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Hoxd and *Gli3* interactions modulate digit number in the amniote limb

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Abstract

During limb development, Sonic hedgehog (SHH) and HOX proteins are considered among the most important factors regulating digit number and identity. SHH signaling prevents the processing of GLI3 into a short form that functions as a strong transcriptional repressor. *Gli3* mutant limbs are characterized by a severe polydactyly and associated ectopic anterior expression of 5′Hoxd genes. To genetically determine the involvement of 5′Hoxd genes in the polydactyly of *Gli3* mutants, we have generated a compound mutant that simultaneously removes the three most 5′-located Hoxd genes and *Gli3*. Remarkably, the limbs that form in the absence of all four of these genes show the most severe polydactyly so far reported in the mouse. The analysis of gene expression performed in compound mutants allows us to propose that the increase in the number of digits is mediated by the gain in function of *Hoxd10* and *Hoxd9*. Our results also support the notion that an adequate balance between positive and negative effects of different Hoxd genes is required for pentadactyly.

Keywords: *Gli3*; *Hoxd*; *Hoxa*; *Shh*; Limb development; Extra-toes; Polydactyly; Posterior prevalence

Introduction

Pentadactyly is the ancestral amniote digit formula. All extant tetrapods descend from an ancestor with a pentadactyl limb, although many species have reduced the number of digits or even lost the limb (Cohn and Tickle, 1999; Cohn, 2001). Despite the multiple variations on the basic pentadactylous pattern, a limit of 5 in the number of digits appears to be constant.

The detailed way in which the number and identity of the digits is established and controlled during limb development is not completely understood and remains a subject of active investigation. In humans, alterations in the number of digits, preferentially polydactyly, are among the most frequent congenital malformations (Lamb et al., 1982; Graham and Ress, 1998).

It is known that the zone of polarizing activity (ZPA), through its production of Sonic hedgehog (SHH), controls distal limb patterning including the number and identity of the digits (Saunders and Gasseling, 1968; Tickle et al., 1975; Riddle et al., 1993). SHH signaling in vertebrates is mediated by the three Gli genes (*Gli1*, *Gli2* and *Gli3*). Of these, *Gli3* is the one that is essential for limb development as evidenced by the polydactylous phenotype of the *Extra-toes* (*Xt*) mutation, which represents the complete loss of function of *Gli3* (Schimmang et al., 1992; Hui and Joyner, 1993; Maynard et al., 2002). In the absence of *Shh*, GLI3 is processed to a short form that acts as a strong transcriptional repressor (GLI3R) (Dai et al., 1999; Aza-Blanc et al., 2000; Wang et al., 2000). Therefore, as a consequence of the posterior secretion of SHH during normal limb development, a gradient of GLI3R is established along the anteroposterior axis of the bud with maximum levels at the anterior border (Wang et al., 2000; Litingtung et al., 2002; Bastida et al., 2004). The molecular study of *Xt* homozygous limbs and several other polydactylous mutations in mice revealed an ectopic spot of *Shh* expression at the anterior border, which was considered responsible for the polydactylous phenotype (Chan et al., 1995; Masuya et al., 1995; Buscher et al., 1997; Masuya et al., 1997). However, the subsequent study of *Shh*;*Gli3* double mutant embryos, phenotypically indistinguishable from *Gli3* mutants, demonstrated that the

Abbreviations: FL, forelimb; HL, hindlimb; P3, third phalanx; P?, undefined phalanx.

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polydactyly in the absence of *Gli3* was independent of *Shh* (Litingtung et al., 2002; te Welscher et al., 2002b). Consequently, two types of polydactylous phenotypes can be distinguished with different underlying molecular mechanisms. Type 1 depends on the ectopic anterior activation of *Shh*, with intact *Gli3* function, and preaxial mirror-image duplication of digits with clear identities. Type 2 polydactyly disrupts *Gli3* function, is *Shh* independent and shows unidentifiable digits (Litingtung et al., 2002; te Welscher et al., 2002b).

It has also been clearly demonstrated that the morphogenesis of the vertebrate digits requires the function of genes of the *HoxA* and *HoxD* clusters (Davis et al., 1995; reviewed by Zakany and Duboule, 1999). During limb development Hoxa and Hoxd genes are activated sequentially in time and space, following their genomic topography, a phenomenon known as temporal and spatial collinearity (Lewis, 1978; Gaunt et al., 1988). Genes located more 3′ in the chromosome (also referred to as anterior or proximal) are expressed earlier and at more anterior locations than genes located more 5′ (also referred to as posterior or distal). The division between anterior and posterior Hoxd genes has not been clearly defined although a separation between Hoxd genes expressed throughout the limb bud and those excluded from anterior cells and capable of inducing *Shh* has been precisely mapped between *Hoxd9* and *Hoxd10* (Tarchini et al., 2006). Interestingly, the forced expression of a posterior gene earlier and/or more anteriorly than normal results in a posteriorization phenotype (Duboule, 1991; Duboule and Morata, 1994), an observation that led to the model of "posterior prevalence" meaning that the product of a posterior gene can inactivate the function of a more anterior one, likely at the posttranscriptional level (van der Hoeven et al., 1996; Herault et al., 1997; Williams et al., 2006).

