Impairment of \textit{Sox9} Expression in Limb Buds of Rats Homozygous for Hypodactyly Mutation

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Abstract. Rat hypodactyly (\textit{hd}) is an autosomal recessive mutation manifesting in homozygotes as reduction or loss of digits II and III. We mapped the \textit{hd} allele to a short segment of chromosome 10, containing 16 genes. None of these genes has been shown to influence limb development yet. \textit{In situ} hybridization showed no changes in several important patterning genes (\textit{Shh}, \textit{Fgf8}, \textit{Bmp2}, 4, 7). However, we found that expression of cartilage condensation marker \textit{Sox9}, and Bmp receptor \textit{Bmpr1b} (acting as an upstream activator of \textit{Sox9} expression) is absent from the subepithelial mesenchyme of the digit condensations II and III. The failure of the chondrogenic condensations to extend towards the subepithelial mesenchyme may reduce the size of digit primordia and underlie the subsequent loss of phalanges and reduction of metacarpals/metatarsals in \textit{hd} rats.

Introduction

The limb of vertebrates serves as a valuable model of embryonic development. Assignment as well as readout of the positional information and coordination of cell proliferation, differentiation, motility, and death must be coordinated for proper formation of the elaborately patterned limb (Niswander, 2003). One of the fundamental aspects of limb development is the formation of the skeleton. During development of the limb skeleton, the limb bud mesenchyme first assembles into precartilage condensations that precede cartilage formation and enchondral ossification (Fell, 1925). The position and shape of the mesenchyme condensations is determined by interplay of various modes of cell communication. Signalling through the major limb patterning centres, apical ectodermal ridge (AER) and zone of polarizing activity (ZPA), as well as segmental patterning induced by \textit{Hox} genes are thought to play a substantial role in this process (Capdevila and Izpisua Belmonte, 2001; Zakany and Duboule, 2007); however, the details of the skeletal element patterning have not been elucidated yet.

Precartilaginous condensations are formed in a proximal-distal order and can be visualized by \textit{Sox9} expression earliest by E9.5 in the mouse forelimb (Ng et al., 1997). \textit{Sox9}, a transcription factor of the high-mobility group (HMG) family, is considered to be a master regulator of chondrogenesis. Conditional inactivation of \textit{Sox9} at varying times during mouse limb development has revealed that it is involved in all important steps of the cartilage formation, i.e. in condensation, proliferation and maturation. Akiyama (Akiyama et al., 2002) generated mouse embryos in which \textit{Sox9} was removed from limb mesenchymal cells prior to the onset of condensation, resulting in total absence of condensations and subsequent development of extremely short limbs without any skeletal components. Inactivation of \textit{Sox9} after mesenchymal condensation led to severe chondrodysplasia, when most of condensed mesenchymal cells did not differentiate into chondrocytes and proliferation of chondrocytes was inhibited.

Differentiation of chondrocytes is accompanied by secretion of molecules such as collagen type II (\textit{Col2a1}), type IX (\textit{Col9a1}), and aggrecan, forming cartilage-spe-
cific extracellular matrix. Sox9 directly induces the expression of Col2a1, a molecular marker of chondrocytes (Bell et al., 1997). Sox9 works in concert with two other transcription factors from the HMG family, Sox5 and Sox6 (Smits et al., 2001).

Bone morphogenetic protein (BMP) signalling (namely BMP2, BMP4, and BMP7) transduced by heterodimers of type I receptors (Bmpr1a, Bmpr1b, ActR1) with type II receptor (Bmpr2) is important for early events in chondrogenesis. Studies have shown that Bmpr1a and Bmpr1b have distinct roles during chondrogenesis. In chick, Bmp signal transduced by Bmpr1b appears to be particularly important for precartilaginous condensation, while Bmpr1a is active during later phases of chondrogenesis (Zou et al., 1997). Mice deficient in Bmpr1b (Yi et al., 2000) are viable, exhibiting defects confined to phalangeal elements and appendicular joints, when initial formation of the digital rays occurs normally, but proliferation of prechondrogenic cells and chondrocyte differentiation are markedly reduced. Bmpr1a conditional knockout mice (Yoon et al., 2005) exhibit generalized chondrodysplasia with long bones shortened and ossification delayed. In double (Bmpr1a−/− and Bmpr1b−/−) mutants (Yoon et al., 2005), all bones that form through enchondral ossification are either absent or malformed, and differentiation of prechondrocytes into chondrocytes is severely affected. Importantly, double mutants do not express Sox9, indicating that BMP signalling acts as an upstream activator of Sox9 expression.

The hd mutation in the rat was described in 1973 (Moutier et al., 1973). Hypodactyl is an autosomal recessive trait – hd/hd homozygotes are affected, +/hd heterozygotes are indistinguishable from wild-type animals at the phenotypic level. The phenotype consists of the reduction or loss of digits II and/or III, in both fore- and hindlimbs. Moreover, males are infertile due to the impairment of the latest step of male gametogenesis – spermiogenesis (Liška et al., 2009).

