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Altered IHH signaling contributes to reduced chondrocyte proliferation in the growth plate of MPS VII mice

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ABSTRACT

Bone elongation is driven by chondrocyte proliferation and hypertrophy in the growth plate. Both processes are modulated by multiple signaling pathways including the Indian Hedgehog (IHH) signaling pathway. Mucopolysaccharidoses (MPS) are a group of lysosomal storage disorders characterized by accumulation of glycosaminoglycans (GAGs) in multiple tissues and organs, leading to a range of clinical symptoms including bone shortening through mechanisms that are not fully understood. Using MPS VII mice, we previously observed a reduction in the number of proliferating and hypertrophic chondrocytes and a reduced gene expression of *Ihh* in the tibial growth plate. We further demonstrate here that IHH secretion by MPS VII chondrocytes was reduced both *in vitro and in vivo*. While normal chondrocytes showed no response to exogenous IHH, proliferation of MPS VII chondrocytes was stimulated in response to exogenous IHH *in vitro*. This was accompanied by an elevated gene expression of patched receptor (*Ptch1*). The results from this study suggested that reduced proliferation in MPS VII growth plate may be partially due to dysfunction of the IHH signaling pathway.

1. Introduction

Postnatal bone elongation results from the proliferation and hypertrophy of chondrocytes in the growth plate. Chondrocytes in the proliferative zone form columns along the longitudinal axis of the bone and undergo rapid cell division. Chondrocytes further from the epiphysis enlarge and undergo hypertrophic differentiation [1,2]. Both processes are regulated by the coordinated activity of multiple proteins, including Indian Hedgehog (IHH) [1,3–5]. IHH is produced by prehypertrophic chondrocytes and its action is mediated through its receptors patched receptor (PTC) and smoothened receptor (SMO) in the growth plate [6,7]. IHH induces chondrocyte proliferation through transcription factors of the glioma-associated oncogene (GLI) family, and inhibits transition of chondrocytes to terminal differentiation *via* the IHH/PTHrP feedback loop [4,5]. Deficiency of *Ihh* in mice leads to reduced chondrocyte proliferation and an expanded hypertrophic zone (HZ) in the growth plate [6], whereas overexpression of *Ihh* in mice leads to upregulated PTHrP expression and a delayed transition of chondrocytes from the proliferative to hypertrophic phenotypes [8]. The effect of IHH on chondrocyte proliferation is thought to parallel that of insulin like growth factor 1 (IGF1) [9] and IGF1 can stimulate secretion of IHH by chondrocytes {Wang, 2006 #198 [10]; IHH is highly expressed by chondrocytes in the growth plate, but has also been found in lungs, liver, kidneys, gastrointestinal tract, ovarian and prostate. There is growing evidence that local production of IHH by chondrocytes is crucial for the regulation of endochondral ossification [3,4,6,11], but there is less evidence for the role of circulating IHH in bone length.

Mucopolysaccharidoses (MPS) are a group of lysosomal storage disorders caused by deficiencies in enzymes that break down glycosaminoglycans (GAGs). Accumulation of undegraded GAG results in failure of multiple tissues and organs, including the skeletal system [12]. Using the MPS VII mouse model, our lab previously found a progressive bone shortening accompanied by an impaired growth plate characterized by reduced proliferation and hypertrophy [13]. MPS VII mice also have a reduced gene expression level of *Ihh* in the growth plate [13,14]; however; the expression pattern of the morphogen IHH in the growth plate and its effect on MPS chondrocyte proliferation in MPS remained unclear. Thus, to investigate how chondrocyte proliferation is regulated in the MPS growth plate, the ability of MPS VII chondrocytes to proliferate in response to exogenous IHH was assessed *in vitro*. In addition, the

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expression pattern of IHH and gene expression levels of components of the IHH signaling pathway were examined in the growth plate.

