ORIGINAL ARTICLE

Genistein increases glycosaminoglycan levels in mucopolysaccharidosis type I cell models

Sandra D. K. Kingma · Tom Wagemans · Lodewijk IJlst · Frits A. Wijburg · Naomi van Vlies

Abstract Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disorder characterized by diminished degradation of the glycosaminoglycans (GAGs) heparan sulfate and dermatan sulfate, which results in the accumulation of these GAGs and subsequent cellular dysfunction. Patients present with a variety of symptoms, including severe skeletal disease. Genistein has been shown previously to inhibit GAG synthesis in MPS fibroblasts, presumably through inhibition of tyrosine kinase activity of the epidermal growth factor receptor (EGFR). To determine the potentials of genistein for the treatment of skeletal disease, MPS I fibroblasts were induced into chondrocytes and osteoblasts and treated with genistein. Surprisingly, whereas tyrosine phosphorylation levels (as a measure for tyrosine kinase inhibition) were decreased in all treated cell lines, there was a 1.3 and 1.6 fold increase in GAG levels in MPS I chondrocytes and fibroblast, respectively ($p < 0.05$). Sulfate incorporation in treated MPS I fibroblasts was 2.6 fold increased ($p < 0.05$), indicating increased GAG synthesis despite tyrosine kinase inhibition. This suggests that GAG synthesis is not exclusively regulated through the tyrosine kinase activity of the EGFR. We hypothesize that the differences in outcomes between studies on the effect of genistein in MPS are caused by the different effects of genistein on different growth factor signaling pathways, which regulate GAG synthesis. More studies are needed to elucidate the precise signaling pathways which are affected by genistein and alter GAG metabolism in order to evaluate the therapeutic potential of genistein for MPS patients.

S. D. K. Kingma · T. Wagemans · F. A. Wijburg · N. van Vlies
Department of Pediatrics and Amsterdam Lysosome Centre
"Sphinx", Academic Medical Center, University of Amsterdam,
Amsterdam, The Netherlands

S. D. K. Kingma
e-mail: s.d.kingma@amc.uva.nl

T. Wagemans
e-mail: a.h.wagemans@amc.uva.nl

N. van Vlies
e-mail: n.vanvlies@amc.uva.nl

S. D. K. Kingma · T. Wagemans · L. IJlst · N. van Vlies
Laboratory of Genetic Metabolic Diseases, Department of Clinical
Chemistry and Pediatrics, Academic Medical Center, University of
Amsterdam, Amsterdam, The Netherlands

L. IJlst
e-mail: l.ijlst@amc.uva.nl

F. A. Wijburg (✉)
Department of Pediatrics, Division of Metabolic Diseases (H7-270),
Academic Medical Centre (AMC), Meibergdreef 9, 1105
AZ Amsterdam, The Netherlands
e-mail: f.a.wijburg@amc.uva.nl

Introduction

The mucopolysaccharidoses (MPSs) comprise a group of lysosomal storage diseases, each caused by a single enzyme deficiency, leading to diminished glycosaminoglycan (GAG) degradation. Consequently, GAGs accumulate in lysosomes, resulting in progressive cellular and organ dysfunction. MPS type I (OMIM 252800), is caused by deficiency of the hydrolase α-L-iduronidase (IDUA, EC 3.2.1.76), resulting in impaired heparan sulfate (HS) and dermatan sulfate (DS) degradation and subsequent GAG accumulation. This leads to progressive cardiac and pulmonary disease, inguinal and umbilical hernias, corneal clouding and severe musculoskeletal disease. In addition, patients with the severe (Hurler) phenotype also suffer from progressive central nervous system (CNS) disease, significantly limiting life expectancy (Muenzer 2011).

The constellation of radiographic abnormalities resulting from defective intramembranous and endochondral bone formation is collectively referred to as dysostosis multiplex. These

skeletal changes may lead to progressive loss of joint motion with contractures, growth arrest, kyphosis, scoliosis, hip dysplasia and hypoplastic vertebral bodies resulting in atlanto-axial instability and spinal cord compression (Muenzer 2011; White 2011). Current therapeutic strategies, such as haematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT), effectively treat many features of MPS I, but have limited effects on bone disease (Aldenhoven et al 2008; Baldo et al 2013; van der Linden et al 2011). Efficacy of HSCT is limited because cartilage cells are derived from mesenchymal stem cells and these are not transplanted in sufficient amounts by HSCT (Koc et al 2002). In addition, circulating enzymes do not easily reach growth plates and other cartilaginous tissue due to the relative avascularity of cartilage. Finally, the relatively large molecular weight of lysosomal hydrolases hampers easy diffusion through the matrix to target cells, which limits the efficacy of both HSCT and ERT on cartilaginous tissues (Aldenhoven et al 2008; Baldo et al 2013; Oussoren et al 2011; Sifuentes et al 2007).

