contribute to the specification of progenitor cell identity. BMPs, in contrast, have no apparent effect on cell proliferation but instead appear to act with FGFs to control the initial selection of thyrotroph and corticotroph progenitor identity. Our results suggest a model in which the coordinated actions of BMPs and FGFs regulate the temporal and spatial progression of progenitor cell differentiation that underlies the emergence of pattern in the developing AP.

The dual roles of FGF8 and BMPs in controlling AP development that are suggested from these studies have parallels with proposed mechanisms of cell patterning in other vertebrate tissues. In the developing limb, FGF8 is expressed in the apical ectodermal ridge (AER) and appears to maintain underlying mesenchymal cells in a proliferative state (reviewed by Tabin, 1995; Tickle, 1995). The patterning of tissues that derive from the limb mesenchyme appears, however, to be dependent upon the actions of other secreted signals, Sonic Hedgehog and possibly BMPs (Niswander and Martin 1993; Francis et al., 1994; Laufer et al., 1994; Yang and Niswander 1995; Zou and Niswander 1996). There is also evidence that the coordinate activities of BMPs and FGF8 control the position of initiation of tooth development (Neubuser et al., 1997). Thus, the integration of FGF and BMP signaling appears to coordinate the timing and pattern of cell differentiation in many vertebrate tissues.

**Coordinated FGF8 and BMP signaling controls the progression of progenitor cell differentiation**

We discuss our results in the context of a model which may provide insight into how the integration of FGF and BMP signaling coordinates the spatial and temporal control of cell differentiation in the embryonic AP. We focus first on thyrotroph differentiation, which we consider in three successive stages: the establishment of an early Isl1+ thyrotroph progenitor state, the progression to an Isl1+/αGSU+ prospective thyrotroph state and the conversion to Isl1+/αGSU+/TSHβ+ definitive thyrotrhps (Fig. 7A).

At early stages of AP development in vivo, all cells in RP express Isl1 and when grown in vitro cells in RP explants maintain Isl1 expression but stop proliferating and initiate expression of αGSU. These observations suggest that RP is initially composed of cells with the properties of thyrotroph progenitors and that these cells have the potential to progress to an αGSU+ state characteristic of prospective thyrotrhps. They also raise the question of how the early thyrotroph progenitor state is established. BMP4 is expressed in the INF at early stages, prior to the onset of FGF8 expression and its expression persists over the period that Isl1 is expressed uniformly within RP. Moreover, in vitro, BMPs can reinitiate Isl1 expression in RP cells (Fig. 7). These findings raise the possibility that an early phase of BMP4 signaling from the INF establishes the thyrotroph progenitor state.

Once the thyrotroph progenitor state is established, it appears that the maintenance of this state can occur independently of BMP signaling, but only in the absence of FGFs. Exposure of RP progenitors to FGFs switches cells from a prospective thyrotroph state to a corticotroph progenitor state. Thus BMPs and FGFs have opponent activities in the control of progenitor cell identity. In the presence of both factors, the action of BMPs is dominant and the thyrotroph progenitor state is maintained (Fig. 7B). The progression of thyrotroph progenitors to cells with markers of prospective thyrotrhps can also occur independently of BMP signaling but again is blocked in the presence of FGFs. At this step, however, FGF signaling is dominant (Fig. 7A). Thus, in the presence of FGFs, thyrotroph progenitors do not exit from the cell cycle and the onset of expression of αGSU is inhibited. The hierarchy of FGF and BMP signaling appears therefore to differ at successive stages of thyrotroph differentiation. The opponent activities of FGFs and BMPs on Isl1 expression in AP development has parallels with studies of tooth patterning which have shown that BMP4 signaling opposes the actions of FGFs in the regulation of Pax9 expression (Neubuser et al., 1997).

What controls the conversion of prospective into definitive thyrotrhps? In contrast to the two earlier steps, FGF signaling fails to inhibit this third step in thyrotroph differentiation. Moreover, this step appears to require a signal that is distinct from BMPs, since E10 RP cells grown in the presence of BMPs do not convert into definitive thyrotrhps. However, ventral Isl1+ cells isolated at E11.5 can generate definitive thyrotrhps, suggesting that this signal is supplied around the time of establishment of the presumptive thyrotroph state. This result argues that the early Isl1+/αGSU+ cells detected in RP explants in vitro are likely to represent the precursors of thyrotrhps rather than of gonadotrophs, a late born αGSU+ cell population which expresses the β subunits of LH and FSH rather than of TSH (Simmons et al., 1990; Japon et al., 1994). Our findings therefore support a model in which the three distinct steps in the differentiation of RP progenitors into thyrotrhps can be delineated (Fig. 7A), with each step exhibiting a differential sensitivity to BMP and FGF signaling.

