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Interactions between *HOXD* and *Gli3* genes control the limb apical ectodermal ridge via *Fgf10*

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Abstract

The development of the vertebrate limb is dependent upon two signaling centers, the apical ectodermal ridge (AER), which provides the underlying mesenchyme with essential growth factors, and the zone of polarizing activity (ZPA), the source of the *Sonic hedgehog* (SHH) product. Recent work involving gain and loss of function of *Hox* genes has emphasized their impact both on AER maintenance and *Shh* transcriptional activation. Here, we describe antagonistic interactions between posterior *Hoxd* genes and *Gli3*, suggesting that the latter product protects the AER from the deleterious effect of the formers, and we present evidence that *Fgf10* is the mediator of HOX-dependent AER expansion. Furthermore, the striking similarity between some of the hereby observed *Hox/Gli3*-dependent morphogenetic defects and those displayed by fetuses with severely altered retinoic acid metabolism suggests a tight connection between these various pathways. The nature of these potential interactions is discussed in the context of proximal–distal growth and patterning.

Keywords: *Hox*; *Gli3*; *Fgf10*; Limb development; Posterior prevalence; Collinearity; Mouse

Introduction

Genetic studies in mice have shown that limb growth and patterning critically depends upon *Hox* genes belonging to paralogy groups 9 to 13 of both the *HoxA* and *HoxD* clusters. Despite structural homology and genomic neighborhood, individual representatives of the different groups have distinct roles in the formation of particular limb regions. For example, in the absence of both *Hoxa13* and *Hoxd13* function, autopods (hands and feet) mostly fail to develop (Fromental-Ramain et al., 1996b; Kondo et al., 1997). Similarly, severe truncations of the zeugopod (forearm, or lower arm) were seen when removing group 11 function (Davis et al., 1995) and, likewise, group 9 deficit mostly affected the stylopod (humerus) (Fromental-Ramain et al., 1996a; Wellik and Capecchi, 2003). These analyses uncovered anatomical defects generally corresponding in space and time to the expression domains of the genes concerned. In developing limbs, both the timing of expression and the position of the functional domains of the various *Hox* genes reflect their linear order along the chromosome (Kmita and Duboule, 2003). However, at the most proximal end of the stylopod *Hox* gene function seems to be somewhat dispensable (Kmita et al., 2005).

The importance and necessity for such a strict temporal-spatial distribution of gene expression domains along the proximo-distal limb axis has been illustrated by several approaches. Extensive rearrangements in the *HoxD* cluster induced limb anatomical defects due to the abnormal expression of *Hox* genes, rather than to their loss of function. When group 13 products were ectopically expressed in growing zeugopods, these segments were strongly affected, reminiscent of group 11 functional deficits. Related examples of forced expression of group 13 or 12 products in developing chick or mouse limbs resulted in similar patterning defects (Goff and Tabin, 1997; Williams et al., 2006). These observations gave support to the existence of functional interactions between *Hox* gene products, following the rule of

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posterior prevalence (Duboule, 1991; Duboule and Morata, 1994), whereby a 'posterior' or 'distal' gene product (e.g. HOXD13) can abrogate the function of a more 'anterior' or 'proximal' gene (e.g. group 11), likely at the post-transcriptional level (Herault et al., 1997; Spitz et al., 2003; van der Hoeven et al., 1996). During limb development, posterior prevalence has been documented at rather late stages, i.e. at times and in domains corresponding to distal pieces of the appendages, and the functional relevance of excluding distal *Hox* gene products from the early limb bud, such as to prevent distal structures to form at proximal locations, has not been assessed.

At the molecular level, posterior prevalence may result from interactions between HOX proteins either with various HOX partners, or with other gene products, leading for instance to the modulation of their functional activities and concurrent impact upon the regulation of target genes (Williams et al., 2006; Zappavigna et al., 1994). Among the few confirmed protein partners of HOX products (Capellini et al., 2006; Chen et al., 2004), the zinc finger domain transcription factor *Gli3* is of particular interest in this context. The *Gli3* gene product is critical for proper limb development, mainly through its antagonistic genetic interaction with *Shh*, as the stimulation of *Shh* signaling prevents the default processing of GLI3 from an activator to a repressor form (Litingtung et al., 2002; te Welscher et al., 2002; Wang et al., 2000), thereby up-regulating *Shh* target genes. In addition to this involvement in *Shh* signaling, *Gli3* and *Hoxd* genes were reported to interact during early limb development, in two different contexts. First, genetic evidence suggested that GLI3 acts as a negative regulator of several *Hoxd* genes, such as *Hoxd13* and *Hoxd12* during early limb budding (Buscher et al., 1997; Zuniga and Zeller, 1999). Secondly, GLI3 was shown to physically interact with the HOXD12 protein during distal limb patterning. In this latter case, the GLI3/HOXD12 interaction modified digit patterning, likely as a consequence of direct protein/protein contacts (Chen et al., 2004).