An alternative strategy for the treatment of bone disease in MPS I might be substrate reduction therapy which aims to reduce GAG synthesis, thus decreasing the accumulation of undegradable material. Such an approach may prevent or halt the pathophysiological cascades initiated by the accumulating GAGs and involve inflammatory processes, dysregulation of osteoclastogenesis and apoptosis of chondrocytes, all resulting in abnormal bone formation and growth (Simonaro et al 2008). Genistein, which is an isoflavone naturally occurring in soy and several other plants, inhibits the activity of tyrosine kinase receptors including the epidermal growth factor receptor (EGFR) (Akiyama et al 1987). Previous studies demonstrated that genistein inhibits GAG synthesis in MPS fibroblasts (FBs), presumably through this mechanism (Jakobkiewicz-Banecka et al 2009; Piotrowska et al 2006). Genistein is well tolerated, appears to be safe also in high doses (Kim et al 2013) and can reach bone tissue (Coldham and Sauer 2000). In this study, we aimed to investigate the effects of genistein on GAG accumulation in induced chondrocytes (ICs) and osteoblasts (IOs) of MPS I patients in order to determine its potential in the treatment of MPS I related bone disease.

Materials and methods

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin and amphotericin were obtained from Lonza (Basel, Switzerland). Fetal bovine serum (FBS) was from Bodinco B.V (Alkmaar, The Netherlands). Bovine serum albumin (BSA), phosphatase inhibitors, insulin, dexamethasone, alcian blue and nuclear fast red were from Sigma Aldrich (St. Louis, MO, USA). Genistein aglycone (>98 % pure) was from Sigma Aldrich or received as a kind gift from Axcentua (Huddinge, Sweden). Recombinant TGF-ß was from MT-diagnostics (Metten-Leur, The Netherlands). Complete mini protease inhibitor cocktail, first-strand cDNA synthesis kit and LC480 SYBR Green Master mix were from Roche Applied Science (Indianapolis, IN, USA). 1-step Para-nitrophenyl-phospate (PNPP) solution was from Thermo scientific (Waltham, MA, USA). Para-nitrophenol was from Merck Schuchardt (München, Germany). CellTiter 96® Aqueous One Solution Reagent was from Promega (Mannheim, Germany). TRIzol was from Invitrogen (Bleiswijk, The Netherlands). Primers were from Biolegio (Nijmegen, The Netherlands) or Sigma Aldrich. $H_2{}^{35}SO_4$ was from Hartmann Analytic GmbH (Huissen, The Netherlands). Antibodies against phosphorylated tyrosin (pTyr), LAMP-1 and tubulin (mouse) were from Cell Signaling Technologies (Danvers, MA, USA), tubulin (rabbit) from Sigma Aldrich and all secondary antibodies from Westburg B.V. (Leusden, The Netherlands). Paraformaldehyde, glutaraldehyde, Triton-X-100, sodium thiosulphate, silver nitrate, eosin, haematoxylin, ascorbate-2-phosphate and glycerol-2-phosphate were of analytical grade.

Cell culture

Informed consent for the use of FBs was obtained from all patients or their parents. FBs were grown in DMEM supplemented with 10 % heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotericin in a humidified atmosphere containing 5 % CO_2 at 37 °C.

Induction of differentiation

Chrondrogenic and osteogenic differentiation of FBs was performed essentially as described earlier (Junker et al 2010), with minor modifications. Osteogenic induction medium contained 5 % FBS instead of 10 %. One day after plating cells, induction medium was added and cells were maintained in induction medium for 3 weeks. Medium was changed twice a week.

Chondrogenic induction was verified by analyzing cell morphology, proteoglycan production (alcian blue staining) and gene-expression of chondrogenic markers. Osteogenic induction was verified by analysis of cell morphology, calcium deposition (von Kossa staining), alkaline phosphatase (ALP) activity and gene-expression of an osteogenic marker.

Isoflavone treatment

After 2 weeks of induction, induction medium of ICs and IOs was supplemented with 50 μM of genistein dissolved in DMSO (final DMSO concentration in medium was 0.1 %). Unsupplemented cells were incubated with induction medium containing only 0.1 % DMSO. Cells were harvested after 1 week of genistein treatment. In the experiments with

undifferentiated FBs, cells were allowed to attach after which genistein was added to the medium. FBs were treated for 2, 4 or 7 days with 50 μM genistein or 2 days with 25 μM genistein.

