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The T-box transcription factor *Tbx15* is required for skeletal development

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Abstract

During early limb development several signaling centers coordinate limb bud outgrowth as well as patterning. Members of the T-box gene family of transcriptional regulators are crucial players in these processes by activating and interpreting these signaling pathways. Here, we show that *Tbx15*, a member of this gene family, is expressed during limb development, first in the mesenchyme of the early limb bud, then during early endochondral bone development in prehypertrophic chondrocytes of cartilaginous templates. Expression is also found in mesenchymal precursor cells and prehypertrophic chondrocytes, respectively, during development of skeletal elements of the vertebral column and the head. Analysis of *Tbx15* null mutant mice indicates a role of *Tbx15* in the development of skeletal elements throughout the body. Mutants display a general reduction of bone size and changes of bone shape. In the forelimb skeleton, the scapula lacks the central region of the blade. Cartilaginous templates are already reduced in size and show a transient delay in ossification in mutant embryos. Mutants show a significantly reduced proliferation of prehypertrophic chondrocytes as well as of mesenchymal precursor cells. These data suggest that *Tbx15* plays an important role in the development of the skeleton of the limb, vertebral column and head by controlling the number of mesenchymal precursor cells and chondrocytes.

Keywords: Limb; Skeleton; Chondrocyte; T-box; Proliferation

1. Introduction

The vertebrate limb has long been employed as a model system to analyze the molecular pathways underlying both patterning as well as cellular differentiation during skeletal development. Limb development starts with the thickening of the lateral plate mesoderm and the formation of limb buds at the flanks of the early organogenesis stage embryo. Mesenchymal cells of the bud will give rise to all the progenitors of the skeletal system as well as to tendons and muscle sheaths. Skeletogenesis starts with the aggregation and condensation of mesenchymal precursor cells to templates that prefigure the shape of the future bones at their appropriate position. Development proceeds by endochondral ossification via a cartilaginous intermediate, a process that contrasts with the direct (intramembranous) ossification of some bones of the skull and the clavicle. Patterning of individual skeletal elements occurs at the level of the condensation, i.e. the number and adhesion properties of mesenchymal cells regulate the size and shape of the future bone. Experimental manipulations primarily done in chick embryos and genetic experiments in the mouse have provided evidence that specific regions of the early limb bud act as signaling centers that regulate limb skeletal patterning along the proximal–distal (PD), the anterior–posterior (AP) and the dorso-ventral (DV) limb axes. Patterning is subsequently refined by the combinatorial action of various classes of transcription factors including *Hox* genes (for recent reviews on limb and skeletal patterning see Karsenty

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and Wagner, 2002; Kronenberg, 2003; Niswander, 2003; Mariani and Martin, 2003; Tickle, 2003).

T-box (*Tbx*) genes constitute a multigene family encoding transcription factors characterized by a highly conserved DNA-binding region, the T-box. T-box genes play crucial roles in the development of diverse tissues and organs both in vertebrates and in invertebrates (Papaioannou, 2001). Analyses both in chick and in mouse suggest that at least seven *Tbx* family members are important regulators of limb development. *Tbx5* and *Tbx4* are a closely related pair of T-box genes with differential expression in the developing forelimb and hindlimb buds, respectively (Gibson-Brown et al., 1996). Mutations in human *TBX5* underlie a developmental disorder, Holt-Oram syndrome, where heterozygous carriers show heart and upper limb defects (Li et al., 1997). Misexpression experiments in chick embryos suggest a role for either factor in specifying forelimb and hindlimb identity, respectively (Rodriguez Esteban et al., 1999; Takeuchi et al., 1999). Loss of function studies in the mouse have precluded a direct analysis of such a role but have instead revealed a requirement for *Tbx4* and *Tbx5* in triggering limb outgrowth. Here, *Tbx4* and *Tbx5* appear to coordinate Wnt and FGF signaling between limb mesenchyme and the overlying ridge ectoderm (Naiche and Papaioannou, 2003; Rallis et al., 2003; Takeuchi et al., 2003).

