Paired-like Repression/Activation in Pituitary Development

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ABSTRACT

Pituitary gland development is controlled by signals that guide expression of specific combinations of transcription factors that dictate serial determination and differentiation events. One class of factors is *paired*-like homeodomain factors. Two that have been investigated are the repressor Hex1/Rpx and activator prophet of Pit-1 (Prop-1), which exert selective roles during pituitary development. The opposing actions of these factors provide one aspect of pituitary organogenesis.

I. Prophet of Pit-1 (Prop-1)

The anterior pituitary gland develops from a midline structure contiguous with the primordium of the ventral diencephalon (Watanabe, 1982; Couly and Le Douarin, 1988). It integrates signals from the peripheral and central nervous systems, regulating production and secretion of critical regulatory hormones, including growth hormone (GH), prolactin, thyroid-stimulating hormone (TSH), gonadotrophins, and adrenocorticotrophic hormones by specific cell types (somatotropes, lactotropes, thyrotropes, gonadotropes, and corticotropes, respectively) (Voss and Rosenfeld, 1992). The initial organ determination involves interaction of the primordial stomadeal ectoderm and the neuroepithelium during head folding at embryonic day (e) 8.5 in the mouse (Simmons *et al.*, 1990). The resulting development of Rathke's pouch (RP) is characterized by expression of multiple homeodomain factors, including the LIM homeodomain factors P-Lim/Lhx3 and Lhx4 (Seidah *et al.*, 1994; Bach *et al.*, 1995; Sheng *et al.*, 1996, 1997) and the OTX-related homeodomain factors P-OTX/Pitx1 (Lamonerie *et al.*, 1996; Szeto *et al.*, 1996) and Pitx2 (Gage *et al.*, 1999; Lin *et al.*, 1999; Lu *et al.*, 1999). Restriction of expression of the *paired*-like homeodomain factor Hesx1/Rpx (Hermesz *et al.*, 1996) to RP also occurs. Following proliferation and early organ expansion, different cell phenotypes arise in a distinct spatial and temporal fashion.

The Snell and Jackson allelic murine dwarf (dw, dw^J) mutations established that the product of the POU domain gene, Pit-1 — expressed in thyrotropes, somatotropes, and lactotropes (Camper *et al.*, 1990; Li *et al.*, 1990) — was required for initial expression of genes encoding secreted hormonal products and receptors for releasing factors. Pit-1 is required later for the continued expression of the *Pit-1* gene itself and the proliferation and survival of these three cell types (Godfrey *et al.*, 1993; Li *et al.*, 1993; Rhodes *et al.*, 1993). A mouse genetic defect, referred to as the Ames dwarf (df), was mapped to chromosome 11 (Bartke, 1965; Buckwalter *et al.*, 1991) and proved to be cell-autonomous (Buckwalter *et al.*, 1991), resulting in a hypoplastic anterior pituitary similar to that of the Snell and Jackson dwarfs. In contrast to the complete absence of somatotropes, lactotropes, and thyrotropes in the Snell mouse, the Ames mouse pituitary gland contains between $\approx 1\%$ (Gage *et al.*, 1996b) and 0.001% (Andersen *et al.*, 1995) of the normal complement of somatotropes as well as a few lactotropes and thyrotropes (Gage *et al.*, 1996b). Failure to detect *Pit-1* transcripts later in ontogeny (Andersen *et al.*, 1995) is consistent with the hypothesis that the Ames dwarf mutation is epistatic to Pit-1.

The region surrounding the df locus was generally mapped using a CAST/Ei $\times$ C57BL/6J intercross and a *Mus spretus* $\times$ C57BL/6J backcross to related CA-repeat markers (Dietrich *et al.*, 1996) to the previously identified locations of the markers Pad-1 and the interleukin (IL) cluster (IL-3, IL-4, and IL-5) (Buckwalter *et al.*, 1991). Four markers provided two proximal and two distal loci that were used to genotype progeny of a large intercross between Cast/Ei and DF/B df/df . The mice were genotyped and phenotypically characterized by size at 4–7 weeks. To obtain more closely linked markers, genetically directed representational difference analysis (GDRDA) (Lisitsyn *et al.*, 1993, 1994) was performed using two subsets of F_2 and F_3 df/df mice with local recombination events, targeting 0.5-centimorgan (cM) and 0.1-cM intervals surrounding df . This technique permitted documentation of a contig. Complementary DNA selection was used to identify the candidate gene, referred to as *Prop-1* (Sornson *et al.*, 1996). This gene encodes a *paired*-like homeodomain factor and a single functional point mutation S83P that decreased DNA binding to the cognate palindromic DNA sites, decreasing the ability to regulate DNA site-dependent genes.