Cell viability

For all the conditions tested, cell viability was measured using CellTiter 96® Aqueous One Solution Reagent (containing MTS tetrazolium). The quantity of produced formazan was measured at 485 nm and is directly proportional to the number of metabolically active cells in culture (Ferdinandusse et al 2009). All measurements were performed in triplicate.

Histology

Cell morphology was analysed with routine haematoxilin/eosin (HE) staining. Alcian blue staining was used to detect proteoglycans and von Kossa staining to detect calcified extracellular matrix (ECM). Cells were fixed in 4 % glutaraldehyde, washed three times with phosphate buffered saline (PBS), and for alcian blue staining: stained 15 min with 10 g/L alcian blue in 3 % acetic acid and counterstained for 9 min using nuclear fast red. For von Kossa staining, fixed cells were stained for 90 min with 50 g/L silver nitrate solution under a 40 W light, and treated for 9 min with 50 g/L sodium thiosulphate.

Alkaline phosphatase activity

To confirm osteogenic differentiation, ALP activity was measured. Cells were detached with a cell scraper in 9 g/L NaCl solution containing a cocktail of protease inhibitors, and disrupted by sonification using a Vibra Cell sonicator (Sonics & Materials Inc., Newtown, CT, USA). Protein concentration was measured in whole cell lysates as earlier described (Lowry et al 1951).

Of cell lysate 50 μl was added to 100 μl of PNPP solution, to a final protein concentration of 0.1 mg/ml. After 10 min of incubation at 37 °C, the reaction was stopped by addition of 100 μl of 1 M NaOH. The absorbance of released para-nitrophenol was measured at a wavelength of 405 nm. ALP activity was calculated using a calibration curve of para-nitrophenol. All assays were performed in duplicate.

Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent. cDNA was produced using a first-strand DNA synthesis kit. qPCR analysis for chondrogenic markers (ACAN, COL2A1, COL10A1, SOX9), the osteogenic marker SPARC (osteonectin) and genes involved in HS synthesis (XYLT1, EXT1, EXT2, NDST1, NDST2, HS2ST1, HS6ST1) was performed using LC480 SYBR Green Master mix. Melting curve analysis and gel electrophoresis were carried out to confirm the generation of a single product. Duplicate analyses were performed for all samples. Data were analyzed using linear regression calculations as earlier described (Ramakers et al 2003). To adjust for variations in the amount of RNA, all values were normalized against the housekeeping gene PPIB (cyclophilin B). Primer sequences are described in Table 1.

GAG analysis

The effect of genistein on GAG levels in FBs, ICs and IOs was determined by measuring heparan and dermatan sulfate-derived disaccharides using HPLC-MS/MS, as described previously (Kingma et al 2013), with one minor modification: instead of 25 μg, 12.5–50 μg of protein was used. In all experiments, the effects of genistein were similar for the different disaccharides, therefore only values of the sum of all GAG derived disaccharides are given.

Sulfate incorporation

GAG synthesis was approximated by monitoring ^{35}S incorporation using an earlier described, modified protocol (Kloska et al 2012). FBs were plated and the next day the medium was supplemented with 50 μM genistein or DMSO only. After 48 h of incubation, half of the medium was removed and fresh medium containing 50 μM genistein and 20 μCi/ml of $H_2^{35}SO_4$ was added. 24 h later, cells were washed six times with PBS and lysed in PBS containing a cocktail of protease inhibitors and 25 g/L Triton-X-100. ^{35}S incorporation was measured in a scintillation counter. Counts were corrected for protein concentration measured in aliquots of the same cell lysates (Lowry et al 1951). ^{35}S incorporation experiments were performed in at least two independent cultures.

Western blot analysis

Cells were detached with a cell scraper in a 9 g/L NaCl solution containing protease and phosphatase inhibitors, and 5 g/L Triton-X-100. Cells were disrupted by sonification and protein concentration was measured (Lowry et al 1951). 40 μg of protein was loaded onto a 10 % SDS-polyacrylamide gel for detection of pTyr or 50 μg for detection of LAMP-1, and after electrophoresis, transferred onto a nitrocellulose membrane. Membranes were blocked in 50 g/L BSA, 0.1 % Tween-20 in Tris buffered saline, pH 7.4, for detection of pTyr or 30 g/L BSA, 0.1 % Tween-20 in PBS for the detection of LAMP-1. Expression levels were normalized for the housekeeping protein tubulin, detected on the same membrane.

Antibodies were diluted as follows: pTyr; 1:400, tubulin (rabbit); 1:1,000, LAMP-1; 1:1,000, tubulin (mouse); 1:5,000 solution, IRDye 800 goat anti-mouse; 1:5,000, IRDye 680 goat anti-rabbit; 1:5,000, IRDye 800 goat anti-rabbit; 1:10,000, IRDye 680 donkey anti-mouse; 1:5,000.