Brachyury (*T*), the prototypical member of the *Tbx* gene family was recently found to play an important role in maintaining the AER. Expression of *T* in the lateral plate mesoderm and later in the mesoderm underlying the AER might put *T* in a regulatory loop of Wnt and FGF signaling between the ridge epithelium and the subridge mesenchyme involved in maintaining a functional AER (Liu et al., 2003).

Tbx2 and *Tbx3* form a second pair of structurally related T-box genes with an important function in limb patterning. *Tbx2* and *Tbx3* are expressed in anterior and posterior edges of limb buds with distinct domains in relation to posterior digit identity (Gibson-Brown et al., 1996). Mutations in human *TBX3* have been found to cause ulnar-mammary syndrome, a condition characterized by absence or deformation of the ulna and posterior digits IV and V (Bamshad et al., 1997). Mice homozygous for a targeted null mutation of *Tbx3* show severe malformation of both forelimbs and hindlimbs characterized by AP-patterning defects (Davenport et al., 2003). Recent misexpression experiments in chick further support a role for *Tbx2* and *Tbx3* in anterior–posterior limb patterning, namely specification of identity of digits III and IV. These experiments suggest feedback and feedforward loops between Tbx2/3 and Shh and BMP signaling cascades to specify posterior digit identity (Suzuki et al., 2004).

Tbx18 and *Tbx15* constitute a third pair of closely related T-box genes expressed during limb development. *Tbx18* is expressed in the proximal region of the limb bud mesenchyme (Kraus et al., 2001). *Tbx18*$^{-/-}$ mice do not show morphological defects in the limbs but exhibit severe malformation of the vertebral column, a phenotype that was traced back to defects in AP-somite polarization (Bussen et al., 2004).

Expression of *Tbx15* (also referred to as *Tbx8* or *Tbx14*; NCBI GeneBank) has been reported in limb buds, in the craniofacial region and in the skin during mouse development (Agulnik et al., 1998; Candille et al., 2004). Analyses of the *droopy ear* mouse mutation, in which *Tbx15* is deleted, and of a targeted null allele of *Tbx15* have revealed a role for *Tbx15* in DV-patterning of the mouse coat (Curry, 1959; Candille et al., 2004). Here, we extend the analysis of the *Tbx15* skeletal phenotype (Curry, 1959), and show that *Tbx15* is dynamically expressed in the developing limb bud as well as in other regions of the embryo associated with mesenchymal condensations during early endochondral bone formation. In mice lacking *Tbx15* most bones of the appendicular skeleton and the upper vertebral column, and some of the skull are reduced in size, and show subtle changes in shape. The scapula is marked by a hole in the center of the blade. Defects in the adult skeleton can be traced to changes in the mesenchymal and cartilaginous anlagen of the skeleton, and may be caused by a reduced proliferation of mesenchymal precursor cells and prehypertrophic chondrocytes.

2. Results

2.1. Expression of Tbx15 in limb and skeletal development

Previous studies described *Tbx15* expression in early limb buds, branchial arches, flanks and the craniofacial region by whole-mount in situ hybridization, and in skin development using section in situ hybridization analysis (Agulnik et al., 1998; Candille et al., 2004). In order to investigate expression in (skeletal) development of limbs in more detail, we hybridized whole embryos from embryonic day (E) 8.5 to 12.5 and sections of whole embryos and individual bones from E10.5 to 18.5 with an antisense riboprobe derived from a full length *Tbx15* cDNA (Fig. 1).

Tbx15 expression is prominent in limb development from E9.0 to 16.5 (Fig. 1A–D,F,H,I,K,L,O–R). Expression is first seen in the developing forelimb buds on E9.0–9.5 (Fig. 1A). On E10.5, expression is found along the proximo-distal extension in the central region of the forelimb buds but not in the anterior and posterior edges (Fig. 1B with inset). This pattern is maintained throughout further limb development (Fig. 1C,D). With formation of digits expression is confined to digits II–IV (Figs. 1P,2Dc). Hindlimb expression follows that of the forelimb buds with a developmental delay of one day (Fig. 1B–D). Section in situ hybridization analysis revealed that expression is confined to the mesenchymal compartment of the developing limb buds. On E10.5, expression is highest in the central core of the forelimb bud (Fig. 1F). With formation of a mesenchymal preskeleton, expression becomes weaker in the condensing cells but is