2. Materials and methods

2.1. Animal husbandry

All experimental procedures were approved by the Women and Childrens Health Network and the University of Adelaide animal ethics committees. Founder animals for MPS VII (*Gus*$^{mps/mps}$ strain), a naturally occurring model of MPS VII comprising a 1 bp deletion on exon 10 resulting in premature stop codon and no detectable residual enzyme activity [15], were obtained from Jackson Labs (stock number 006407). MPS and normal (homozygous) mice were bred from heterozygous parents and affected pups identified by PCR and restriction enzyme digest as previously described [13,16,17]. Mice were housed in a 14/10 light/dark cycle with food and water *ad libitum*. Mice were humanely killed at experimental end points by carbon dioxide asphyxiation followed by cervical dislocation before blood collection.

2.2. ELISAs on serum

Blood was collected from normal and MPS VII mice at 14-day, 1-, 2- and 6-month of age and stored at 4 °C overnight before serum was separated by centrifugation at 1500 *xg* for 10 min and stored at -80 °C. Measurements of circulating IHH in normal and MPS VII mice was performed using a commercially available ELISA kits (mouse Indian Hedgehog ELISA kit, MBS706033, MyBioSource Inc., San Diego, USA).

2.3. Immunohistochemistry

Hind limbs of normal and MPS VII mice ($n = 5$ for each group) were fixed in 10% neutral buffered formalin, decalcified in Immunocal^{Tm} and embedded in paraffin. Immunohistochemistry was carried out on 5 μm sections after deparafinization and antigen retrieval in 10 mM sodium citrate buffer at 60 °C overnight. Anti IHH N-terminus antibody (AF1705, R&D system, Minneapolis, USA) was used as primary antibody at a 1:20 dilution. Blocking solutions and biotinylated secondary antibody were as per the Cell and Tissue Staining Kit (anti-rabbit, R&D systems, Minneapolis, USA). Sections were counterstained with Mayer's hematoxylin (Prosci Tech, QLD, Australia) and evaluated under an Olympus BX51 microscope (Olympus Australia Pty. Ltd., Gulfview Heights, SA) fitted with Soft Imaging System's Colorview III camera and analySIS® LS software at Adelaide Microscopy. Distribution of IHH in the growth plate was determined by counting the number of chondrocytes stained positive in each zone using Olympus analySIS® LS Research Olympus Soft Imaging Solutions (version 3.1) software. The number of chondrocytes within a 200 μm wide strip that encompassed all zones was determined and repeated twice more. Chondrocyte number in each zone was averaged to give the final value per animal and presented as a percentage of the total number of cells per area counted.

2.4. Response of chondrocytes to IHH

The proximal tibia growth plate was dissected out from day 14 normal and MPS VII mice (Fig. 2.1) free of perichondrium, primary ossification centre and secondary ossification centre Growth plate was incubated in 1% (*w/v*) pronase and 5000 U/mL penicillin and 5000 μg/mL streptomycin in Hams F-12 at 37 °C for 1 h. Tissue was washed with PBS and incubated in 0.08% (w/v) collagenase, 5% FCS and 5000 U/mL penicillin and 5000 μg/mL streptomycin in Hams F-12 at 37 °C for at least 15 h. Cells were filtered through a 50 μm nylon filter and collected by centrifugation at 1500 rpm for 5 min. Cell number was determined using the Countess® automated cell counter and 0.4% trypan blue.

To assess determine cell proliferation, chondrocytes were seeded at 6.25×10^4 cells/cm^2 in 96-well plate and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 5000 U/mL penicillin, 5000 μg/mL streptomycin, 1% FCS. Recombinant human/mouse IHH (cat#31705-HH/*CF*, R&D systems, MN, USA) was reconstituted in dH_2O at 100 μg/mL and added to chondrocyte cultures to give a final concentration of 25 ng/mL. Chondrocytes were maintained for 4 h (0 day), 2-, and 4 days. The negative control consisted of cells incubated in DMEM containing 5000 U/mL penicillin, 5000 μg/mL streptomycin plus 1% FCS. The positive control consisted of cells incubated in DMEM containing 5000 U/mL penicillin, 5000 μg/mL streptomycin plus 10% FCS. After 4 h (0 day), 2- and 4-day, the CyQUANT® Direct Cell Proliferation Assay Kit (Molecular Probes, Invitrogen) was used to determine cell proliferation. Cells were lysed according to manufacturer's instruction, and total cellular nucleic acid was measured using florescence (485 nm excitation, 535 nm emission, 0.1 s) on a VICTOR^2 automated plate reader (Perkin Elmer). Measurements at 2- and 4-day were normalized to measurements at 4 h (0 day).

