Original Article

X-Chromosome Inactivation Analysis in Different Cell Types and Induced Pluripotent Stem Cells Elucidates the Disease Mechanism in a Rare Case of Mucopolysaccharidosis Type II in a Female

(mucopolysaccharidosis II / Hunter syndrome / iduronate sulphatase deficiency / X-chromosome inactivation / induced pluripotent stem cells)

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Abstract. Mucopolysaccharidosis type II (MPS II) is an X-linked lysosomal storage disorder resulting from deficiency of iduronate-2-sulphatase activity. The disease manifests almost exclusively in males; only 16 symptomatic heterozygote girls have been reported so far. We describe the results of X-chromosome inactivation analysis in a 5-year-old girl with clinically severe disease and heterozygous mutation p.Arg468Gln in the *IDS* gene. X inactivation analysed at three X-chromosome loci showed extreme skewing (96/4 to 99/1) in two patient's cell types. This finding correlated with exclusive expression of the mutated allele. Induced pluripotent stem cells (iPSC) generated from the patient's peripheral blood demonstrated characteristic pluripotency markers, deficiency of enzyme activity, and mutation in the *IDS* gene. These cells were capable of differentiation into other cell types (cardiomyocytes, neurons). In MPS II iPSC clones, the X inactivation ratio remained highly skewed in culture conditions that led to partial X inactivation reset in Fabry disease iPSC clones. Our data, in accordance with the literature, suggest that extremely skewed X inactivation favouring the mutated allele is a crucial condition for manifestation of MPS II in females. This suggests that the X inactivation status and enzyme activity have a prognostic value and should be used to evaluate MPS II in females. For the first time, we show generation of iPSC from a symptomatic MPS II female patient that can serve as a cellular model for further research of the pathogenesis and treatment of this disease.

Introduction

Mucopolysaccharidosis II (Hunter syndrome, MPS II, OMIM 309900) is an X-linked lysosomal storage disorder caused by deficiency of iduronate-2-sulphatase activity (IDS, EC 3.1.6.13). Iduronate-2-sulphatase encoded by the *IDS* gene (Xq27-q28) catalyses the first step in the sequential degradation of heparan sulphate and dermatan sulphate, and its deficiency leads to the lysosomal accumulation of these glycosaminoglycans (Neufeld and Muenzer, 2001).

Children with MPS II have a normal appearance at birth and the disease manifests usually in late infancy or toddler age. The signs and symptoms include coarse facial features, short stature with joint stiffness, dysostosis multiplex, hepatosplenomegaly, and cognitive decline. The prognosis depends on the severity of the disease,

and is poor especially in children with severe form associated with death in the second decade of life (Neufeld and Muenzer, 2001).

Enzyme replacement therapy improves the visceral disease; however, it has no effect on the CNS. The first study on intrathecal idursulfase-IT in children has been published recently (Muenzer et al., 2016) and lentiviral isogeneic haematopoietic stem cell gene therapy was described as a promising approach for correction of neuronal manifestation in MPS II mice by ameliorating lysosomal storage and autophagic dysfunction in the brain (Wakabayashi et al., 2015).

Recently, the possibility of using autologous induced pluripotent stem cells (iPSC) instead of haematopoietic stem cells for cell-based therapy received a great deal of attention. In female heterozygotes with X-linked diseases, individual cells are either functionally normal or deficient based on the origin of inactivated X chromosome (mutant or normal), and the X-chromosome inactivation (XCI) remains conserved in daughter cells. Selected iPSC clones or differentiated cells with favourable XCI skewing could possibly serve as suitable material for cell therapy without the need for gene manipulations (Bhatnagar et al., 2014).

The prevalence of MPS II is estimated to be 0.43–1.09 per 100,000 live births in five different countries (Poupetova et al., 2010). The vast majority of MPS II patients are males, while only 16 symptomatic MPS II female patients have been reported in the literature so far.