Expression of Prop-1 commences after initial establishment of pituitary structure on e10.5. The expression initially is observed dorsally but subsequently involves most cells in the anterior pituitary gland. The df mutation caused dysmorphogenesis of RP at e12–e12.5, with convolution of the lumen and a failure of expression of the caudomedial Pit-1 lineage. A delayed appearance of gonadotropes was noted but corticotropes appeared as expected. Temporally extended expression of the *paired*-like homeodomain repressor Hesx1/Rpx1 also was noted. More recently, subtractive hybridization techniques have suggested

targets, perhaps including components of the Wnt pathway, which may be under control of Prop-1 (Douglas *et al.*, 2001).

II. Rpx/Hesx1 in Pituitary Development

In the anterior neural ridge (ANR), from which the pituitary is derived, a series of transcription factors initially induced in the ANR remain present following the invagination of oral ectoderm to form RP, which subsequently gives rise to the pituitary gland (reviewed in Sheng and Westphal, 1999, and Dasen and Rosenfeld, 2001). Following RP formation, opposing dorsal fibroblast growth factor/bone morphogenetic protein 4 (FGF/BMP4) and ventral Sonic hedgehog (Shh)/BMP2 gradients impart positional and proliferative signals to the pituitary progenitor field, acting to induce combinatorial patterns of transcription factor gene expression. As many mediators of the early signaling events appear capable of acting as either transcriptional activators or repressors, a critical issue has been to define the mechanisms by which these transcriptional codes positively and/or negatively influence downstream gene programs. We examined this with respect to Prop-1 and Rpx/Hesx1.

Multiple transcription factors in pituitary development act in sequential fashion to mediate the appearance of six hormone-producing cell types, which include the homeodomain factors expressed in the anterior neural plate as well as *Pitx1/2*, *Pax6*, and *Hesx1/Rpx*. Upon invagination of the oral ectoderm, the LIM homeodomain factor *Lhx3* is induced on e9.5 in the nascent RP and is required for initial organ commitment and growth (Sheng *et al.*, 1996). Subsequently, a second *paired*-like homeodomain factor, *Prop-1*, appears on e10.5 and is required for determination of four ventral cell types, including the Pit-1-dependent lineages (somatotropes, lactotropes, and thyrotropes) and gonadotropes (Sornson *et al.*, 1996; Wu *et al.*, 1998). Additional factors — including Pit-1, steroidogenic factor-1 (SF-1), and GATA-2 — are required for cell-type specification within these four lineages (reviewed in Dasen and Rosenfeld, 2001).

Based on the analysis of Ames dwarf (*df*) mice, which bear a hypomorphic mutation in the homeodomain of the *Prop-1* gene, and more severe human mutations (Cogan *et al.*, 1998; Fluck *et al.*, 1998; Wu *et al.*, 1998; Deladoey *et al.*, 1999; Pernasetti *et al.*, 2000), Prop-1 is necessary to activate gene programs required for ventral proliferation and determination of four cell lineages. Hesx1, in contrast, is typical of critical transcriptional repressors that regulate the development of multiple placodally derived anterior structures (Dattani *et al.*, 1998; Martinez-Barbera *et al.*, 2000; Thomas *et al.*, 2001). Attenuation of *Hesx1* expression in the developing pituitary coincides with the Prop-1-dependent progression of the pituitary, suggesting that temporal regulation of *Hesx1* expression is essential for deployment of the Prop-1-dependent gene activation program (Gage *et al.*, 1996b; Hermes *et al.*, 1996; Sornson *et al.*, 1996).

