Negative Smad Expression and Regulation in the Developing Chick Limb

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Abstract

The inhibitory or negative Smads, Smad6 and Smad7, block TGFβ superfamily signals of both the BMP and TGFβ classes by antagonizing the intracellular signal transduction machinery. We report the cloning of one Smad6 and two Smad7 (Smad7a and Smad7b) chick homologs and their expression and regulation in the developing limb. Smad6 and Smad7a are expressed in dynamic patterns reflecting the domains of BMP gene expression in the limb. Activation and inhibition of the BMP signaling pathway in limb mesenchyme indicates that negative Smad gene expression is regulated, at least in part, by BMP family signals.

Introduction

Bone morphogenetic proteins (BMP) are members of the transforming growth factor-β (TGFβ) ligand superfamily. BMPs have diverse essential roles during limb development which include establishment of the apical ridge and zone of polarizing activity, as well as in the regulation of cell death, chondrogenesis, myogenesis, digit identity and fracture repair [1–12]. TGFβ/Activin signaling is as well as in the regulation of cell death, chondrogenesis, myogenesis, digit identity and fracture repair [1–12]. TGFβ/Activin signaling is also implicated in digit tip-specification [5]. These roles necessitate precise control both of ligand expression and the responsiveness of target cells, neither of which is well understood. The negative Smad genes (Smad6 and Smad7), antagonize block TGFβ superfamily signaling [13–16]. Thus, negative Smad activity could influence the spatial and temporal extent, as well as the magnitude, of BMP or TGFβ/Activin signaling during limb development.

In previous studies limited descriptions of Smad6 expression in the developing chick limb have been reported [5,17–19]. Smad6 mRNA expression was also found to be upregulated in interdigital regions following Bmp5 protein application [19]. However more detailed analyses that include developmental timecourses of Smad7 expression or the dependence of negative Smad expression on BMP signaling have not been described. Thus while some information is available about Smad6 in the developing limb, how the negative Smad gene expression patterns and their expression levels are regulated, and how they might contribute to the dynamic control of BMP or TGFβ/Activin signaling during limb development remains to be determined.

Results and Discussion

Cloning of chicken negative Smad genes

In order to study negative Smad gene function in chick limb bud development, we cloned homologs of Smad6 and Smad7 from HH st12–15 whole chicken embryo and HH st20–24 chicken limb bud cDNA libraries. Multiple cDNAs encoding three distinct open reading frames were identified. Comparison with known Smad protein sequences indicates that one encodes a Smad6 homolog (Genbank Accession FJ417094), while the other two encode Smad7 homologs (cSmad7a and cSmad7b; Genbank Accession FJ417093 and FJ417092 respectively; Figure 1). The cSmad6 gene sequence is identical to that previously described [18]. The two Smad7 cDNAs are equally related to other Smad7 genes, and are themselves 93% identical at the nucleotide level within their open reading frames and 80% identical in their 3’ untranslated regions. The conceptual translations of their open reading frames are 98% identical. Neither Smad7a nor Smad7b has major deviations from other Smad7 amino acid sequences, and both terminate in a conserved c-terminal motif lacking phosphorylatable serines.

Most vertebrate species have only one Smad7 homolog. Therefore we compared the Smad7a and Smad7b sequences with cDNA and chicken genomic sequences present in public databases to ask whether either or both were described previously. The Smad7b cDNA contains a complete open reading frame that encompasses a previously described cSmad7 partial open reading frame (Genbank Accession AF230192), and maps to a contig that is not associated with a particular chromosome within the sequenced chicken genome (Contig: NW_001472907.1). The Smad7a cDNA contains a partial open reading frame that is lacking only the four amino terminal amino acids as determined by comparison with other Smad7 proteins. This sequence maps to the predicted genomic Smad7 locus on the Z chromosome (XM_427238). Thus there are two chicken Smad7 loci: the cDNA we call Smad7a is equivalent to a genomic “Smad7” locus on the Z chromosome. The sequence we call Smad7b is equivalent to a
Figure 1. Chicken negative Smad genes. A. Phylogenetic relationship of chicken negative Smad proteins to other Smads. Conceptual translations of the chicken Smad6, Smad7a and Smad7b gene open reading frames were compared to those of other vertebrate negative Smad, positive Smad and common Smad genes. The chick gene names are underlined. x: Xenopus, m: mouse, h: human, r: rat, c: chick. B. Comparison of cSmad7a (7a) and cSmad7b (7b) translated open reading frames. Blank positions in the cSmad7a sequence are identical to cSmad7b, dashes are absent and differences are indicated. Vertical arrowhead marks the beginning, and vertical arrow marks the end, of the translated sequence conserved between cSmad7a and genomic contig NW_001482773.1. The vertical arrow marks the beginning of the translated sequence conserved between cSmad7a and the genomic cSmad7 locus on the Z chromosome. Underline indicates the MH2 domain that is conserved amongst all Smad proteins. Note absence of serine residues among last four amino acids.