Three of these affected females had structural abnormality of the X chromosome impairing *IDS* expression of the wild-type allele, while two others had both *IDS* alleles defective. In the remaining 11 patients, the cause of MPS II manifestation was the presence of one mutated *IDS* allele in combination with highly skewed XCI leaving only the mutated allele active (Jurecka et al., 2012; Pina-Aguilar et al., 2013; Lonardo et al., 2014); other cases are reviewed in Tuschl et al. (2005) and Scarpa et al. (2011).

Here, we describe the first MPS II female patient in the Czech Republic. We show extremely skewed XCI favouring the mutated allele as the apparent epigenetic cause of the clinical manifestation. For the first time we demonstrate iPSC generated from the MPS II patient cells. This cellular model will serve for future research into the pathogenesis and treatment of MPS II.

Material and Methods

Ethics

The study was approved by the ethics committee of the General University Hospital in Prague (The Ethics Committee Approval number 41/12) and was conducted in agreement with institutional guidelines. Written informed consent was obtained from both adult study participants. On behalf of the patient, written informed consent was obtained from her parents.

Molecular analyses

Genomic DNA was extracted from whole blood and from the urinary sediment using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). DNA from buccal swabs and total RNA were isolated using a QIAamp DNA Micro Kit (Qiagen) and a BiOstic Blood Total RNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA), respectively. Reverse transcription of RNA to cDNA was performed using a High Capacity RNA to cDNA Kit (Applied Biosystems, Carlsbad, CA).

PCR and reverse transcription PCR (RT-PCR) products of the *IDS* (GenBank NC_000023.11, NM_000202.6) and *LAMP2* (NM_002294.2) genes were generated according to standard PCR protocols using primers shown in Table 1. The genotypes were analysed by Sanger sequencing using a Big Dye Terminator v3.1 Cycle Sequencing Kit and a 3500xL Genetic Analyzer (Applied Biosystems).

For amplicon-based deep sequencing, singleplex PCR and RT-PCR products were pooled and prepared under standard protocols using the NexteraXT kit and the MiSeq reagent kit (2×250), respectively. Paired-end sequence reads were generated using the MiSeq platform (Illumina, San Diego, CA). Sequencing data were demultiplexed and trimmed for low quality and duplicates using MiSeq reporter v.2.4. Secondary analysis of the cDNA data was performed using TopHat v2.0.13 (Kim

Table 1. Primer sequences used for PCR amplification

Gene		Fragment/ Exon	Sequence
IDS	cDNA	Fr. 4 U	TAATACGACTCACTATAG GGACCTTGTGGAACTTGTGT
		Fr. 4 L	TGAAACAGCTATGACCATG AAACGACCAGCTCTAACTCC
	gDNA	ex. 9a U	TAATACGACTCACTATAG TCCTGCTATTTGATTGGATG
		ex. 9a L	TGAAACAGCTATGACCATG GTCTATGGTGCGTATGGAAT
		ex. 9b U	TAATACGACTCACTATAG CGATTCCGTGACTTGGA
		ex. 9b L	TGAAACAGCTATGACCATG ATGGGTAATCACAAAACGAC
LAMP2	cDNA	Fr.1 U	GGTCGGTGGTCATCAGTGCT
		Fr.1 L	ATTCTGATGGCCAAAAGTTCAT
	gDNA	ex.2 U	TAATACGACTCACTATAG TTTAGAGCTGGTTGAACTTC
		ex.2 L	TGAAACAGCTATGACCATG TCTAAAGGATAAAGTCAATTAAA

Upper (U) and lower (L) primers (excepting LAMP2 cDNA) contain a T7 and an RP sequence, respectively, at the 5′ end.

et al., 2013). The human hg19 genome sequence was used as a reference.