Mice carrying the *Extra-toes* (*Xt*) mutation lack the function of *Gli3*. These mice have a range of anomalies, among which a severe polydactyly of both fore-and hindlimbs, likely due to the de-repression of *Hox* genes, and concurrent ectopic expression of *Shh*, at the anterior margin of the developing limb (Buscher et al., 1997). In order to assess whether the wild-type pentadactyly was indeed due to a *Gli3*-dependent anterior repression of *Hox* genes, in other words whether the polydactyly observed in *Xt* mutant mice is dependent upon the gain of *Hox* gene function(s), we crossed *Xt* mice with mice carrying either a full, or a partial, deletion of the *HoxD* cluster (Zakany et al., 2001, 2004). Here, we show that removing all *Hoxd* gene function, in addition to *Gli3* in the developing autopod, does not significantly reduce the number of digits when compared to mice mutant for *Gli3* alone.

In striking contrast, however, the combination of the *Gli3* mutant allele with a partial deletion of the *HoxD* cluster (deletion of *Hoxd1* to *Hoxd10* included) gave mice with heavily truncated limbs, a situation drastically different from the phenotype observed with the same deletion, but in the presence of *Gli3* function. In this latter case, gain of function of the remaining 'posterior' *Hoxd* genes lead to an ectopic *Shh* domain anteriorly and consequent bilateral symmetry of an otherwise weakly truncated limb (Zakany et al., 2004). This observation indicates that widespread and early expression of *Hoxd13* and *Hoxd12* can severely impair stylopod development, but only when *Gli3* function is either reduced or removed, suggesting that *Gli3* function protects against the prevalent function of posterior genes over their more anterior neighbors. Such severe limb truncations involved defects in the apical ectodermal ridge (AER), likely due to a dramatic decrease of *Fgf10* expression in limb bud mesenchyme. We discuss the potential roles of these various players in the growth and patterning of the limbs.

Materials and methods

Mouse stocks, crosses and genotyping of mid-gestation embryos and near-term fetuses

The mouse lines carrying the *HoxD* cluster alleles used in this study were produced by *loxP/Cre*-mediated site-specific recombination. *del(1–13)* is an approximately 100-kb large deletion encompassing from the *Hoxd1* to the *Hoxd13* loci. In this deletion, the entire *HoxD* function is lost (Zakany et al., 2001). *Del(1–10)* was generated by targeted meiotic recombination (Herault et al., 1998) using *del(1–13)* as one of the parental alleles to produce an approximately 70-kb large targeted deletion from *Hoxd1* to *Hoxd10* included (Zakany et al., 2004). The two deficiencies have the same breakpoint near *Hoxd1*. All *HoxD* alleles were genotyped in a multiplex PCR reaction, using the 5′-CCACCCTGCTAAATAAACGCTG-3′ *Hoxd11* forward primer, and the 5′-GGTTGCCTCTTTTCCTCTGTCTC-3′ *Hoxd10* reverse primer for wild-type and the 5′-CTATTCAAAGGTGGGGAGCAGTC-3′ *Hoxd1* reverse primer for mutant allele. *Gli3 XtJax* allele was genotyped with the 5′-TACCCCAGCAGGAGACTCAGATTAG-3′ forward and 5′AAACCCGTGGCTCAGAGCAAG-3′ reverse primers, while the *Gli3* wild-type allele with the 5′-GGGTGAACAGCATCAAAATGGAG-3′ forward and 5′-ATAGCCATGTGGTGGTGCCCATG-3′ reverse primers.

Heterozygous males or females of either *HoxD* deficiencies were crossed over *Xt* heterozygous males or females to obtain compound heterozygous *Xt/+; del(1–13)/+* and *Xt/+;Del(1–10)/+* males and females. Both compound mutants were obtained in near Mendelian proportions, and most individuals of both genotypes displayed characteristic digit defects in forelimbs: oligodactyly in *Xt/+;del(1–13)/+*(Fig. 1E) and polydactyly in *Xt/+;Del(1–10)/+*(Fig. 1F). As *del(1–13)* homozygous animals are semi-lethal post-natally and both *Del(1–10)* and *Xt* homozygous animals are lethal at birth, we collected the F2 progeny from *Xt/+;del(1–13)/+* and *Xt/+;Del(1–10)/+* parents on the 18th day post-fertilization (E18) in order to minimize losses of individuals with compound genotypes. Genomic DNA was extracted from tail biopsies or yolk sac (E10, see below) and genotyped by PCR reactions, using the specific primers indicated above.

RNA in situ hybridization

To evaluate early limb development in the various genotypic classes, F2 embryos were collected on the morning of the 10th day of development (E10) and processed for whole mount RNA in situ hybridization following standard procedures (see e.g. www.eumorphia.org/EMPReSS/servlet/EMPReSS Doc. Number: 13_003). Yolk sac samples were collected individually and genomic DNA was isolated for genotyping, whereas individually fixed embryos were stored at minus 20 °C in methanol. Once genotypes were established, representatives of the selected genotypes were grouped and processed together for any given probe. Forelimb buds of all specimens were photographed and the same magnifications are shown. Probes were as originally described: *Fgf8* (Crossley and Martin, 1995), *Fgf10* (Bellusci et al., 1997), *Gli3* (Hui and Joyner, 1993), *Hoxd13* (Dolle et al., 1993), *Meis1* (Saleh et al., 2000) and *Shh* (Echelard et al., 1993). After the in situ hybridization patterns were documented, the embryos were homogenized, genomic DNA was extracted and the genotypes were further verified.