III. Functional Antagonism Between *Hesx1* and *Prop-1* in Pituitary Organogenesis

The reciprocal expression and actions of *Prop-1* and *Hesx1* suggested that their functional interactions might regulate pituitary organogenesis. *Hesx1* expression initially marks the oral ectoderm and invaginating RP but then becomes restricted to RP (excluding the ventral rostral tip), where it is maintained until e13.5. In contrast, *Prop-1* expression is undetectable until e10.5–e11, becomes maximal during the ventral migration of pituitary lineage precursors beginning at e12.5, and remains detectable between e14.5–e15.5. Following e15.5, expression is maintained only at low levels (Figure 1). *Prop-1* and *Hesx1* can each bind effectively to a well-described palindromic site (Wilson *et al.*, 1993) as cooperative homodimers or heterodimers, with *Prop-1* acting as an activator but not as a repressor (Sornson *et al.*, 1996). *Hesx1* acts only as a repressor that can inhibit *Prop-1* activation function. Both the N-terminal and homeodomain (HD) regions of *Hesx1* can independently act as repressors.

Based on the requirement for *Hesx1* for development of anterior organs (Dattani *et al.*, 1998; Martinez-Barbera *et al.*, 2000; Thomas *et al.*, 2001), we investigated defects in pituitary development in *Hesx1* gene-deleted mice. A striking, although infrequent ($\approx 5\%$), phenotype (*Hesx1*^{−/−} mice) was a complete lack of the pituitary gland (Figure 2). Initial thickening of oral ectoderm and minimal activation of *Lhx3* initially was observed at e12.5 but the pituitary gland was absent by e18.5. Thus, we tested whether *Prop-1* would be capable of phenocopying the pituitary arrest observed in *Hesx1*^{−/−} mice. When *Prop-1* expression was targeted in transgenic mice under control of a promoter expressed only in oral ectoderm, RP, and first branchial arch (Treier *et al.*, 1998,2001), the mice exhibited absent anterior pituitary glands with no initial induction of *Lhx3* expression but no abnormalities of developing ventral diencephalon (Figure 3).

FIG. 1. *In situ* hybridization showing reciprocal temporal expression of *Hesx1* and *Prop-1* during the initial phases of pituitary organogenesis. RP indicates Rathke's pouch.

FIG. 2. In the most-severe class of *Hesx1* mutants, the anterior pituitary (AP) is absent by embryonic day (e) 18.5. Hematoxylin and eosin (H&E) staining. Arrowhead indicates where the gland should be found.

FIG. 3. Analysis of *Pitx1/Prop-1* transgenic founder embryos shows the absence of the AP when *Prop-1* is temporally misexpressed. H&E staining. Arrowhead indicates where the developing organ should be found.

Thus, either absence of *Hesx1* or the premature expression of *Prop-1* can block pituitary organogenesis.

IV. Multiple Pituitary Patterning and Growth Defects in *Hesx1* Mutant Mice

Unexpectedly, however, the highest percentage of pituitaries with *Hesx1* gene deletion resulted in the formation of multiple oral ectoderm invaginations, generating supernumerary pituitary glands with dramatic cellular overproliferation of all the hormone-producing cell types.

Hesx1 mutants exhibited increased domains of *Lhx3* and *Prop-1* expression (Figure 4). The expression domains of *FGF8* and *FGF10* in the infundibulum were expanded rostrally, consistent with previous studies demonstrating that

FIG. 4. Immunohistochemistry of *Hesx1* mutant embryos revealing multiple RPs as invaginations of oral ectoderm expressing the pituitary markers Prop-1 and Lhx3.

Lhx3 expression can be regulated by FGF signaling (Ericson *et al.*, 1998; Treier *et al.*, 1998), indicating that *Hesx1* is required for maintaining the proper domains of *FGF* expression.

V. *Hesx1* Recruits the Groucho-related Corepressor Transducin-like Enhancer of Split

The coregulatory apparatus that mediates *Hesx1*-dependent repression is unknown, although the nuclear receptor corepressor (N-CoR) has been linked to repression mediated by the homeodomain (Laherty *et al.*, 1998; Xu *et al.*, 1998). Another class of corepressors that has been linked to many homeodomain factors is the mammalian orthologues of the *Drosophila* protein Groucho (reviewed in Chen and Courey, 2000). Groucho is distantly related to the yeast corepressor Tup1, which binds several components of the core transcriptional apparatus — including Srb7, Srb10, Srb11, and Med6 — as well as histone deacetylases (Kuchin and Carlson, 1998; Gromoller and Lehming, 2000; Watson *et al.*, 2000; Wu *et al.*, 2001). Both Groucho and Tup1 contain WD40 repeats, a well-characterized protein-protein interaction domain that has been linked to repression mediated by associations with DNA binding proteins, including transcription factors and histones.