X-chromosome inactivation analysis

Two independent methods were used to determine the XCI pattern: a DNA methylation-based assay and a transcript expression analysis. The methylation status of polymorphic repeat regions was examined at two loci, *AR* and *CNKSR2*, using digestion with methylation-sensitive enzyme *Hpa*II, as described elsewhere (Racchi et al., 1998; Musalkova et al., 2015). The DNA of the patient's father was used as a male control.

The transcriptional assay was performed similarly as described previously (Mossner et al., 2013). The RT-PCR product containing the single-nucleotide polymorphism (SNP) was used for quantification of SNP allele frequencies, which reflected the XCI ratios. The *LAMP2* gene polymorphism c.156A>T (rs12097) was selected for the assay as *LAMP2* is subject to XCI (Cotton et al., 2013), and the patient is heterozygous for this polymorphism. Instead of pyrosequencing (Mossner et al., 2013), amplicon sequencing using the Illumina platform was applied. The same procedure was used for quantification of the wt/mutated allele in the *IDS* gene transcript.

Generation of iPSC lines

The iPSC lines were generated from mononuclear cells isolated with Histopaque (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions from peripheral blood of the presented MPS II female patient. Isolated peripheral blood mononuclear cells (PBMC) were frozen in 10% dimethyl sulphoxide (DMSO) in inactivated foetal bovine serum (FBS, BenchMark™ Fetal Bovine Serum, Gemini Bio-Products, West Sacramento, CA) and kept in liquid nitrogen until further use. Thawed PBMCs were cultured in complete LGM medium (Lonza, Walkersville, MD) containing 0.4 µg/ml of purified no azide/low endotoxin NA/LE Mouse Anti-Human CD3 (BD Biosciences, San Jose, CA), 0.4 µg/ml of Mouse Anti-Human CD28 (BD Biosciences), and 50 ng/ml of interleukin 2 (IL2; Abbiotec, San Diego, CA) and were plated onto CD3-coated 6-well plates for five days. Reprogramming of the cells into iPSCs was performed using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. In brief, the cells were transduced at an appropriate multiplicity of infection (MOI) with each of the three reprogramming vectors (KOS MOI = 5, hc-Myc MOI = 5, hKlf4 MOI = 3) in the complete LGM medium. The free virus was removed by replacing the medium on the second day. The cells were transferred onto the layer of feeder cells (irradiated mouse embryonic fibroblasts) in the presence of HES medium (KnockOut™ESC/iPSC Medium Kit) with 8 ng/ml basic fibroblast growth factor (bFGF; Life Technologies, New York, NY) on the 4th day after the transduction. The selected colonies were picked and placed in 12-well plates containing feeder cells and then passaged with Accutase (STEMCELL Technologies Inc.,

Vancouver, BC, Canada) and the StemPro EZPassage tool (Invitrogen, Carlsbad, CA, USA) until the creation of stable iPSC lines.

Expression of pluripotency markers in generated iPSC was confirmed by immunostaining as previously described (Lian et al., 2013). Primary antibodies used in this study were directed to Oct3/4 (Santa Cruz Biotechnology, Dallas, TX, cat# sc-5279), SSEA4 (Invitrogen, cat# 41-4000), anti-TRA-1-81 (Invitrogen, cat# 41-1100), Sox2 (Santa Cruz Biotechnology, cat# sc-365823), CD63 (Abcam, Cambridge, UK, cat# ab1318), heparan sulphate (Amsbio, Abingdon, UK, Cat# 370255-1) and Lin-28 (Proteintech Group, Chicago, IL, cat# 11724-1-AP). The cells were incubated with Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies (Molecular Probes, Invitrogen) and the nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen).

Naïve iPSC lines were established by cultivation of generated iPSC in NHSM medium containing leukaemia inhibitory factor (LIF) and small molecule inhibition of ERK1/ERK2 and GSK3β signalling, which induce conversion of primed iPSC toward naïve pluripotency, as described previously (Gafni et al., 2013).