The generation of an extensive series of mutations in the *HoxA* and *HoxD* clusters including inversions, deletions, duplications and compound mutations has shed light on the regulation and function of Hox genes during limb development (Kmita et al., 2002; Spitz et al., 2003; Kmita et al., 2005; Tarchini and Duboule, 2006). Hoxd genes are activated in two consecutive waves under different transcriptional control. The first phase of Hoxd gene expression, essential for the development of the limb up to the forearm, relies on two opposite regulations: one, the early limb control region (ELCR), located at the telomeric end of the complex, and the other located at the centromeric end of the complex (Zakany et al., 2004; Tarchini and Duboule, 2006). The ELCR acts as a timer activator relying on relative distance to the promoter while the centromeric enhancer acts by preventing expression in anterior cells (Tarchini and Duboule, 2006). The second phase of Hoxd activation, regulated by a global control region (GCR) located centromeric to the cluster, is essential for digit development (Spitz et al., 2001; Spitz et al., 2003).

The *HoxA* and the *HoxD* gene clusters are also required for the initiation of *Shh* expression in the ZPA (Kmita et al., 2005). Furthermore, the study of a series of *Hoxa*/*Hoxd* double mutant mice showed that correct *Shh* expression depends on quantitative and qualitative combinations of Hoxa and Hoxd genes of the paralogous groups 10 to 13 (Tarchini et al., 2006). Therefore, the notion that anterior-posterior patterning of the limb bud is a consequence of the intrinsic colinearity of Hox gene expression has emerged (Tarchini et al., 2006).

Interestingly, the 5′-located Hoxd genes are broadly and ectopically expressed in the anterior limb bud mesoderm of *Xt* mutants, indicating that GLI3R represses their transcription (Zuniga and Zeller, 1999; Litingtung et al., 2002; te Welscher et al., 2002b). Since overexpression of these genes cause preaxial polydactyly (Morgan et al., 1992; Goff and Tabin, 1997; Knezevic et al., 1997; Kmita et al., 2002), it has been proposed that the polydactyly in *Xt* mutants is mediated by the ectopic anterior expression of posterior Hoxd genes. Interestingly, it has been recently shown that GLI3 and HOXD12 interact genetically and physically, and that this interaction modulates GLI3R function (Chen et al., 2004). This finding indicates that HOXD proteins may function semiquantitatively to regulate digit pattern and identity through the interaction with GLI3 (Chen et al., 2004). The graded posteroanterior level of 5′ HOXD proteins during their second phase of expression in the developing limb can be envisaged as functioning to counteract the transcriptional function of GLI3R, therefore potentiating SHH function (Chen et al., 2004).

To directly determine the involvement of 5′Hoxd genes in the polydactyly of *Gli3* mutants, we have generated a compound mutant that simultaneously removes the three most 5′-located Hoxd genes and *Gli3*. SHH prevents the processing of GLI3 while HOXD12 and HOXD13 convert its function from a transcriptional repressor to a transcriptional activator (Wang et al., 2000; Chen et al., 2004). Therefore, removal of the 5′ Hoxd genes in the absence of *Gli3* may also help to determine other functions of 5′ Hoxd genes not related with their interaction with GLI3.

Remarkably, the limbs deficient in all four of these genes are extremely polydactylous, arguing against most 5′-located Hoxd genes mediating the polydactyly characteristic of the *Xt* phenotype. Compound mutants differ from *Gli3* mutants in the number, length and chondrogenic differentiation of the digits, indicating that posterior Hoxd genes have functions independent of GLI3. Based on the molecular study of the compound mutant, we propose that the polydactylous phenotype is mediated by the gain of function of HOXD10 and HOXD9. Our results also support the notion that an adequate balance between positive and negative effects of different Hoxd genes is required for pentadactyly.

Materials and methods

Mice mutant genotyping

The *Extra-toes* ($Gli3^{Xt.J}$ Jackson allele; Hui and Joyner, 1993), the $Hoxd^{Del(11–13)}$ (Zakany and Duboule, 1996) and the *Shh* (Chiang et al., 1996) mutant lines were maintained in a mixed background. The $Hoxd^{Del(11–13)}$ allele is the deletion of the *Hoxd13* and *Hoxd12* locus plus the insertion of the *lacZ* reporter gene within the *Hoxd11* gene, thus representing the loss-of-function of *Hoxd13*, *12* and *11* (Zakany and Duboule, 1996).

The embryos were obtained by caesarean from pregnant mice and dissected in cold phosphate buffered saline (PBS). Genotyping was performed by PCR as described (Hui and Joyner, 1993; Chiang et al., 1996; Zakany and Duboule, 1996). Noon of the day the vaginal plug was observed was considered as