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previously reported ‘Smad7’ cDNA, which is not encoded by the genomic Smad7 locus.

BLAST analysis of cSmad7a sequences with public databases identified two distinct genomic sequences with similarities to cSmad7a. Nucleotides 541–616 of cSmad7a are identical to sequences in an unassigned 1363 nucleotide genomic contig (Contig: NW_001492773.1), while cSmad7a nucleotides 615–1235 and genomic DNA sequences at the ‘Smad7’ locus have only one mismatch. These data suggest the cDNA spans at least two genomic fragments. Analysis of the conceptual protein sequences is consistent with this idea: The cSmad7a ORF encodes a protein of at least 384 amino acids, while the predicted genomic cSmad7 protein sequence is 222 amino acids long. cSmad7a aa207–384 (the translation of nt615–1235) and genomic cSmad7 aa45–222 are identical, consistent with the nucleotide alignments. Protein BLAST analysis of aa1–44 of genomic cSmad7 does not identify any protein other than genomic cSmad7, while similar analysis of cSmad7a aa1–206 or the unassigned genomic contig (NW_001402773.1) identifies numerous Smad7 homologs. These data imply that the first 45 aa predicted by the conceptual

![Figure 2](image_url). Negative Smad gene expression patterns during chick forelimb development. Expression patterns of cSmad6 (A–H), cSmad7a (I–O) and cSmad7b (P–T) in the developing chick forelimb. Developmental stages as indicated. Note cSmad6 expression domain extends as development proceeds to beneath apical ridge (black arrowhead). Expression of both cSmad6 and cSmad7a is present in distal mesenchyme before being restricted to interdigital regions (black asterisk), then further restricted to areas around the digit tip (white arrowhead). cSmad7b is not expressed in the forelimb mesenchyme until HH st29/30 and is then restricted to the cartilage condensations (white arrow). Signal in body and head in (P) is non-specific background. Images are of limb dorsal surface. Anterior to the top; distal to the right. Scale bars indicate 500 μm.

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translation of genomic cSmad7 are incorrect, and that the unassigned contig belongs in the genomic sequence in this region.

As cSmad7a is similar to cSmad7b through their most amino terminal amino acids, and genomic regions of identity to cSmad7a extend 5’ only to cSmad7a nucleotide 541, there is likely at least one additional sequence missing from the genomic sequence. The cSmad7a and cSmad7b cDNAs show multiple differences scattered throughout their sequences, and are most closely conserved with sequences at different chromosomal locations. Thus cSmad7a and cSmad7b are encoded by different genes, and are not splice or allelic variants. To the best of our knowledge, the chicken is the first example of an amniote species with two Smad7 genes.

Negative Smad gene expression during limb development

cSmad6 Expression. We examined cSmad6 expression by whole mount in situ hybridization from HH stages 18–34, focusing on the developing limbs. At HH st18 expression is observed in the fore and hindlimb buds in small anterior and posterior mesenchymal domains (Figure 2A–C). As limb outgrowth proceeds expression extends around the mesenchymal margin directly abutting the ectoderm and apical ridge (Figure 2D). Weak expression is also detected in the apical ridge through approximately HH st25 (data not shown). As limb outgrowth continues distal mesenchymal expression intensifies beneath the apical ridge, and is maintained along the anterior and posterior margins. From HH st28 cSmad6 expression becomes restricted to the autopod, primarily in interdigital regions (Figure 2E, F). From HH st32 autopodal expression is peridigitally restricted (Figure 2G, H).

