Developmental Biology 385 (2014) 67–82

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Forward genetics defines Xylt1 as a key, conserved regulator of early chondrocyte maturation and skeletal length

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ARTICLE INFO

Article history:
Received 16 August 2013
Received in revised form
11 October 2013
Accepted 14 October 2013
Available online 23 October 2013

Keywords:
Forward genetics
Xylt1
Dwarfism
Chondrocytes
Skeletal development
Proteoglycans
CSPGs
HSPGs
Glycosaminoglycans

ABSTRACT

The long bones of the vertebrate body are built by the initial formation of a cartilage template that is later replaced by mineralized bone. The proliferation and maturation of the skeletal precursor cells (chondrocytes) within the cartilage template and their replacement by bone is a highly coordinated process which, if misregulated, can lead to a number of defects including dwarfism and other skeletal deformities. This is exemplified by the fact that abnormal bone development is one of the most common types of human birth defects. Yet, many of the factors that initiate and regulate chondrocyte maturation are not known. We identified a recessive dwarf mouse mutant (*pug*) from an *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen. *pug* mutant skeletal elements are patterned normally during development, but display a ~20% length reduction compared to wild-type embryos. We show that the *pug* mutation does not lead to changes in chondrocyte proliferation but instead promotes premature maturation and early ossification, which ultimately leads to disproportionate dwarfism. Using sequence capture and high-throughput sequencing, we identified a missense mutation in the *Xylosyltransferase 1* (*Xylt1*) gene in *pug* mutants. Xylosyltransferases catalyze the initial step in glycosaminoglycan (GAG) chain addition to proteoglycan core proteins, and these modifications are essential for normal proteoglycan function. We show that the *pug* mutation disrupts Xylt1 activity and subcellular localization, leading to a reduction in GAG chains in *pug* mutants. The *pug* mutant serves as a novel model for mammalian dwarfism and identifies a key role for proteoglycan modification in the initiation of chondrocyte maturation.

Introduction

One of the defining features of vertebrates is their internal skeleton. While the global organization of the skeleton is conserved across vertebrates, changes in the number, size, or shape of specific skeletal elements correspond to newly acquired or modified structures induced through evolutionary pressure. Within a species, however, the organization and morphology of skeletal elements are much more constrained; suggesting that tight regulation of skeletal development is essential for an animal's reproductive fitness. Most skeletal elements in the appendicular and axial skeleton of the vertebrate body are formed through the process of endochondral ossification that involves the formation of a cartilage anlage, within which chondrocytes undergo a maturation process before being replaced by mature bone. This process begins with the condensation and aggregation of mesenchymal cells, which then differentiate into chondrocytes (Shimizu et al., 2007). As the cartilage anlage grows, the chondrocytes become arranged into morphologically distinct zones of resting, proliferative, prehypertrophic, and hypertrophic chondrocytes, called growth plates (Goldring et al., 2006). Progression through the growth plates occurs as chondrocytes mature, resulting in the most mature cells located near the center of the skeletal element. While bone is laid down in the center of the anlage by invading osteoblasts, the element continues to grow on either end within the growth plates via highly regulated proliferation of a subset of the chondrocyte pool. This process, if misregulated, leads to skeletal abnormalities that can affect the size, shape and strength of skeletal elements (Baldridge et al., 2010).

Multiple signaling pathways regulate chondrogenesis, and tight regulation of their signaling output is critical for normal skeletal development. For example, achondroplasia, the most common

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http://dx.doi.org/10.1016/j.ydbio.2013.10.014

form of human dwarfism, is associated with excess Fibroblast growth factor (Fgf) signaling due to activating mutations in *Fgfr3*. This results in the rhizomelic shortening of limb bones, or a more severe shortening of proximal limb elements compared to distal elements (Deng et al., 1996; Ornitz, 2005; Rousseau et al., 1994; Segev et al., 2000). Signaling molecules such as Indian Hedgehog (*Ihh*) and Parathyroid hormone related protein (*PTHrP*) (Retting et al., 2009; Shimizu et al., 2007) also regulate chondrogenesis and maturation. A loss of either *PTHrP* or its receptor, *PTH1R*, results in reduced skeletal element size and premature maturation, while a loss of *Ihh* results in reduced skeletal elements size due to delayed maturation and a disorganization of the growth plate (Karaplis et al., 1994; Lanske et al., 1996; Vortkamp et al., 1996). Though these signaling pathways each have specific roles, they do not act in isolation; crosstalk between the major pathways helps to maintain the proper rate and coordination of chondrocyte maturation to create an appropriately sized and patterned skeleton (Amizuka et al., 2004; Kronenberg, 2003; Minina et al., 2002).

