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Msx genes are important apoptosis effectors downstream of the Shh/Gli3 pathway in the limb

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ABSTRACT

In tetrapods, the anteroposterior (AP) patterning of the limb is under the control of the antagonistic activities of the secreted factor Sonic hedgehog (Shh) and Gli3R, the truncated repressor form of the transcription factor Gli3. In this report, we show that *Msx1* and *Msx2* are targets and downstream effectors of Gli3R. Consequently, in *Shh* null mutants, *Msx* genes are overexpressed and, furthermore, partially responsible for the limb phenotype. This is exemplified by the fact that reducing Msx activity in *Shh* mutants partially restores a normal limb development. Finally, we show that the main action of the *Msx* genes, in both normal and $Shh^{-/-}$ limb development, is to control cell death in the mesenchyme. We propose that, in the limb, *Msx* genes act downstream of the Shh/Gli3 pathway by transducing BMP signaling and that, in the absence of Shh signaling, their deregulation contributes to the extensive apoptosis that impairs limb development.

Introduction

In amniotes, anteroposterior (AP) development of the limb is controlled by a small posterior domain of the limb mesenchyme called the zone of polarizing activity (ZPA). Its action is mediated by the secreted factor Sonic hedgehog (Shh) (Echelard et al., 1993; Riddle et al., 1993). The pivotal role of Shh in AP limb patterning is exemplified by the analysis of the $Shh^{-/-}$ null mutant. In this mutant, distal limb development is severely impaired and all posterior structures of both the zeugopod (intermediate elements giving rise to the radius and ulna in the forelimb, and the tibia and fibula in the hindlimb) and the autopod (distal elements giving rise to the wrist and the fingers in the forelimb, and the ankle and toes in the hindlimb) are missing or severely disfigured. At the molecular level, the main role of Shh during limb development is to counteract the activity of the transcription factor Gli3 and, in particular, of the truncated form of the Gli3 protein, Gli3R, that acts as a transcriptional repressor. First, Shh signaling represses *Gli3* transcription, such that *Gli3* mRNA is transcribed according to a gradient that increases from the posterior (little finger or digit 5) to the anterior (thumb/big toe or digit 1). Second, Shh is necessary to prevent the post-transcriptional proteolysis of the full-length Gli3 protein (Gli3FL) into its truncated form (Gli3R) (Wang et al., 2000). The primary importance of Gli3 downstream of Shh signaling in limb AP patterning has been assessed by studies of genetic interactions between the two genes. Contrary to the $Shh^{-/-}$ null mutant, the $Gli3^{-/-}$ null mutant displays overgrowth of the autopod leading to polydactyly (up to eight digits). Strikingly, disruption of one or both *Gli3* alleles in mice lacking Shh signaling restores limb distal development and digit formation in a dosage-dependent manner (Litingtung et al., 2002; te Welscher et al., 2002). More recently, it has been shown that replacing a single wild-type *Gli3* allele by a mutant allele producing only the Gli3FL protein also rescues the *Shh* null mutant limb phenotype to a great extent (Wang et al., 2007). All these results indicate that the main role of Shh during limb development is to counteract Gli3R activity. Nevertheless, while the mechanism allowing Shh to modulate Gli3 activity is well documented, the downstream targets of the Shh/Gli3 pathway have only been partially identified. Due to their involvement in AP limb patterning (Lallemand et al., 2005), *Msx* genes, which encode homeodomain-containing proteins, might be such targets. In the mouse, the *Msx* family comprises three members: *Msx1*, *Msx2* and *Msx3*. Whereas *Msx3* expression is confined to the neural tube, *Msx1* and *Msx2* are widely expressed especially at sites of development that depend on ectoderm-mesoderm interactions (Alappat et al., 2003; Bendall and Abate-Shen, 2000). Since their discovery, *Msx1* and *Msx2* have been proposed to play a role in limb development as they are expressed from the onset of limb bud outgrowth in both ectoderm and mesoderm (Davidson et al., 1991; Robert et al., 1991). Due to the redundancy between the two genes, neither *Msx1* nor *Msx2* null mutants exhibit a limb phenotype. However, the limbs of the double