To assess the secretion of IHH by chondrocytes, cells were seeded at a density of 1×10^5 cells/cm^2 on 24-well plates and maintained in DMEM containing 5000 U/mL penicillin, 5000 μg/mL streptomycin and 1% FCS. Medium were collected after 4 h (0 day), 2- and 4 days and assayed for IHH expression using commercially available ELISA kits as above.

2.5. Gene expression analysis

RNA was isolated from micro-dissected proliferative zone of normal and MPS VII mice tibiae as previously described [13]. The concentration of RNA was determined using a Thermo Scientific NanoDrop 1000 and its operating software, version 3.8.1 (Thermo Scientific,VIC, Australia). RNA was reverse transcribed into cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Maryland, USA). Quantitative real-time PCR was performed as previously described [13]. Data were normalized to cyclophilinA (*CycA*) and the fold change to normal calculated using the $\Delta\Delta$Ct method [18]. The following primers were used: *Ptch1* forward, 5′-CGGGACTATCTGCACCGGC-3′ and *Ptch1* reverse, 5′- AAACTTCGCTCTCAGCCACAG-3′; *Smo* forward, 5'-AAGCTCGTGCTCTGGTCCG-3′ and *Smo* reverse, 5′- TCCACTCGGTCATTCTCACAC-3′; *CycA* forward, 5′- AGCATACAGGTCCTGGCATC-3'and *CycA* reverse, 5′- TTCACCTTCCCAAAGACCAC-3′.

2.6. Statistics

Statistical significance was determined by a two-way ANOVA with a Tukey's HSD post-hoc analysis using GraphPad Prism version 7.0 (GraphPad Software Inc., California, USA) or a student's *t*-test using Microsoft Excel 2016. Statistical significance was assumed when $p < 0.05$.

3. Results

In normal mice, the circulating IHH level was 54.0 ± 16.5 ng/ml at 14 days of age and decreased with age (Fig. 1) such that at 6 months of age, the serum IHH level was no longer detectable. Circulating IHH level in MPS VII mice followed a similar pattern as that of normal mice and declined with age (Fig. 1). The circulating IHH level of MPS VII decreased to 37%, 57% and 67% of those of normal mice at day 14, 1 month and 2 months of age, respectively, but did not reach statistical significance. At 6 months of age, serum IHH level in MPS VII mice was undetectable.

To study the distribution of IHH through the MPS VII growth plate, immunohistochemistry was performed on the tibial growth plates of normal and MPS VII mice at day 14, 1 and 2 months of ages. IHH is secreted by pre-hypertrophic chondrocytes and diffuses through the growth plate to regulate proliferation and hypertrophy [6]. Immunostaining of IHH was observed in day 14 normal and MPS VII growth plate (Fig. 2A), but was absent from the growth plate of both normal and MPS VII mice at 1 and 2 months of ages, suggesting that production of IHH in the growth plate decreased with age in both normal and MPS VII

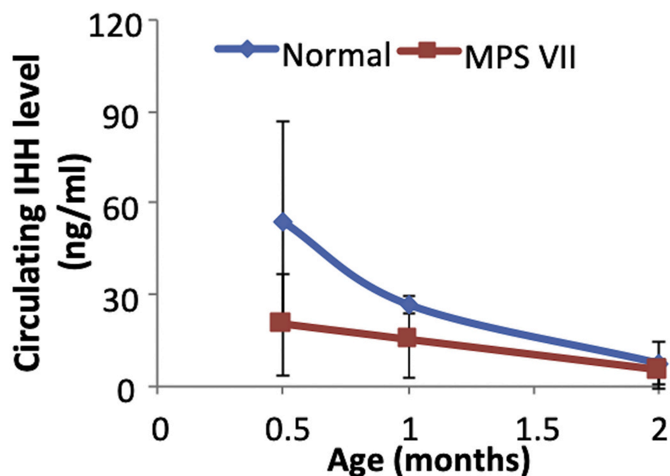

Fig. 1. Circulating IHH levels in MPS VII mice were not significantly different from those of normal. Circulating IHH level in normal and MPS VII mice at 14 days, 1 month and 2 months of age. Blood samples were collected from normal and MPS VII mice through cardiac puncture. Results are presented as mean ± S. D. of 5 mice.

mice.