We also examined cSmad6 expression by section in situ hybridization on HH st29 forelimb (Figure 3A–E) and hindlimb (Figure 3F–I) cryosectioned tissue. We detected expression in interdigital mesenchyme (Figure 3A,F,I), perichondrium (Figure 3B,D,F), hypertrophic chondrocytes (Figure 3A,B), forming joints (Figure 3A,F–H), interstitial mesenchyme (Figure 3A,F,G), subepidermal mesenchyme (Figure 3A,D,E) and epidermis (Figure 3F,G).

cSmad6 is expressed in other regions of the developing chick embryo including the heart, feather buds, neural tube, vascular endothelium and facial primordia, including the branchial arches and nasal placodes (data not shown). These data are consistent with, and extend, previously described cSmad6 expression patterns [18].

cSmad7 Expression. We examined cSmad7a and cSmad7b expression by whole mount in situ hybridization from HH stages 17–34, again focusing primarily on expression in the developing limb...
Expression is also observed in a small mesenchymal domain in the forelimb to the anterior hindlimb (data not shown; Figure 2I). extending from a small mesenchymal domain in the posterior bud.

**Negative Smads are expressed in regions of highest BMP expression.** Since BMP family signals are potential regulators of negative Smad gene expression, we compared BMP and BMP antagonist gene expression patterns to those of *cSmad6* and *cSmad7a*. We focused on *cSmad7a* rather than *cSmad7b*, as *cSmad7b* is expressed in the limb from HH st 19 through at least HH st32. Taken together the *Bmp2*, *Bmp4* and *Bmp7* expression domains bear a remarkable similarity to those of *cSmad6* and *cSmad7a* (Figure 4A-F; data not shown) [20]. For example, in early limb development the BMP genes, *cSmad6* and *cSmad7a* are expressed in peripheral, but not central, mesenchyme (Figure 4A-E), and later each is expressed interdigitally. In contrast, *Gremlin*, a BMP-dependent antagonist of BMP signaling essential for maintaining signaling pathways regulating limb patterning, is expressed in central mesenchyme (Figure 4F) [21–25].

**Regulation of Smad6 and Smad7a expression in the developing limb**

These gene expression patterns led us to test whether negative Smad gene expression might be regulated by BMP signals in the limb. We used retroviral misexpression and recombinant protein application approaches to modulate BMP signaling in vivo. To ectopically activate the BMP signaling pathway in the limb, we infected limb tissue with RCAS retroviruses that express constitutively active forms of the type I BMP receptors, *BMPR Ia* and *BMPR Ib* (*BMPR Ia<sup>CA</sup> and *BMPR Ib<sup>DN</sup>* or applied recombinant BMP protein directly to limb tissue [26]. HH st19–20 limb buds were infected with the activated receptor viruses and *cSmad6* and *cSmad7a* expression was assessed up to 72 hours later. Using a virus-specific probe to monitor the extent of infection, we observed extensive but incomplete staining by 24 hours post-infection, and that by 48 hours post-infection had spread throughout the limb mesenchyme (data not shown). In the majority of cases both *cSmad6* and *cSmad7a* were upregulated in their normal expression domains after 24 hr and were also ectopically induced in limb territories such as the central and proximal mesenchyme (Figure 5D; Table 1). Heparin beads soaked in recombinant BMP2 protein were grafted into HH st20 proximal mesenchyme (Figure 5D; Table 1). Heparin beads surrounding the implanted bead at levels higher than those detected in the normal expression domain (Figure 5E; 6 embryos affected of 7 tested; n = 6/7). *cSmad7a* expression is also induced adjacent to the implanted beads, although to a lesser extent than *cSmad6* (Figure 5F; n = 5/7). These data indicate that the negative Smad gene expression can be induced throughout the limb mesenchyme by activating the BMP signaling pathway.