Determination of enzyme activities

Total leukocytes were isolated from the blood anticoagulated with EDTA within 24 h of drawing using the method described by Skoog and Beck (1956). The iPSC were re-plated onto Geltrex® (Life Technologies, Grand Island, NY)-coated 6-well plates and cultivated in mTeSR™1 medium (STEMCELL Technologies Inc.) for two passages for the removal of the feeder cells. The homogenates were prepared by sonication in water. The protein concentration was determined using the method described by Hartree (1972). Iduronate-2-sulphate sulphatase activity was assayed using fluorogenic substrate 4-methylumbelliferyl-α-L-iduronate-2-sulphate according to the method described by Voznyi et al. (2001).

Results

Clinical description

A 5-year-old girl was born in term as a first child of healthy, non-consanguineous Ukrainian parents. Postnatal adaptation and early development were uneventful. She used five words and started to walk at the age of 13 months. Snoring, hearing impairment and delayed speech were recognized at the age of two years. A mild improvement in hearing was observed with a hearing device; however, the speech problems persisted. Coarse facial features, gingival hyperplasia, mild hepatosplenomegaly, sternal protrusion, claw hands, lumbal hyperlordosis, large joint contractures and mild mental retardation were noted during the third year of life. At the age of 3.5 years, clinical and laboratory analyses revealed mild paleocerebellar symptoms, mild mitral regurgitation, and "dysostosis multiplex" on the X-ray survey.

Table 2. X-chromosome inactivation at three X-chromosome loci compared with a mutant alelle in the IDS gene

Sample	Locus			
	Methyl sensitive		Transcript analysis	
	AR (Xq12) %	*CNSKR2* (Xp22.12) %	*LAMP2* (Xq24) A:T %	*IDS* (Xq28) G:A %
Proband (leukocytes)	98:2[a]	99:1[a]	100:0[b]	0,5:99,5[b]
Proband (buccal swabs)	96:4	n.d.[c]	n.d.[c]	n.d.[c]
Mother (leukocytes)	n.i.[d]	70:30	n.d.[c]	n.d.[c]

[a] Maternal chromosome is preferentially inactivated, [b] transcript analysis using the amplicon-based deep sequencing: number of reads Q>30 *LAMP2* (c.156A>T, rs12097) A: 3910, T: 2. The A allele was inherited from the patient's father.
IDS (c.1403G>A) G: 18, A: 3668. Only the mutated allele is expressed. [c] not determined, [d] non-informative marker

No corneal clouding was present. Urinary excretion of heparan and dermatan sulphates were increased (60.5–65.7 g/mol creatinine; controls < 15.5). The IDS activity was markedly decreased in leukocytes (0.46 nmol/4 h/mg, control range: 28.1–70.4 nmol/4 h/mg) and serum (19 nmol/4 h/ml, control range: 167–475 nmol/4 h/ml). Heterozygous mutation c.1403G>A (p.Arg468Gln) was identified in the *IDS* gene. The girl is treated with enzyme replacement therapy, the glycosaminoglycan excretion decreased (19.5–22.5 g/mol creatinine), but she still has speech problems, delay of fine motor functions and a moderate delay of the gross motor development.

X-chromosome inactivation and mutation analysis in patient′s tissues

Examination of the methylation status at the *AR* locus (Xq12) showed extreme skewing in both blood leukocytes and buccal swabs; the maternal allele was almost completely inactivated. Complete inactivation of the maternal allele was also observed in *CNKSR2* and apparent homozygosity for the c.156A allele inherited from the patient's father was detected in the *LAMP2* transcript. Deep sequencing of the *IDS* gene transcript revealed exclusive expression of the mutated allele (Table 2).