The phenotypes of *Hesx1* mutants are consistent with *Hesx1* acting as an *in vivo* transcriptional repressor. Domain mapping revealed that both the N-terminus and homeodomain regions exhibited repression activity. Comparing *Hesx1* sequences from several vertebrate species revealed the presence of two conserved motifs outside the homeodomain. The N-terminus contains a motif

similar to the eh1 motif originally characterized in the *Drosophila* repressor engrailed (Smith and Jaynes, 1996) and one similar to the WRPW motif found in several bovine helix-loop-helix (bHLH) proteins (Paroush *et al.*, 1994), both of which are genetically and biochemically linked to the Groucho class of corepressors (Jimenez *et al.*, 1997; Tolkunova *et al.*, 1998). Affinity chromatography revealed a strong and specific interaction between Hesx1 and transducin-like enhancer of split (TLE) corepressors in the mammalian orthologs of Groucho.

Both *Hesx1* and *TLE1* proved to be coexpressed within RP between e9.5 and e12, with expression becoming rapidly extinguished beginning at e13.5, coincident with the appearance of anterior lobe pituitary cell types. Both the N-terminal tetramerization (Q and GP) domain and WD40 repeat region of TLE were required for interaction with Hesx1/Rpx. Full transcriptional repression required the GP and WD40 domains. Thus, as in the case of the yeast repressor Tup1, the repression and interaction domains appear to be separable, although the WD40 repeat proved to be required for both functions. Finally, repression by the N-terminal domain of Hesx1 was inhibited by α TLE-1 immunoglobulin G (IgG), indicating that it alone could serve as a component required for Hesx1-dependent repression.

In an attempt to address the functional significance of TLE1 and Hesx1 actions in pituitary development, we generated transgenic mice expressing *Hesx1* under the control of a promoter (Pitx1) that targeted misexpression to ventral pituitary cell types, or a promoter alpha glycoprotein hormone subunit (α GSU) to maintain *Hesx1* expression in specific cell lineages through later stages of development. In these transgenic animals, we observed only minimal phenotypes, characterized by a modest reduction in some cell lineages. In similar experiments, TLE1 expression alone was found to have minimal effects. However, when we generated transgenic animals that maintained expression of both *Hesx1* and *TLE1* in pituitary development, these mice were characterized by the near-complete absence of ventral anterior pituitary cell types, with an ontogeny and dysmorphogenesis quite similar to that observed in *Prop-1*-defective Ames dwarf mice (Gage *et al.*, 1996a; Sornson *et al.*, 1996). Thus, there was an absence of *Prop-1*-dependent cell lineages as determined by loss of expression of the *Pit-1*, *GH*, *TSH β* , and α GSU genes, while expression of *Prop-1* and proopiomelanocortin (*POMC*) were maintained (Figure 5A and B). Indeed, the ventral aspect of RP expressed a uniform field of *POMC*-expressing cells.

However, when specific interaction between transgenically expressed TLE1 and Hesx1 was abolished through mutation of the eh1 domain, no defects occurred in the appearance of pituitary cell types, nor was organ morphology altered (Figure 5B). Thus, specific interactions between TLE1 and Hesx1 are required for Hesx1 repression *in vivo*; in this case, to prevent the appearance of the Pit-1 and gonadotrope lineages.

FIG. 5. Analysis of *Pitx1/Hesx1:Pitx1/TLE1* double-transgenic founder embryos. (A) Loss of anterior pituitary and morphological similarity to pituitary defects in Ames *df/df* mice, except when the *Pitx1/Hesx1* transgene encodes a mutated eh1 domain, which cannot interact with TLE1. H&E staining. (B) *In situ* hybridization showing loss of growth hormone (*GH*), *Pit-1*, and ventral *Prop-1*, while expression of proopiomelanocortin (*POMC*) appears unaffected and *Lhx3* and *Prop-1* continue to be expressed in the ectoderm of RP. Asterisk indicates the dorsal pouch ectoderm.

