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Increased *Col10a1* expression is not causative for the phenotype of Phex-deficient *Hyp* mice

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ABSTRACT

X-linked hypophosphatemic rickets (XLHR) is a severe disorder of phosphate homeostasis and skeletal mineralization caused by mutations of *PHEX*, encoding a bone-specific endopeptidase. Phex-deficient *Hyp* mice have been extensively studied to understand the molecular bases of XLHR, and here it was found that *Fgf23*, encoding a major phosphaturic hormone, was transcriptionally activated in bone-forming osteoblasts. We and others could additionally show that *Col10a1* expression is increased in *Hyp* osteoblasts and bones, thereby raising the possibility that ectopic production of type X collagen could contribute to the impaired mineralization of the *Hyp* bone matrix. Here we show that an additional deficiency of the *Col10a1* gene does not overtly affect the skeletal phenotype of *Hyp* mice. More specifically, *Col10a1*-deficient *Hyp* mice displayed severe disturbances of skeletal growth, bone mass acquisition and bone matrix mineralization, and they were essentially indistinguishable from *Hyp* littermates. This was confirmed by non-decalcified histology and bone-specific histomorphometry quantifying all relevant parameters of growth plate maturation, trabecular bone architecture and osteoid accumulation. Taken together, our results show that increased *Col10a1* expression in Phex-deficient osteoblasts is not a major cause of the XLHR phenotype, which was an important issue to address based on the previous findings.

1. Introduction

Phosphate homeostasis is tightly linked to skeletal mineralization, not only because low serum phosphate concentrations (hypophosphatemia) can result in decreased bone matrix mineralization (osteomalacia), but also because the most critical regulators of renal phosphate handling are expressed in bone [1]. More specifically, there is hallmark genetic evidence, that Fgf23 is the most relevant regulator of phosphate homeostasis in mice and humans [2]. Of note, Fgf23 is predominantly expressed by matrix-embedded terminally differentiated osteoblasts (osteocytes) and it primarily acts in the kidney, where it regulates the expression of at least three relevant target genes, *Slc34a1* (encoding a sodium-phosphate cotransporter), as well as *Cyp27b1* and *Cyp24a1* (encoding hydroxylases activating or inactivating vitamin D) [3]. The key role of Fgf23 in phosphate homeostasis is underscored by the fact that activating *FGF23* mutations cause autosomal dominant hypophosphatemic rickets, whereas inactivating *FGF23* mutations cause an autosomal recessive hyperphosphatemic disorder known as tumoral calcinosis [4,5]. Importantly, high circulating FGF23 levels are also found in other genetic and acquired disorders of renal phosphate loss, such as autosomal recessive and X-linked hypophosphatemic rickets, as well as tumor-induced osteomalacia [2,6].

The most common form of inherited hypophosphatemic rickets, with an incidence of 1 in 20,000 newborns, is XLHR, where the underlying genetic defect was identified in 1995 [7]. The affected gene was termed *PHEX* (Phosphate-regulating gene with homologies to endopeptidases on the X-chromosome), and since a mouse model (termed *Hyp*) with a spontaneous deletion within the murine *Phex* gene was already available, it was possible to obtain some important insights into the pathophysiology of XLHR [8]. More specifically it was found that the hypophosphatemia in *Hyp* mice is caused by a circulating phosphaturic factor, later identified as Fgf23, and not by an intrinsic renal defect [9–11]. Likewise, Phex is not expressed in the kidney, but by terminally differentiated osteoblasts, also known as the main producers of Fgf23 [12,13]. The severe impairment of skeletal mineralization in *Hyp* mice is partially explained by their hypophosphatemia, yet there is steadily increasing evidence for a cell-autonomous defect of Phex-deficient osteoblasts and an Fgf23-independent role of Phex in skeletal mineralization [14–19]. Since the Phex protein shares

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homologies to a family of endopeptidases it has been speculated that Phex acts by cleaving specific substrates that could act downstream of Phex to control serum phosphate levels and/or bone mineralization [20]. Importantly however, Fgf23 is not cleaved and thereby inactivated by Phex, unlike it was initially hypothesized [20,21]. Instead, *Fgf23* was found transcriptionally activated in Phex-deficient bone cells, thus implying that the XLHR pathologies are related to changes in gene expression [22,23].

In this regard it is important to state that two studies have been performed to analyze the impact of Phex-deficiency on gene expression in an unbiased approach. First, RNA was isolated from long bones of wildtype and *Hyp* mice and subjected to genome-wide expression analysis [23]. Here it was found that three genes were expressed at more than 5-fold higher rate in *Hyp* mice, namely *Fgf23*, *Thbs4* and *Col10a1*. Second, we have previously applied genome-wide expression analysis to compare primary calvarial osteoblasts from wildtype and *Hyp* mice at day 25 of differentiation [19]. Here we identified four genes with more than 5-fold higher expression in *Hyp* osteoblasts, namely *Mlana*, *Aqp5*, *Fgf23* and *Col10a1*. Regardless of the potential limitations associated with global expression analyses, it was remarkable that both studies identified *Fgf23* and *Col10a1* as Phex-regulated genes, and while the contribution of Fgf23 to the XLHR pathology has already been proven, it was quite important to address the relevance of *Col10a1* induction as well. More specifically, our hypothesis was that the ectopic presence of type X collagen, endoded by the *Col10a1* gene, in the bone matrix would interfere with the mineralization process and thereby explain, at least in part, the osteomalacia phenotype of *Hyp* mice. Moreover, since *Hyp* mice additionally display hypomineralization of growth plate cartilage with persistence of cartilage remnants in the primary spongiosa [24], it remained to be established, if increased type X collagen production by hypertrophic chondrocytes would contribute to the growth defects of *Hyp* mice. For that purpose we generated *Col10a1*-deficient *Hyp* mice and analyzed their skeletal phenotype.