At day 14, immunostaining of IHH was observed across the three zones of the growth plate, but was predominately observed in the proliferative zone (PZ) and HZ of the normal growth plate (Fig. 2A, supplementary figure); however, in MPS VII, IHH was distributed mainly in the HZ but to a significantly less extent in the PZ as compared to normal growth plate ($p < 0.05$, Fig. 2B), indicating an impaired distribution of IHH in MPS VII growth plate.

In vitro, IHH secretion by normal and MPS VII chondrocytes at day 0 was 0.04 pg/mL and 0.26 pg/mL, respectively. An increase in IHH secretion by normal chondrocytes was observed over time of culture (Fig. 2C). A similar increase in IHH secretion by MPS VII chondrocytes was observed but at a significantly reduced level, indicating an impaired secretion of IHH by MPS VII chondrocytes.

Cell proliferation of normal chondrocytes exhibited minimal response to exogenous IHH *in vitro* (Fig. 3A). However, IHH significantly increased cell proliferation by cultured MPS VII chondrocytes on day 2 and day 4 *in vitro*.

Expression of IHH receptors patched (*Ptch1*) and smoothened (*Smo*) was examined using cartilage tissue isolated from proliferative zone of normal and MPS VII mice tibiae by real-time PCR analysis. In the PZ (Fig. 3B), *Smo* expression was not obviously changed as compared to normal; however, *Ptch1* expression was significantly elevated at 2-fold of normal ($p < 0.05$).

Fig. 2. IHH production was greatly reduced in MPS VII growth plate. (A) Immunohistochemical staining for IHH on normal and MPS VII mice at P14, 1 month and 2 months of age. (B) Distribution of IHH in the growth plate of P14 mice were presented as percentage of positive stained cells to total cells in each zone. Scale bar = 20 μm. *denotes to significant differences between normal and MPS VII mice ($p < 0.05$, student's *t*-test). (C) Level of IHH produced by normal and MPS VII chondrocyte in culture. # denotes significant difference of IHH secretion between normal and MPS VII chondrocytes. (Two-way ANOVA, Turkey post-hoc, $p < 0.05$). Results were presented as the mean ± S.D. of 3 replicates of pooled chondrocytes.