We mapped hd previously to chromosome 10 (Křenová et al., 1999). Rat hd is therefore distinct from mouse Hd, caused by a 50 nucleotide deletion in exon 1 of the Hoxa13 gene (Post et al., 2000). Besides the different mode of inheritance and different phenotype, its mapping excluded Hoxa13 mutation as the molecular cause, since the HoxA cluster is located on rat chromosome 4. We previously investigated the possibility of Shbg (sex hormone-binding globulin) gene as the positional and molecular candidate because of the localization on the non-recombinant segment of chromosome 10 and the infertility seen in hd/hd males. Shbg was free of the coding sequence variation that could be associated with hd. The apparent expression of Shbg was stronger in hd/hd mutants, but this phenomenon might be due to a higher proportion of Shbg-producing Sertoli cells in the mutants (Liška et al., 2004).

We present here a fine mapping of hd to 464 kb, leading to Shbg exclusion. None of the 16 genes in the region has been implicated in limb development yet. Therefore, we screened limb buds of the mutants by whole-mount in situ hybridization (ISH) for expression of a panel of genes with known role in limb development. Many of the key patterning genes (including Fgf8 and Shh) remain unchanged in the mutants; however, markers of chondrogenic condensations, Sox9 and Bmpr1b, do not extend distally to the subepithelial layer in regions marking digit primordia II and III. This may be causative for the impairment of digit formation with subsequent reduction defects.

Material and Methods

Animals

All experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997), which is in compliance with the European Community Council recommendations for the use of laboratory animals 86/609/EEC. All experiments were approved by The Charles University Animal Care Committee. hd is propagated as WHD (Wistar hypodacty- lous) strain by brother-sister mating of hd/hd females with (fertile) +/hd males. Congenic brown Norway rat (BN)-hd and spontaneously hypertensive rat (SHR)-hd rats were derived by cross-intercross mating of hypo- dactyloous WHD females to BN/Cub or SHR/Otalpcv males, respectively, using DNA-marker-assisted selection. Phenotype was scored visually.

Fine mapping (positional cloning)

Combined backcross (N = 497) and intercross (N = 353) between WHD and BN was employed. From 497 intercross animals, 50 were derived from BN-hd congenic production. The same is true for 33 out of the 353 backcross animals. Tail DNA was genotyped by PCR amplification of microsatellite markers selected from public databases or derived from the rat chromosome 10 sequence using Pompous (Fondet et al., 1998). Primer3 was used for primer design (Rozen and Skaltsky, 2000). The linkage map was constructed using MapManager QTX (Manly et al., 2001) separately for backcross, intercross, and congenic backcross- and intercross-like families and merged manually to form an integrated map.

Skeletal staining

Cartilage was stained by alcian blue, bone by alizarin according to Mundlos (2000). Whole-mount in situ hybridization

Whole-mount ISH was performed as described in Stricker et al. (2006).

Results

Fine mapping of hd mutation

Using a combined backcross-intercross approach, we were able to refine the mapping of hd to 0.15 cM interval, which corresponds to a 463,782 bp segment of the
rat genome, assembly 3.4 (Fig. 1A). This segment contains 16 genes and gene predictions (Fig. 1B). Shbg is localized 116,992 bp downstream of the 3' end of the non-recombinant segment (not shown), thus excluding the Shbg gene as a candidate.

Mutants show reduction or loss of the distal portion of digits II and III

hd mutation affects only the autopod of both fore- and hindlimbs (Fig. 2); stylopod and zeugopod of hd/hd mutants are comparable with wild-type controls (data not shown). Carpal and tarsal parts of the autopod are likewise unchanged in the mutants. Further, we found normal morphology of thumb and fingers and toes IV and V. However, digit II was usually nearly missing, with reduced or rudimentary metacarpal/metatarsal bone remaining. Digit III was more variably affected—it was often relatively preserved, or shortened and malformed or missing, like digit II, although relatively normal metacarpals/tarsals were formed. Notably, reduced digit III often displayed missing or greatly reduced ossification centres in phalanx 2 and 3. The latter forms earlier, by a different mechanism (Casanova and Sanz-Ezquerro, 2007; Montero and Hurle, 2007), at the tip of the distal phalanges.

Expression of known patterning genes is not changed in mutants

Using whole-mount ISH, we assessed the expression of a panel of genes known to be important for limb pat-
terning and morphogenesis. AER signalling was not altered in mutants, as can be demonstrated by expression of Fgf8, an established AER marker (Fig. 3A). ZPA signalling, conveyed by Shh, was also unchanged in the mutants, as inferred by Shh expression (Fig. 3B). Several members of the Bmp family are necessary for chondrogenic development of limb skeletal elements including digits. We therefore analysed the expression of Bmp2, 4, and 7, which do not show apparent pattern alterations in hd/hd limb buds (Figs. 3C-E).