To test whether negative Smad gene expression in the limb is dependent on BMP signals, we infected developing limb tissue with RCAS viruses that express either a dominant negative *BMPR Ib* gene (*BMPR Ib<sup>DN</sup>* or the BMP signaling antagonist noggin [2,26], *BMPR Ib<sup>DN</sup>* virus infection down regulates, but does not completely abolish both *cSmad6* and *cSmad7a* expression (data not shown; Table 1). Viral misexpression of noggin reduces expression of *Smad6* to undetectable levels by 48 hours post-infection (n = 9/13; Figure 5G). In contrast while *cSmad7a* expression is reduced, it is still detectable, even at 72 hours post-infection (n = 8/10; Figure 5H). Thus *Smad6* expression in the chick limb mesenchyme apparently depends completely on BMP signaling, while *Smad7a* expression is partially dependent on similar signals.
Figure 5. Regulation of negative Smad gene expression by BMP signaling. Expression patterns of cSmad6 in contralateral (A, C, E, G) or manipulated (A', C', E', G') limbs and of cSmad7a in contralateral (B, D, F, H) or manipulated (B', D', F', H') limbs. (A'–D', G'–H') virus infection at HH St17–20, fixed after 48 hr. (E'–F') BMP2 protein soaked bead implanted into proximal mesenchyme at HH St20, fixed at 15 hrs. Experimental manipulation as indicated on panels. Note ectopic expression of cSmad6 and cSmad7a following BMPR IaCA and BMPR IbCA misexpression (white arrowheads); position of heparin sulphate bead (black arrowhead) and resulting ectopic expression (black arrow). Reduction in limb size in C' and D' is due to cell death induction caused by BMPR IaCA misexpression and is not a photographic artifact. All images show dorsal surface. Anterior to top, distal to right. Scale bars 500 μm.
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Table 1. Summary of virus effects on negative Smad gene expression.

| Virus     | Undetectable cSmad6 expression | cSmad7a expression |
|-----------|---------------------------------|--------------------|
|           | Normal | Reduced | Ectopic | Normal | Reduced | Ectopic |
| BMPR IaCA | -      | -       | 15/15    | 1/7    | -       | 6/7     |
| BMPR IbCA | -      | 4/27    | 7/27     | 16/27  | 3/9     | 6/9     |
| Noggin    | 9/13   | -       | 4/13     | -      | 2/10    | 8/10    |
| BMPR IbDN | -      | 9/15    | 6/15     | -      | 4/8     | 4/8     |

Number of limbs displaying result/total number of limbs examined.
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Materials and Methods

Ethics Statement
Animals were handled in accordance with Columbia University guidelines.

Cloning and Sequencing
Chicken Smad6 and Smad7 homologs were obtained by screening Hamburger and Hamilton (HH) st12–15 chicken embryo [27,28] and st20–24 chicken limb cDNA libraries [29,30] with probes derived from mouse and Xenopus Smad6 and Smad7 genes [31,32]. Overlapping clones for each gene were sequenced using standard dye termination chemistry. DNA and protein sequences were compared to the non-redundant Genbank databases and published Smad sequences using both NCBI Blast [33] and DNASStar MegAlign v4.00 software. Genomic comparisons were made to the Gallus gallus Genome Build 2.1. Sequences with the following Genbank accession numbers were used to generate the phylogenetic tree in Figure 1: hSmAD1: Q15797, hSmAD2: Q15796, hSmAD3: Q92940, hSmAD4: S71811, hSmAD5: Q99717, hSmAD6: Q43541, mSmAD6: AF010133, xSmAD6: AF055529, lSmAD7: AAB81354, mSmAD7: 2460040, rSmAD7: AAC25962; xSmAD7: AAC09303, mSmAD8: AAF77079. Putative chicken Smad6 and Smad7a and Smad7b gene assignments were made based on similarities of the predicted protein sequences with published family members and sequences submitted to Genbank. Accession number for cSmad6: FJ417094; cSmad7a: FJ417093; cSmad7b: FJ417092.

Embryology and in situ hybridization
Fertile White Leghorn chicken eggs (SPAFAS, Farmington, CT) were incubated at 38°C in a humidified, forced air incubator and embryos collected at appropriate developmental stages. Experimental manipulations were performed on HH st15 to HH st21 right limb buds; the left limb bud served as a control. Embryos were harvested through 72 hr postmanipulation and were fixed in 4% paraformaldehyde and processed for in situ hybridization analysis. Embryos were incubated for approximately 15 hours, fixed in 4% paraformaldehyde and processed for in situ hybridization analysis.

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