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homozygous mutant (*Msx1*$^{-/-}$;*Msx2*$^{-/-}$) are severely affected (Lallemand et al., 2005). In particular, limb development is impaired along the AP axis, suggesting that *Msx* genes are involved in the genetic cascade controlling patterning along this axis. In these mutants however, the complexity of the limb phenotype makes the analysis of the precise role of the *Msx* genes in AP morphogenesis difficult. During the early stages of limb development (9.0–10.5 dpc), the absence of Msx activity leads to agenesis of the anterior region of the AER and, as a consequence, of the underlying mesenchyme including the digit 1 territory. Thus, the AP morphology of the limb is modified in a way that precludes further analysis. As an example, the use of genetic markers expressed in a polarized manner along the AP axis becomes uninformative, because either the region where they are normally expressed is missing (e.g. *Pax9*) or, on the contrary, only posterior tissues where they are highly expressed remain (e.g. *Hoxd11*, *Hoxd12*). Similarly, apoptosis, which is particularly important in the anterior region at later stages (Fernández-Terán et al., 2006), cannot be studied in a limb bud that is truncated anteriorly.

We therefore relied on the analysis of gene interactions between *Shh*, *Gli3*, *Msx1* and *Msx2* to get insight into the role of *Msx* genes in AP limb development. In this report, we show that *Msx* genes are targets of Gli3R in the limb. They are further effectors for part of Gli3 function and, in this process, are required for the extensive cell death that leads to the deleterious *Shh* mutant limb phenotype. Indeed, removing *Msx* functional alleles in the *Shh* mutant context, where all posterior digits are lost, correlatively with extensive Gli3R formation, reduces apoptosis and partially restores digit formation. Finally, we demonstrate that a major function of *Msx* genes in normal limb development is to control cell death in the anterior apoptotic domain.

Materials and methods

Mouse strains and genotyping

Mouse strains and genotyping protocols have been described previously (Blanc et al., 2002; Genestine et al., 2007; Houzelstein et al., 1997; Lallemand et al., 2005). The null *Shh* mouse mutant strain was obtained by crossing a conditional *Shh* male mutant (Dassule et al., 2000) with a female of the *PGK-Cre* deleter strain (Lallemand et al., 1998). Genotyping of the *Shh* null mutants was performed by PCR using the following primers: Shh-R: GAG AAG AGA TCA AGG CAA GCT CTG GC; Shh-Wt-F: ATG CTG GCT CGC CTG GCT GTG GAA GC; Shh-mut-F: AGA AGC TGT GGG CTT TTC TGG CTC C. As *Shh* and *Msx1* loci lie within 5 cM on chromosome 5, in the *Shh*^{Hx} strain used in this work, the *Shh*^{Hx} mutation (Blanc et al., 2002) and the *Msx1*^{lacZ} mutant allele (Houzelstein et al., 1997) were associated by genetic crossing. *Shh*$^{Hx/+}$ embryos were then identified by the presence of the *Msx1* mutant allele. To associate *Msx* and *Shh* mutations, recombinant *Shh;Msx1* double heterozygotes carrying the two mutations on the same chromosome were produced and subsequently mated with *Msx2* heterozygotes to obtain *Shh;Msx1;Msx2* triple heterozygotes. *Shh;Msx1;Msx2* compound and triple null mutant embryos were then obtained by intercrossing *Shh;Msx1;Msx2* triple heterozygotes. All strains were maintained in an outbred NMRI genetic background.

In situ hybridization, cell death analysis and skeleton preparation

In situ hybridization and skeleton preparations were performed as previously described (Lallemand et al., 2005). RNA probes were generated from the following DNA fragments: *Fgf8* was a complete cDNA sequence obtained by PCR; *Msx1* and *Msx2* were partial cDNAs corresponding to the coding sequence of the first exon of the respective gene. It should be noted that, in the *Shh*^{Hx};*Msx1*^{lacZ} recombinant strain, the fusion *Msx1-lacZ* RNA and the wild-type RNA were similarly detected by the *Msx1* probe. *Bmp4* was a gift from B. Hogan, *Hoxd11* and *Hoxd13* from P. Dollé, *Grem1* from R. Zeller, *Pax9* from R. Balling and *Sox9* from U. Rüther.