2. Materials and methods

2.1. Animals

Hyp mice (C57Bl/6 genetic background) were obtained from the Jackson Laboratory (#000528), while *Col10a1*-deficient mice were described previously [25]. Genotypes were determined by genomic PCR with the following primer combinations. While the presence of the *Phex* exon 21 was detected using 5′-CGA CCA GGA TGA GGG AAG AAA G -3′ and 5′-CAC TTG TTC TCT GGC AGC TTC TG-3′ (486 bp product), the *Hyp* mutation was detected by 5′-CAG GGG ACT GTC CAC AAG GGG G-3′ and 5′-GGG TGC AAA GGT GTT GTG AAT GGA A-3′ (669 bp product). For *Col10a1* genotyping we used 5′-ATA CCT TCT CGT CCT TGC TT-3′ and 5′-ACA CAA AGG AGA TAT TGG CC-3′ for the wildtype allele (417 bp product) and 5′-ATA CCT TCT CGT CCT TGC TT-3′ and 5′-AGG GGA GGA GTA GAA GGT GG-3′ for the mutant allele (535 bp product). All animal experiments were approved by the animal facility of the University Medical Center Hamburg-Eppendorf and by the ‘‘Amt für Gesundheit und Verbraucherschutz’’ (Org529).

2.2. Immunohistochemistry

Immunohistochemistry was performed on decalcified and paraffin-embedded tibia sections from wildtype and *Hyp* mice. For immunohistochemical detection sections were deparaffinized, rehydrated, and pre-treated for antigen unmasking with pepsin for type X collagen or 0.2% hyaluronic acid for type I collagen. To block endogenous peroxidase activity and non-specific antibody binding, sections were incubated with 3% hydrogen peroxide for 15 min and with 5% BSA for 30 min. Antibodies against type I collagen (1:600, Cedarlane) or type X collagen (1:20, Quartett) were applied over night at 4 °C. Biotinylated goat anti-rabbit IgG (1:200, Dako Cytomation) and rabbit anti-mouse IgG (1:200, Dako Cytomation) for 30 min were used as a secondary antibody, followed by incubation of 30 min with streptavidin/HRP (1:200, Dako Cytomation). Peroxidase activity was detected using DAB as a chromogenic substrate (Dako Cytomation). Sections were counterstained with hematoxylin/eosin, dehydrated, and mounted.

2.3. Skeletal analysis

After their initial analysis by contact X-ray (Faxitron X-ray Corp.), the vertebral bodies L2 to L5 and one tibia from each animal were dehydrated and embedded non-decalcified into methylmetacrylate for sectioning. A fixed-blade rotation microtome was used to cut 4 μm thick sections. These sections were either stained with toluidine blue or by the von Kossa/van Gieson procedure as described [26]. In brief, for toluidine blue staining the sections were incubated for 30 min at ambient temperature in 1% toluidine blue solution (pH 4.5). The Kossa/van Gieson staining was achieved by initially incubating the sections in 3% silver nitrate for 5 min followed by additional 5 min in 5% sodium thiosulfate. Van Gieson solution (0.25% acid fuchsine, 0.5% nitric acid, 10% glycerine, and picric acid to saturation) was used to counterstain the sections for 20 min. The resulting staining shows mineralized bone matrix in black and non-mineralized osteoid in red. Static histomorphometry was performed on Kossa/van Gieson stained sections using the Bioquant Osteo software (BIOQUANT Image Analysis Corporation, Nashville, USA). Additional histomorphometry for the analysis of osteoid parameters was carried out on toluidine blue-stained sections using the OsteoMeasure system (Osteometrics, Decatur, USA) following the guidelines of the American Society of Bone and Mineral Research [27].

2.4. Statistical analysis

All data are presented as means ± standard deviation. Statistical analysis was performed with Prism v6.01 (Graphpad Software, Inc., La Jolla, USA) using an one-way ANOVA followed post hoc by Turkey's range test for multiple comparisons. The *p*-value threshold for significance was set at 0.05.

3. Results

We first performed immunohistochemistry to address the question, if type X collagen is detectable in the bone matrix of Phex-deficient *Hyp* mice. When we analyzed tibia sections from 6 weeks old wildtype mice, we found intensive staining for type X collagen only in hypertrophic cartilage, as expected. In contrast, there was additional staining for type X collagen in the primary spongiosa of 6 weeks old *Hyp* mice, consistent with *Col10a1* over-expression (Fig. 1A). To analyze, whether this difference is attributable to the presence of cartilage remnants in the primary spongiosa of *Hyp* mice, we analyzed consecutive sections incubated with antibodies against type X collagen or type I collagen. Here we found that areas being positive for type X collagen appeared negative for type I collagen (Fig. 1B). Most importantly, the bone matrix stained with the antibody against type I collagen did not display an intense staining for type X collagen. Taken together, these results suggested that the primary spongiosa of *Hyp* mice contains a larger number of cartilage remnants compared to wildtype littermates, but that the bone matrix of *Hyp* mice does not contain the same amount of type X collagen compared to hypertrophic cartilage.