Normal endochondral ossification also depends upon a distinct and complex extracellular matrix (ECM) comprising cartilage-specific collagens, elastin, and proteoglycans. The ECM functions both as a structural component for the skeleton, especially at the joints, but also as a regulator of signaling molecule diffusion and function within the growth plate. Signaling factors must traverse and interact with the extracellular matrix in order to induce their effects. *Ihh*, expressed in prehypertrophic chondrocytes, signals both short-range to activate its downstream targets such as *Patched1* (*Ptch1*) and *Gli1*, but also long-range to regulate the expression of *PTHrP* at the heads of the developing skeletal elements (Gritli-Linde et al., 2001; Karaplis et al., 1994; Karp et al., 2000; Kobayashi et al., 2005; Mak et al., 2008; St-Jacques et al., 1999). This long-range signaling is critical to modulate maturation of the resting pool of chondrocytes (Kronenberg, 2003; Lanske et al., 1996; Vortkamp et al., 1996). *Runx2* also functions downstream of *Ihh* in order to regulate maturation from prehypertrophic to hypertrophic chondrocytes, and is required for bone formation (Enomoto et al., 2000; Enomoto-Iwamoto et al., 2001; Takarada et al., 2013; Takeda et al., 2001). Similarly, during endochondral ossification, *Fgf7, 8, 9, 17*, and *18* are expressed primarily in the perichondrium, or the fibrous layer of cells surrounding the condensed chondrocytes, and signal to *Fgfr3* expressed on proliferative and resting chondrocytes (Hung et al., 2007; Liu et al., 2002; Ornitz, 2005). Despite a large body of work on human bone diseases and animal models, the relationship between skeletal development and defects in the ECM are understudied.

To identify factors that regulate skeletal element length, we took a forward genetics approach and studied a recessive mouse mutant, *pug* that was identified from a *N*-ethyl-*N*-nitrosourea mutagenesis screen. Homozygous *pug* animals display a dwarfism phenotype, with shortened long bones compared to their wild type or heterozygous littermates. However, this is not due to changes in the proliferation of chondrocytes, but rather to their early maturation. The premature progression of *pug* chondrocytes through the maturation process occurs prior to changes in *Ihh* and *Fgf* signaling, suggesting chondrocyte maturation initiates independently of these signaling pathways. We further show that the underlying genetic defect in *pug* mutants is a missense mutation in the *Xylosyltransferase 1* (*Xylt1*) gene. Xylosyltransferases catalyze a rate-limiting step in the addition of glycosaminoglycan (GAG) side chains to proteoglycans (Cuellar et al., 2007; Esko et al., 1985; Kearns et al., 1993; Ponighaus et al., 2007; Prante et al., 2006), which is critical for proteoglycan function within the extracellular matrix. We show that *Xylt1* is specifically expressed in chondrocytes during embryonic development and, as in other studies, Xylt1 protein localizes within the *cis*-Golgi where it functions as a xylosyltransferase (Nuwayhid et al., 1986; Schön et al., 2006a). We find that *pug* is a hypomorphic allele of *Xylt1* that results both in reduced Xylt1 activity and altered Xylt1 subcellular localization. We further show that GAG chain levels are reduced in *pug* mutants and these changes likely underlie the dwarfism phenotype. The defect in proteoglycan processing and subsequent premature maturation in *pug* mutants highlights a key role for Xylt1 and glycosaminoglycans in the regulation of chondrocyte maturation during skeletal development.

Materials and methods

Mouse strains

The *pug* mutant was identified as part of a mutagenesis screen (described in (Liem et al., 2009)). Briefly, C57BL6/J males were mutagenized with *N*-ethyl-*N*-nitrosourea (ENU) and crossed to FVB/NJ females. F1 males were further mated to FVB/NJ females to generate G2 females. Embryos from F1 x G2 crosses were analyzed for recessive phenotypes. The *pug* mutant was identified at embryonic day (e) 18.5 based on shorter limbs compared to littermates. PTHrP-LacZ mice were crossed to *pug* animals and genotyped as described previously for *LacZ* (Chen et al., 2006).

Mapping of the pug mutation

Using a whole genome single nucleotide polymorphism (SNP) panel (Moran et al., 2006), *pug* was mapped to Chromosome 7 and the *pug* interval was subsequently narrowed to between SNP markers rs31909733 (122.29 Mb) and rs6261444 (130.1 Mb) via meiotic recombination mapping. The *pug* mutation has been crossed >10 generations onto the FVB/NJ background, which removed more than 99.9% of the original mutagenized C57BL/6J background, supporting the idea that the *pug* phenotype is monogenic. Mutant characterization was carried out at various stages of crossing into the FVB/NJ background.

Sequence capture

A Nimblegen mouse Sequence Capture 385 K array was designed to contain oligos complementary to the exons within the *pug* genomic locus (NCBI37/mm9 Chr7: 122,297,973–130,145,967 Mb), minus repetitive sequences. Genomic DNA from a *pug* homozygote was isolated and then sheared by sonication, and adaptors were ligated to the resulting fragments. The adaptor-ligated templates were fractionated by agarose gel electrophoresis and fragments of the desired size were excised. Extracted DNA was amplified by ligation-mediated PCR, purified, and hybridized to the Sequence Capture array. The array was washed, and bound DNA was eluted, purified, and amplified by ligation-mediated PCR (similar to methods employed in (Choi et al., 2009)). The capture and sequencing experiments were performed at the W.M. Keck Foundation for Biotechnology Resources at Yale. This array also contained sequences from Chromosomes 4 & 11, unrelated to the *pug* locus. For details about these sequences, please contact the authors.

Sequencing and mutation analysis

Captured libraries were sequenced on an Illumina Genome Analyzer II as single-end, 75-bp reads using previously described methods (Choi et al., 2009). Illumina reads were first trimmed based on their quality scores to remove low-quality regions using the program Btrim (Kong, 2011). A cutoff of 20 for average quality scores within a moving window of size 5-bp was used. Minimum