VI. Conclusions

The sequential actions of transcriptional repressors and activators and their required coregulatory machinery on overlapping sets of gene targets serve as a gene-regulatory strategy in mammalian organogenesis, exemplified here in anterior pituitary development. Two highly related *paired*-like homeodomain factors — *Hesx1/Rpx* and *Prop-1* — with distinct but overlapping patterns of expression over the entire period of pituitary organogenesis (including initial commitment, patterning, and cell-type determination) exert temporally specific roles (Figure 6). *Hesx1*, influenced by the actions of the linked modifier genes

FIG. 6. Model for the temporal switch of a homeodomain repressor for an activator, mediated by the coordinate recruitment of distinct corepressor complexes in pituitary organogenesis. Hesx1 requires the recruitment of coordinately expressed TLE1 for early repression events involved in the induction of RP and to antagonize *in vivo* activation by Prop-1. Downregulation of *Hesx1* is required to temporally control Prop-1 function in proliferation and determination of four pituitary cell lineages.

based on the genetic background, is required for early organ commitment and cell-determination events. Hesx1, with TLE1, serves to prevent Prop-1 from initiating the program required for asymmetric division and proliferation of the Pit-1 and gonadotrope lineages. Conversely, premature expression of Prop-1 can block pituitary organogenesis, phenocopying the effects of *Hesx1*-gene deletion. These data suggest that the switch in binding of a *paired*-like homeodomain repressor for a *paired*-like homeodomain activator results in alteration in the expression of key target genes and prevents organogenesis. Maintained expression of Hesx1, with the TLE1 corepressor, subsequently can block the activation of Prop-1-dependent genes required for appearance of four anterior pituitary cell types. Thus, opposing actions of related repressors and activators, potentially binding to overlapping sets of gene targets, provide critical temporal control of organ development. Interestingly, later persistent expression of *Prop-1* under control of the α GSU promoter caused decreased gonadotrope differentiation and increased adenomatous hyperplasia (Cushman *et al.*, 2001). This indicates that properly extinguishing Prop-1 also may be an important later step in *paired*-like homeodomain-mediated organogenesis.

Hesx1 appears to contain two repressor domains, located in the N-terminal and homeodomain regions of the protein, each recruiting a distinct corepressor complex. The corepressors recruited by the N-terminus includes one mammalian Groucho orthologue, TLE1, that appears to be required for Hesx1-dependent repression. *In vivo*, only coexpression of both *Hesx1* and *TLE1* are sufficient to phenocopy the effects of *Prop-1* gene deletion. The strong association between

TLE1 and Hesx1 is mediated by a highly conserved helical motif (FXLXXIL) present in the Hesx1 N-terminus, analogous to interactions of Nkx, Six, and certain Pax homeodomain factors with other TLE family members (Eberhard *et al.*, 2000; Muhr *et al.*, 2001).

The domains required for effective repression function appears to depend upon cellular and promoter context. Under specific circumstances, the Hesx1/Rpx homeodomain region is alone capable of mediating repression, utilizing a corepressor complex that includes mSin3A/B, histone deacetylases (HDACs) 1 and 2, but not of the high-affinity N-CoR/TBL1/HDAC3 complex (Guenther *et al.*, 2000, Underhill *et al.*, 2000) to the Hesx1 homeodomain. The Hesx1 N-terminal domain binding TLE permits cooperative binding of N-CoR/HDAC1/Sin3A/B to the homeodomain, a strategy that might be quite common with respect to many homeodomain repressors, and provides an additional linkage between TLEs and N-CoR/mSin3 complexes in the actions of strong homeodomain repressors.

Thus, coordinated regulation of a repressor (Hesx1) and corepressor (TLE1) serves as a determinant of organogenesis and the temporal control in pituitary cell lineage generation. The sequential repression and activation of a common set of regulatory genes may prove to be an underlying strategy in the temporal code of pituitary organ development, with initial repression required for organ commitment and proliferation, and subsequent activation for commitment of specific cell lineages.

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