**Mutants exhibit a distinct pattern of Bmpr1b and Sox9 expression**

The initial Sox9 expression pattern before distinct digit condensations are formed (13.5 dpc) is unchanged (Fig. 4A). Whole-mount ISH revealed differences in the expression of Sox9, a marker of chondrogenic condensation of the digit anlage. Normally, the expression at 14.5 dpc extends in the digit mesenchyme along the whole proximo-distal axis of the autopod, reaching the subepithelial layer (known as the progress zone). In hd mutants, this pattern is preserved for digits IV, V and digit I. However, for digit primordia II and III, there is a significant gap between the distal tip of the Sox9-positive primordium and the limb margin (AER) (Figs. 4B,C). Identical finding is replicated by Bmpr1b expression at 14.5 dpc, which does not reach the normal distal boundary with the progress zone (Fig. 4D,E). This altered expression pattern is concomitant with the formation of Sox9-positive digit primordium. The gross limb phenotype can already be recognized in mutants at this stage (14.5 dpc) in forelimbs.
A 13.5 dpc  14.5 dpc
Fgf8  
+/hd  hd/hd  

B  
+/hd  hd/hd
Shh

C 12.5 dpc  13.5 dpc
Bmp2  
+/hd  hd/hd  

D  
+/hd  hd/hd  
Bmp4

E 13.5 dpc  14.5 dpc
Bmp7  
+/hd  hd/hd  

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Discussion

During our fine linkage mapping experiments, we found that Shbg, our previously suggested candidate gene, is recombined from the hd locus. It is still possible that the non-recombinant segment contains a cis-acting (non-coding, regulatory) sequence capable of long-range activation/inhibition of Shbg expression. However, Shbg is situated with its 3' end towards the non-recombinant segment with four other genes in both orientations interfering (Efnb3, Wrap53, Tp53 and Atp1b2). Such hypothetical long-range signalling sequence would therefore likely influence these genes, too, which would be demonstrated by appropriate phenotypic consequences. E.g. Ephrin B3 (encoded by Efnb3) is necessary for proper neuronal control of locomotor activity such as walking (Kullander et al., 2003). Such a phenotype was not observed in hd mutants. The same is true for Tp53 – hd mutants do not have increased tumour incidence.

We revealed 16 positional candidates for hd here. None of these genes was implicated in limb development, nor in any developmental process as far as we know, including cell proliferation and cell death. Our approach to dissect the hd phenotype was based on scanning the expression of known patterning genes. We believe this can get us closer to the mechanistic understanding of the hd phenotype pathogenesis.

Understanding hd may offer a unique insight into limb development. First, there is as yet an unanticipated gene involved in the pathogenesis of this phenotype, as none of the candidate genes was associated with limb development as far as we know. Second, the phenotype itself is unique. Although we can find many examples of digit or autopod reduction in many models as well as in the clinic (Post et al., 2000; Chiang et al., 2001; Schwabe and Mundlos, 2004; Ogino, 2007; Gao et al., 2009), none of them is, to the best of our knowledge, similar to hd in details.

Expression of Bmps is unchanged in hd mutants, so the hd mutation appears to impair autopod development downstream of Bmp. On the other hand, digit formation is affected upstream of Sox9, as Bmpr1b, upstream activator of Sox9 expression (Yoon et al., 2005) shows an altered expression pattern parallel to that of Sox9. Therefore, despite normal Bmp levels, the absence of Bmpr1B in the apical portion of the putative digital rays II and III leads to the absence of Sox9 expression and subsequent lack of cartilaginous and bone formation. Mutations of human BMPR1B cause brachydactyly type A2 (Lehmann et al., 2003). Interestingly, the index finger is shortened in patients with this syndrome, and digit II is reduced or missing in hd mutants. However, the question arises whether there is direct influence of hd upon Bmpr1b expression. If such influence existed, it would be spatially restricted to the distal portion of the digital ray. A plausible hypothesis may be formed in conjunction with the progress zone model (Summerbell et al., 1973; Tabin and Wolpert, 2007), where the digits grow distally by recruitment of cells from the mesenchyme layer just beneath the epithelium. This model was updated recently by introducing the concept of phalanx-forming region (Suzuki et al., 2008) or digit crescent (Montero et al., 2008), a specialized group of cells at the tip of each digit, expressing Bmpr1b and Sox9, that recruits the mesenchyme cells from the progress zone and promotes their differentiation into the digit condensations. The Bmpr1b and Sox9 expression pattern in mutants suggest that possibly these cells are recruited from the progress zone, but they are not properly instructed to become part of the digit anlagen, remaining undifferentiated instead. Perhaps these cells later adopt the “interdigital fate” and are lost by apoptosis, like the normal interdigital mesenchyme.

In conclusion, we present here fine mapping of hd mutation with resulting 16 positional candidate genes. We propose a connection between impairment of limb development in hd homozygotes and definitive formation of the prechondrogenic condensations in apical portion of digits II and III.

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