As the maternal allele is completely inactivated and only the mutated *IDS* is expressed, it can be deduced that the patient inherited the mutated allele from her father. However, Sanger sequencing did not identify the mutation c.1403G>A in the patient's parents. To detect possible somatic mosaicism in the parental tissues, DNA isolated from three cell types (peripheral leukocytes, urinary sediment cells and buccal swabs) was subjected to amplicon-based deep sequencing. The number of reads corresponding to the mutated allele was below the detection limit (< 0.5%) when the total sequence depth was higher than 1300 in all analysed tissues. Thus, the somatic mosaicism was not demonstrated, while germline mosaicism could not be excluded as meiotic cells from the parents were not available for analysis.

Generation of iPSC lines

Two of the multiple generated patient's iPSC lines have been used for the study. The clones were positive

for the characteristic pluripotency markers Oct3/4, SSEA4, anti-TRA-1-81, Lin28, and Sox2 (Fig. 1). The patient's iPSC lines also expressed slightly higher amounts of heparan sulphate (Fig. 2); however, the quantitative determination of GAGs in the cell lysate using dimethyl methylene blue (DMB) with spectrophotometric detection (Lopez-Marin et al., 2013) did not show significant differences. The IDS activities in two
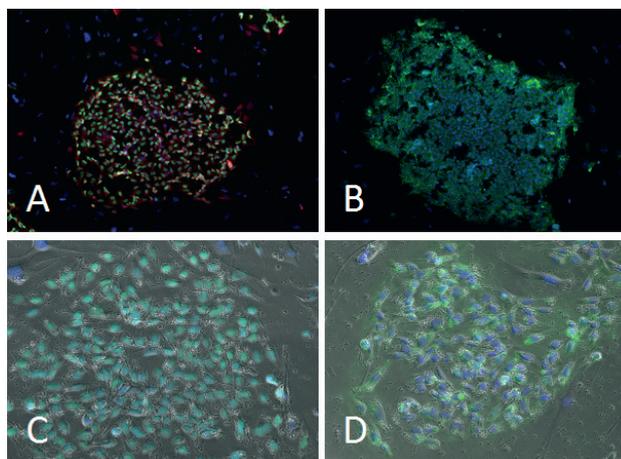
Fig 1. Characterization of iPSC colonies – pluripotency markers. **A:** Lin28 (red), Oct3/4 (green), DAPI (blue); **B:** SSEA4 (green), DAPI (blue); **C:** Sox2 (green), DAPI (blue) and **D:** anti-TRA-1-81 (green), DAPI (blue) in phase contrast image. A, B – 10× objective, C, D – 20× objective.
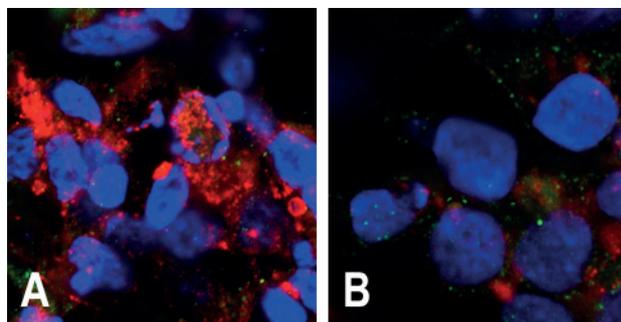
Fig 2. Detection of heparan sulphate in iPSC generated from an MPS II patient. Heparan sulphate (red), CD63 (green), DAPI (blue). **A:** MPS II patient; **B:** control; images were recorded with a laser scanning confocal microscope, 60× objective, NA 1.40

of the patient's iPSC clones were 1.49 and 2.32 nmol per mg of protein per hour, compared to 17.15 and 21.32 nmol/mg/h, respectively, in the controls. The potency to differentiate to cardiomyocytes and neuronal cells has been confirmed using previously published protocols (Stacpoole et al., 2011; Lian et al., 2013).

Analysis in two iPSC clones cultured in usual conditions showed the same XCI pattern with preferential inactivation of the maternal allele (98 : 2). Using cell culture conditions described to lead to the naïve state of iPSC with reset XCI did not result in a changed XCI ratio in the MPS II clones; however, in case of the Fabry disease heterozygote used as a control, the XCI ratio changed from 97 : 3 to 80 : 20 (Fig. 3).