Our results further suggest that the establishment of the corticotroph progenitor state initially requires the extinction of Isl1 expression by RP cells in response to FGF signaling. However, as with thyrotroph progenitors, the progression to definitive corticotrophs is blocked by FGF signaling. Thus, corticotroph differentiation may exhibit a biphasic dependence on FGF signaling; an early phase which requires FGF signaling and a later phase which requires the evasion of FGF signals. These late phase appears not to require the extrinsic factor implicated in the establishment of the definitive thyrotrhph state (Fig. 7A), since early corticotroph progenitors convert to definitive corticotrophs when grown alone in vitro.

**Temporal and spatial constraints on FGF and BMP signaling underlie the patterning of the anterior pituitary**

Our findings suggest also that the early patterning of the AP depends on temporal and spatial constraints on FGF and BMP signaling. We discuss below how such constraints might regulate the pattern of thyrotroph and corticotroph generation and maintain a pool of undifferentiated progenitor cells (Fig. 7B).

Cells in RP appear initially to represent an uniform population of thyrotroph progenitors. The subsequent restriction of thyrotroph progenitors to the ventral domain of the AP may be imposed by the gradual switch in the signaling
properties of the INF, from BMP4 to FGF8. Once FGF8 is expressed, however, cells in the ventral domain of RP, may be exposed to this factor since a high level of Lhx3 expression can be detected ventrally. If this is the case, how then is the thyrotop progenitor state maintained by ventral AP cells? Our results raise the possibility that the expression of BMP2 and BMP7 by the VJM provides a local source of BMPs that maintains the thyrotop progenitor state despite exposure of cells to FGFs. Between E11.5 and 12.5, however, Lhx3 expression is down-regulated and αGSU expression is initiated by ventral Isl1+ cells, suggesting that by this time cells have escaped the influence of FGF signaling and thus, may no longer depend on BMPs provided by the VJM (Fig. 7B).

Corticotrophs are generated in a domain immediately dorsal to that of thyrotops and derive from progenitors that have extinguished expression of Isl1, presumably in response to FGF8 signaling from the INF. However, corticotroph progenitors are initially evenly distributed in the dorsal and intermediate domains of the AP. The restriction in the differentiation of corticotrophs to the intermediate domain of the AP is likely to reflect the subsequent escape of more ventrally located corticotroph progenitors from a dorsal source of FGF signaling. In addition, since BMPs appear to induce thyrotop progenitors at the expense of corticotroph progenitors, the establishment of a corticotroph progenitor population may require evasion of BMP signaling. At early stages this could be achieved by the loss of BMP4 from the INF and by a limitation in the range of action of BMPs derived from the VJM and later, by a loss of competence of corticotroph progenitors to respond to BMPs by reinitiating expression of Isl1.

**Cell proliferation, tissue growth and pituitary pattern**

At early stages, cells in the most dorsal region of the AP remain proliferative and hormone cell types do not differentiate. The maintenance of a pool of dorsal progenitors is likely to reflect the persistence of FGF signaling. Our studies do not address the mechanisms by which later generated hormone cell types, lactotrophs, somatotrophs and gonadotrophs, arise from the dorsal proliferative progenitor cell population. However, the perpetuation of FGF signaling is maintained by ventral AP cells? Our results raise the possibility that the expression of BMP2 and BMP7 by the VJM provides a local source of BMPs that maintains the thyrotop progenitor state despite exposure of cells to FGFs. Between E11.5 and 12.5, however, Lhx3 expression is down-regulated and αGSU expression is initiated by ventral Isl1+ cells, suggesting that by this time cells have escaped the influence of FGF signaling and thus, may no longer depend on BMPs provided by the VJM (Fig. 7B).

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likely to expand the population of dorsal progenitors further, such that more distantly located progenitor cells progressively escape FGF signaling, and initiate terminal differentiation. Transcription factors such as 

Prop1 and Pit1 are required for the generation of most hormone cells that eventually differentiate within this dorsal domain (Ingraham et al., 1988; Li et al., 1990; Lin et al., 1994; Sornson et al., 1996; Treier and Rosenfeld, 1996). However, the identity of factors that might control the subtype identity of these cells remains unclear.

One final implication of our findings is that the FGF-driven promotion of cell proliferation is itself an important element in AP development. On the assumption that the range of FGF8 signaling is restricted spatially, any expansion in the size of the progenitor population will lead, in a self-limiting manner, to the escape of more distant cells from the influence of FGF8 signals. Thus, the FGF-driven expansion in the progenitor cell population in the AP may contribute both to the timing of terminal differentiation and to the resulting pattern of hormone cell types. A similar FGF-driven promotion of cell proliferation has been suggested to control the timing of mesenchymal cell differentiation along the proximodistal axis of the developing limb (reviewed by Tabin, 1995) and the temporal progression of neural differentiation in the mesencephalon (Crossley et al., 1996a; Lee et al., 1997). FGF signaling may therefore have a general role in coordinating temporal and spatial aspects of tissue patterning in vertebrates.

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REFERENCES


Patterning of the anterior pituitary