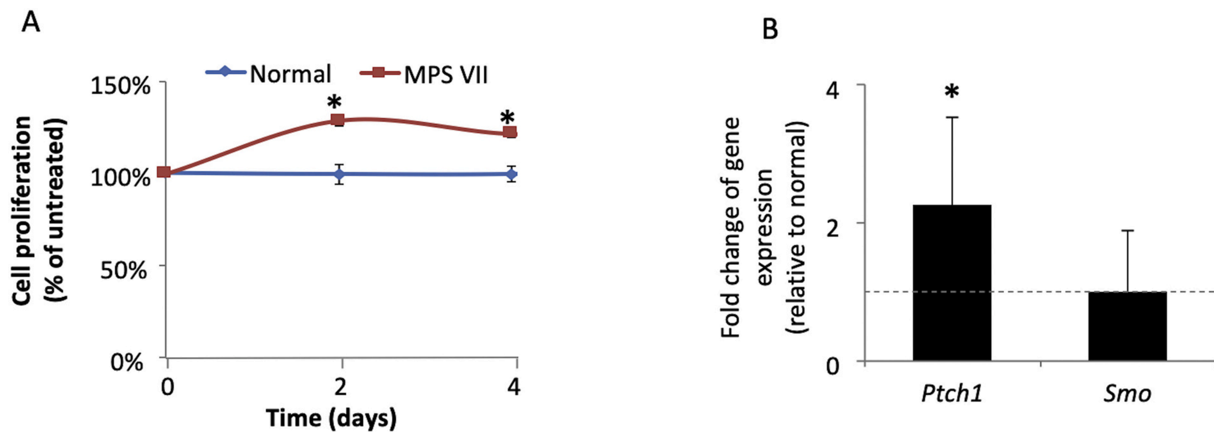

Fig. 3. Exogenous IHH stimulated cell proliferation of MPS VII chondrocytes. (A) Cell proliferation of normal and MPS VII in response to IHH (25 ng/ml) stimulation. Results were presented as the mean ± S.D. of 3 replicates of pooled chondrocytes. * denotes to significant difference of cell proliferation between IHH-treated normal and MPS VII chondrocytes ($p < 0.05$, student's t-test). (B) Gene expression of IHH receptors patched (*Ptch1*) and smoothed (*Smo*) in the PZ of the growth plate, of P14 normal and MPS VII mice. Results were presented as mean ± S.D. of 5 mice.

4. Discussion

We previously demonstrated that chondrocyte proliferation was reduced in the murine MPS VII growth plate along with a reduction in the transition to hypertrophic phenotype [13]. This is associated with a reduction in *Ihh* gene expression and a concomitant increase in *Gli3* expression [13,14]. In this study, we investigate details in the relationship between IHH signaling and delayed proliferation and hypertrophy in MPS VII growth plate, whereupon we found that IHH secretion by chondrocytes was reduced in MPS VII growth plate, accompanied with an elevated gene expression of patched receptor (*Ptch1*).

Circulating IHH decreased with age in both normal and MPS VII mice and although a reduced level of serum IHH was observed at day 14, the reduction was not significant at this or any other age. A direct function for circulating IHH on linear bone growth has not been demonstrated; although Hedgehog signaling has been shown to contribute to hepatic IGF1 production [30], and it is possible that circulating IHH contributes partially to bone shortening in MPS VII mice through modulating hepatic IGF1 production. This function of IHH occurs predominately during the early postnatal period; or even prenatal period as IHH is an essential morphogen for embryonic development [19].

A reduction of IHH expression was previously noted in the whole MPS VII growth plate at the transcriptional level [13,14], along with an increase in the gene expression of *Gli3*, the downstream repressor of IHH function [13]. The current study extends these observations to demonstrate that the level of IHH protein secreted by chondrocytes was also greatly decreased in both the growth plate and in cultured chondrocyte of MPS VII mice. Our results suggest that although circulating IHH has minimal direct effect on bone growth, local IHH in the growth plate may play a more important role.

Chondrocytes entering hypertrophic differentiation express IHH. IHH is distributed through the growth plate to regulate proliferation and hypertrophy [6,20]. In the MPS VII growth plate, immunostaining of IHH in the HZ was not different to that of normal but was significantly decreased in the PZ. Reduced synthesis of IHH by MPS VII chondrocytes leads to reduced distribution of IHH through the growth plate and particularly the PZ and IHH may not reach its target PZ chondrocytes. In addition to a reduced production of IHH, the distribution of IHH may also be affected by its interaction with the gags that accumulate in MPS. IHH binds to directly to CS GAG to form an IHH gradient in the growth pate which may also protect IHH from degradation [21]. CS is the main GAG present on the major cartilage proteoglycan aggrecan, an integral component of cartilage xtracellular matrix. The main function of aggrecan is to absorb compressive loads via its ability to bind water but it also functions to bind a range of biologically active factors [26]. In MPS VII CS turnover is impaired leading to a build-up of partially degraded CS in the extracellular matrix. This accumulation of CS may interfere with the binding of IHH to proteoglycan-associated CS altering the formation of normal IHH gradients within the growth plate. IHH also binds to HS GAG [21,27] and HS GAG can compensate for CS GAG (and vice versa) raising the possibility that HS and CS can substitute for each other in regulating IHH signaling [28,29]. Thus MPS that accumulate CS or HS or both could potentially display growth plate abnormalities due to aberrant IHH signaling. However, MPS III (types A, B, C and D) that accumulate HS only do not display growth abnormalities [12], which may be due to the lower amount of HS in the growth plate compared to CS. Thus it is unlikely that HS plays a significant role in abnormal IHH signaling in the MPS growth plate. MPS I, II and VI are DS accumulating MPS (along with HS in MPS I and II) with growth plate dysfunction similar to MPS VII a defining symptom. To our knowledge there is no published evidence about the interaction between IHH and DS GAG. It is possible that CS may accumulate in the cartilage of these MPS types. Measurement of CS and IHH in the growth plate of these three MPS types would help elucidate if a similar mechanism affects bone growth in MPS I, II, VI and VII or is specific to MPS VII alone.