Discussion

In this report we describe a 5-year-old girl with severe deficiency of IDS activity and clinical manifestation of the disease corresponding to the severe form of Hunter syndrome in boys. The disease in our patient is caused by the heterozygosity for the recurrent mutation p.Arg468Gln (Brusius-Facchin et al., 2014) in combination with highly skewed XCI resulting in exclusive expression of the mutated allele.

To reduce the risk of incorrect interpretation of the results due to chromosomal crossover or due to failure of the individual loci to correlate with XCI we performed XCI analysis using two independent methods at three X-chromosome loci. The results of methyl-sensitive methods (*AR*, *CNKSR2*) agreed well with the results of the transcription-based assay using the *LAMP2* gene polymorphism. Thus, unlike Swierczek et al. (2012), we did not observe any discrepancy among the used methods.

To date, two states of iPSC are known, referred to as primed and naïve, which differ mainly in XCI. Several attempts have been undertaken to generate a more naïve state (with two active X chromosomes in female cells) in established primed iPSC (with one inactive X chromosome) by different media formulation. In our study the XCI was analysed using the methyl-sensitive method (*AR*) in iPSC cultured in standard media and in naïve state-inducing media (Gafni et al., 2013). Cells derived from the MPS II patient did not show any change in the skewed XCI ratio in any culture conditions. There are three explanations for this finding: 1) the X chromosome remains inactive in the iPSC without going through the "reset" state when both X chromosomes in female cells are active, 2) the X chromosome is non-randomly inactivated after resetting XCI during iPSC reprogramming, or 3) a fraction of cells reached the stage of reprogramming in which both X chromosomes are active, but the used methyl-sensitive method is not able to detect these cells (Briggs and Reijo Pera, 2014). In any case, the iPSC derived from the MPS II patient differ from those derived from the Fabry disease heterozygote used as a control line, which responded to media change by partial change of the XCI ratio from 97 : 3 to 80 : 20.

Extreme skewing of XCI in favour of the mutated allele (> 95/5) is a common feature shared by symptomatic MPS II female patients (Scarpa et al., 2011). In accordance with this XCI status, symptomatic MPS II in females is associated with profound IDS deficiency comparable to affected hemizygous males, confirming the recessive nature of the disease. A similar strict correlation is not found in Fabry disease, another X-linked lysosomal storage disorder, where the overwhelming majority of heterozygotes have intermediate levels of the deficient enzyme and develop some symptoms of the disease, although later in life than the hemizygous males (Echevarria et al., 2016). According to the threshold model of Conzelman and Sandhoff, low levels of enzyme activity are compatible with normal levels of substrate degradation unless they cross a threshold, which, in lysosomal diseases, is usually lower than 10 % of the normal activity (Conzelmann and Sandhoff, 1983). It is important to note that in the tissues of heterozygotes of X-linked disorders, there are patches of deficient cells following the pattern of inactivation of the non-mutated chromosome. This illuminates the development of tissue pathology in Fabry heterozygotes, while carriers for autosomal recessive lysosomal diseases are free of it. The absence of symptoms in the majority of MPS II heterozygotes may be explained by the low threshold of the enzyme activity compatible with normal degradation of the substrate or by good uptake of the enzyme by the deficient cells from the surrounding cells. Notably, cultured fibroblasts from MPS II patients are able to cross-correct the IDS deficiency, unlike cultured skin fibroblasts from the Fabry patients (Fuller et al., 2015).

Our results and the review of the literature show that the clinical manifestation of MPS II in heterozygous females is associated with conditions leading to near monoallelic expression of the mutant allele and severe IDS deficiency, most often due to extremely skewed XCI. This suggests that girls with skewed XCI diagnosed postnatally might benefit from enzyme replacement therapy (ERT), which, if introduced early, may significantly improve further clinical course of the disease (Tylki-Szymanska et al., 2012; Tajima et al., 2013).