Cell death analysis was performed using an anti-active caspase3 antibody (BD Pharmingen) according to the manufacturer's protocol.

In all experiments, at least two embryos of each genotype were analysed. For cell death analysis, fore- and hindlimbs from each side were sectioned and all sections were analysed.

Western blotting

Proteins extracts were from whole limb buds (both fore- or hindlimbs of a single embryo of each phenotype for one experiment) or anterior and posterior halves of limb buds of 11.5 dpc old embryos (six halves of fore- or hindlimbs pulled together). Fore- and hindlimbs were analysed separately. Each experiment was carried out with one embryo of each genotype and done in duplicate.

Tissues were homogenized with protease inhibitors and boiled. An aliquot of each sample was used for protein quantification (Bradford test). Equivalent amounts of proteins (10 μg) were subjected to SDS-PAGE on a 7% Tris–HCl gel and proteins were transferred to a nitrocellulose membrane which was incubated with rabbit polyclonal anti-Gli3 antibody (1:100; Santa Cruz Biotechnology, ref: sc-20688). After incubation with an HRP-conjugated anti-rabbit IgG secondary antibody (1:10,000; Pierce Biotechnology, ref: 31402), the signal was detected using SuperSignal® West Pico chemiluminescent substrate (Pierce) and Amersham Hyperfilm ECL.

β-galactosidase staining

This was performed as previously described (Houzelstein et al., 1997). To enhance contrast between high and low *LacZ*-expressing regions, embryos were fixed at least 2 h and stained at 4 °C to slow down staining apparition. Staining was regularly controlled and stopped before saturation. To minimize variations of *Msx1*^{lacZ} expression due to development stage variations, only *Msx1*^{lacZ} and *Shh*^{Hx};*Msx1*^{lacZ} embryos from the same litter were compared. These litters were obtained by crossing *Msx1*^{lacZ} females with *Shh*^{Hx};*Msx1*^{lacZ} males. Embryos homozygous for the *Msx1*^{lacZ} were discarded.

Results

In the limb, Msx gene expression correlates anteriorly with Gli3R level

Shh exerts a repressive activity both on *Gli3* transcription and on the proteolysis of Gli3FL into Gli3R. As a consequence, the anterior-most region of the limb bud, corresponding to the presumptive territory of digit 1 (thumb or big toe), is characterized by the highest expression level of *Gli3* transcripts and the highest Gli3R:Gli3FL ratio (Wang et al., 2000). In the *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ double homozygous mutant, the main abnormalities along the AP axis are confined to the anterior-most region (Lallemand et al., 2005), suggesting a correlation between Gli3R concentration and Msx activity. To check this hypothesis, we first analyzed *Msx1* and *Msx2* expression in different mouse mutant strains in which the Shh/Gli3 cascade is affected (Fig. 1 and Supplementary Fig. S1). In *Gli3*$^{-/-}$ embryos, both *Msx* genes were downregulated (Fig. 1A–D and Supplementary Fig. S1) but, whereas almost no *Msx2* transcripts could be detected in an anterior domain of the limb bud of the mutant (Fig. 1C, D), downregulation of *Msx1* was less striking, consisting in a reduction of the expression domain at the level of the presumptive digit 1 territory (Fig. 1A, B). In both cases, however, the apical and posterior domains were not affected. Thus, the absence of *Gli3* affects *Msx* gene expression only in a region where the Gli3R form is predominant. Similarly, in *Hemimelic extra-toes* (*Shh*^{Hx}) heterozygous mutants, where *Shh* is expressed ectopically anteriorly (Blanc et al., 2002) and prevents Gli3FL processing into Gli3R (our unpublished results), *Msx1* expression is