In the IHH signaling pathway, patched receptor (PTC) suppresses the activity of smoothened receptor (SMO) by triggering degradation or intracellular vesicle trafficking. When IHH ligand binds to PTC, SMO is activated for downstream transcription of IHH target genes, such as GLI family. We previous found that there was increased expression of *Gli3* and reduced chondrocyte proliferation in the MPS VII growth plate [13], possibly due to the decreased expression of IHH by MPS VII chondrocytes. Although expression of *Smo*, the gene encoding SMO, in MPS VII PZ was not apparently different to that of normal PZ at the transcriptional level, the upregulation of *Ptch1,* the gene encoding PTC, at the transcription level may be a response to the decreased IHH in the MPS VII growth plate. Overexpression of PTC can inhibit the translocation of cyclin B1 from progressing to mitosis, demonstrating an anti-proliferative function of PTC [22]. Our previous study found that less chondrocytes were able to progress to mitosis in MPS VII growth plate [13]. Thus, it is possible that the reduced proliferation in MPS VII growth plate is partially attributed by elevated *Ptch1*.

While the addition of IHH had no effect on normal chondrocyte proliferation *in vitro*, it significantly stimulated proliferation of MPS VII chondrocytes. Gene expression of *Ihh* was observed in the growth plate of mice at 3- and 8-day [23] and was at maximum level at 14-day [24]. The observation of significant IHH reduction at 14-day in MPS VII growth plate coincided with the reduction of chondrocyte proliferation

at 14-day [13]. Sonic hedgehog (SHH) protein, a variant of IHH, stimulated proliferation of chondrocytes isolated from 3 day old mice growth plate, but did not affect the proliferation of chondrocytes from 3 week old mice [25]. The function of SHH on proliferation is thus age dependent and is more effective on young chondrocytes. In the present study, normal and MPS VII chondrocytes were isolated from the growth plates of day 14 mice, which falls between the above two ages. The unresponsiveness of normal chondrocytes to IHH *in vitro* may therefore be attributed to the age of mice. In contrast, although MPS VII chondrocytes were obtained from age-matched animals, gene expression analysis of MPS VII chondrocytes in the PZ revealed an elevated level of *Ptch1,* which encodes IHH receptor PTC. Thus, exogenous IHH in MPS VII chondrocytes culture may bind to the increased PTC, which leads to stimulation of chondrocyte proliferation.

Future studies could focus on expression of IHH, either circulating or local in the growth plate at earlier postnatal or prenatal ages, as IHH plays important roles in bone development during the prenatal and early postnatal period.

5. Conclusion

Our study extends previous findings of reduced *Ihh* expression in MPS VII growth plate [13,14] to demonstrate a reduced production of IHH protein by MPS VII growth plate chondrocytes. Despite the reduction in IHH production, MPS VII chondrocytes remained responsive to the addition of exogenous IHH, suggesting that stimulation of cell proliferation is possible given increased local availability of IHH in the growth plate. In conclusion, our findings showed that distribution of IHH across the growth plate was altered in MPS VII, which suggested that the reduced proliferation in MPS VII growth plate may be partially due to dysfunction of IHH signaling pathway. We also highlighted that IHH signaling pathway may be a potential target to develop therapies for correcting long bone growth for MPS.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2020.100668.

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