To our knowledge, we generated the first iPSC model from a symptomatic MPS II heterozygote and generally from any MPS II patient. These models are valuable for further research of MPS II pathogenesis and testing of therapeutic approaches in various cell types differentiated from iPSC clones and relevant to the disease. The iPSC lines from the presented case can also be used for general research of the mechanisms leading to extremely skewed XCI ratios, as the MPS II clones we have studied retained their XCI skewing under naïve state culture conditions in contrast with control clones. Hypothetically, the iPSC clones from heterozygotes of X-linked disorders with favourable XCI and thus functionally normal can serve as a source of autologous material (haematopoietic progenitors, neurons, cardiomyocytes, etc.) for cell-based therapy (Bhatnagar et al.,
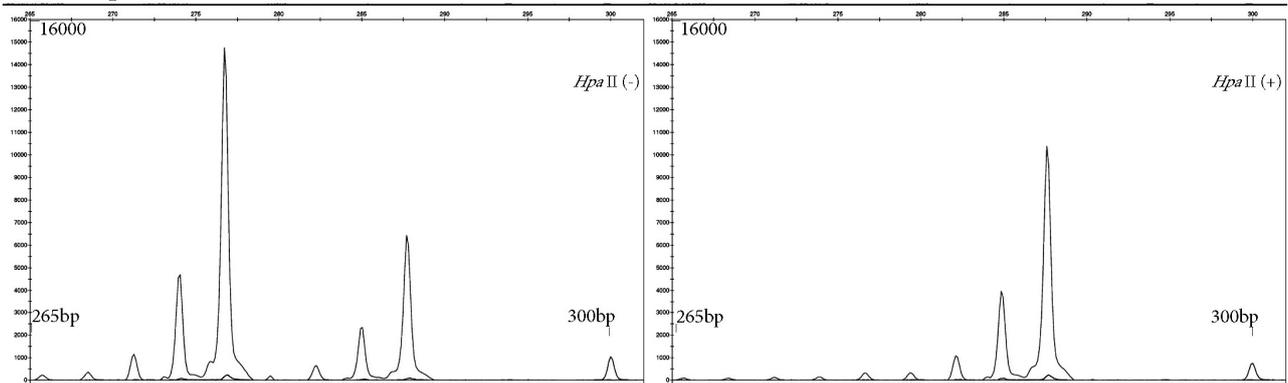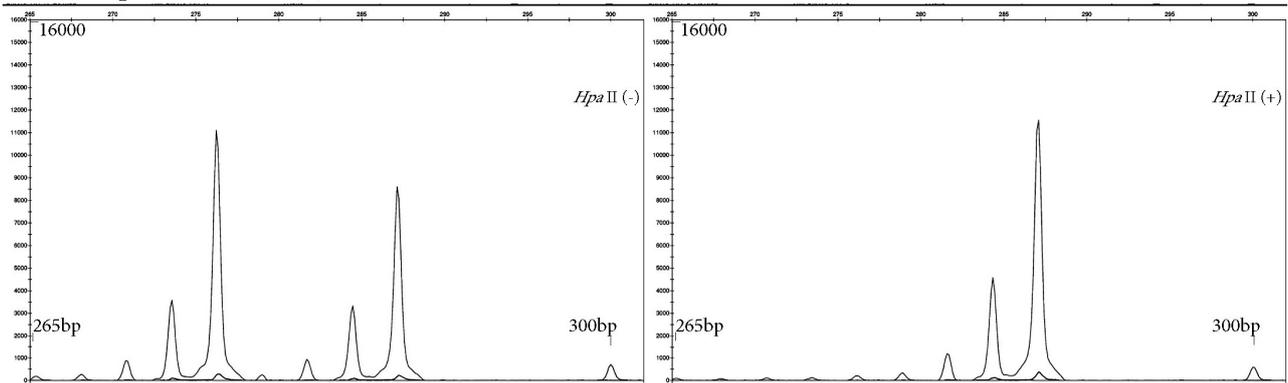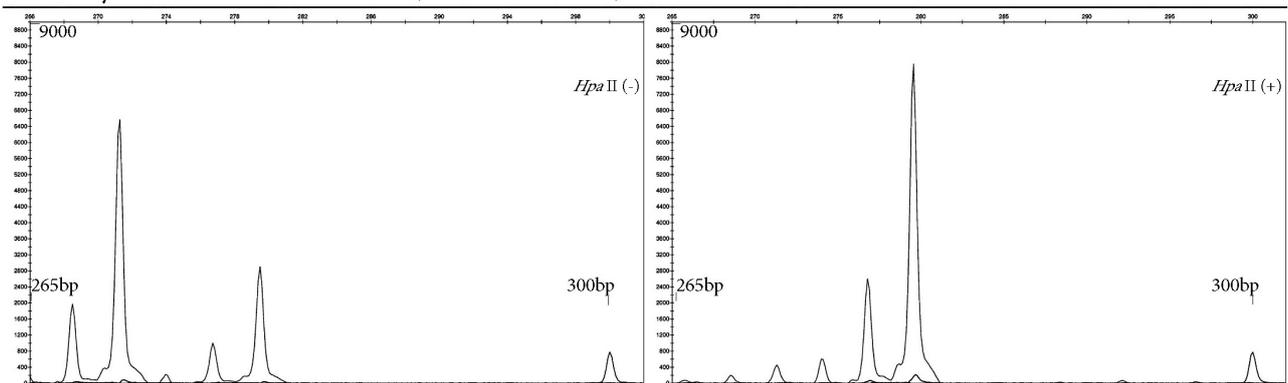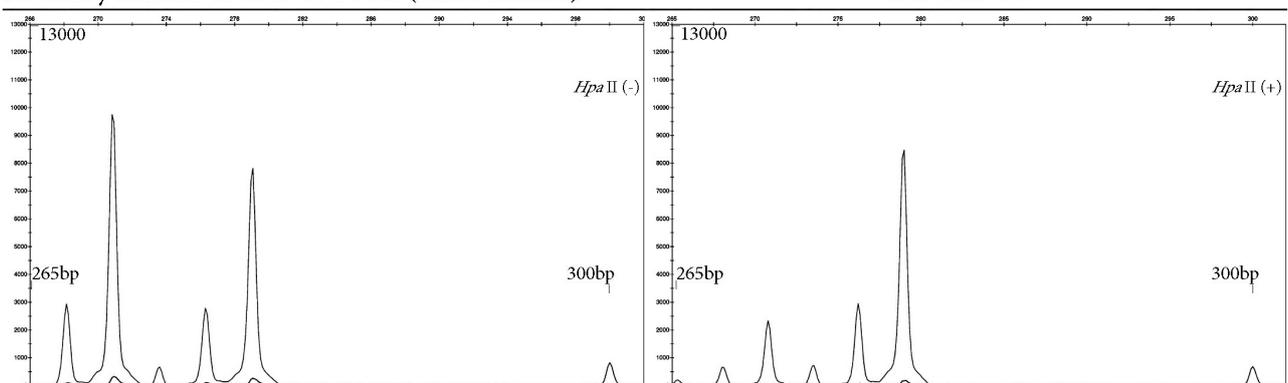
Fig 3. X-chromosome inactivation analysis in the patient's iPSC generated from the MPS II patient and a Fabry disease heterozygote serving as control. Standard and naïve culture conditions cells are compared. Methylation status analysis of the *AR* alleles before (*Hpa*II-) and after (*Hpa*II+) digestion is shown. The 300-bp peak belongs to the GeneScan 500 ROX size standard. The analysis was conducted using GeneMapper software (Applied Biosystems).

2014). These types of therapies, however, will need to await development of safe therapeutic protocols.

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