Table 1 Primer sequences used for RT-qPCR

Gene	Forward sequence	Reverse sequence
ACAN	TTTGTCCAGCGCCACATGCC	GCTCCGCTTCTGTAGTCTGC
Col2A1	TCACGTACACTGCCCTGAAG	CCTCCTATGTCCATGGGTGC
Col10A1	TTCTCTAACTCTACCCCACC	CAAGAGAGGCTTCACATACG
EXT1	TGTCGTCGTCATTGAAGGAG	GTTGTTGAAAGCACCGTGTC
EXT2	GTCTGAGTGGACGAATGAAGTG	ATCCCCAGGCATTTTGTAGG
HS2ST1	CGATGCTCTTCTTGGAAAACC	CCATTGTATGTCGCTGCTCA
HS6ST1	TGGCTCTTCTCCCGCTTCTC	GGGTCTCGTAGCAGGGTGAT
NDST1	TCACCTTCAACCTGGGCTAC	ACGGACTGGTTGTGGAAAAG
NDST2	CGCTGGCTGACTTACTACCC	TGGCACCAAAATCCCTTATC
PPIB	TGGAGAGCACCAAGACAGACA	CTTCTCCACCTCGATCTTGC
SOX9	GGTGCGCGTCAACGGCTCC	AAGGGCCGCTTCTCGCTCTCG
SPARC	GAGAATGAGAAGCGCCTGGAG	ATGGGGATGAGGGGAGCAC
XYLT1	TTGCGCCTCTGACCTTCTCG	TGCGTTCCTCCGTGCCTGTT

Statistical analysis

Statistical analysis was performed using Students *t*-test. Significance was assumed where *p* values were less than 0.05.

Results

Evaluation of chondrogenic and osteogenic induction

After induction, HE staining revealed that both ICs and IOs lost the elongated shape typical of FBs (Fig. 1a–c). In addition, while FBs grew neatly in a monolayer (Fig. 1a), ICs were forming ridges with empty spaces in between (Fig. 1b). In contrast, IOs grew in multiple layers with high cellular density (Fig. 1c). Alcian blue staining revealed the ECM of ICs to be rich in proteoglycans (Fig. 1d–e). Furthermore, ICs had a 275 fold, 53 fold, 25 fold and 48 fold increase in gene expression of *ACAN*, *COL2A1*, *COL10A1* and *SOX9*, respectively, as compared to FBs (Fig. 1f). Von Kossa staining revealed a calcified ECM around IOs (Fig. 1g–h). Furthermore, there was a 3 fold increase in gene expression of *SPARC* (Fig. 1i) and a 27 fold increase in ALP activity (Fig. 1j), as compared to FBs.

These results suggest successful transdifferentiation of FBs into ICs and IOs.

Cell viability

At the end of each experiment, cell viability was analyzed. In ICs and IOs, cell viability was more than 80 % in genistein-treated cells compared to untreated cells (results not shown). Cell viability was more than 90, 70 and 60 % in FBs treated for 2, 4 and 7 days, respectively (results not shown).

GAG analysis

As expected, analysis of GAG levels revealed significantly higher levels in MPS I cells, as compared to healthy control cells (Fig. 2). There was no effect of genistein on GAG levels in healthy control ICs (Fig. 2a). In contrast, a significant increase of GAG levels was observed when MPS I ICs were treated with genistein (Fig. 2a). In IOs, there was no significant difference in GAG levels between genistein-treated and untreated cells (Fig. 2b).

MPS I FBs treated for either 2, 4, or 7 days with 50 μM genistein, showed significantly higher GAG levels compared to untreated MPS I FBs (Fig. 2c–e). When MPS I FBs were treated for 2 days with 25 μM genistein, there was no effect of genistein on GAG levels (results not shown).

To exclude that the unexpected increases in GAG levels were due to the source of genistein, FBs were treated for 7 days with 50 μM of genistein from another manufacturer (Axcentua). Again, no decrease in GAG levels after genistein treatment was observed in either control or MPS I FBs (results not shown).

Sulfate incorporation

GAG synthesis was approximated by monitoring ^{35}S incorporation. In accordance with the increase in GAG levels measured by HPLC-MS/MS, sulfate incorporation was significantly increased (almost three fold) in MPS I FBs treated with genistein compared to untreated MPS I FBs (Fig. 2f). There was no difference between treated and untreated healthy control FBs.

Lysosomal abundance

LAMP-1 protein levels were determined by western blot as a measure of lysosomal abundance. No differences were
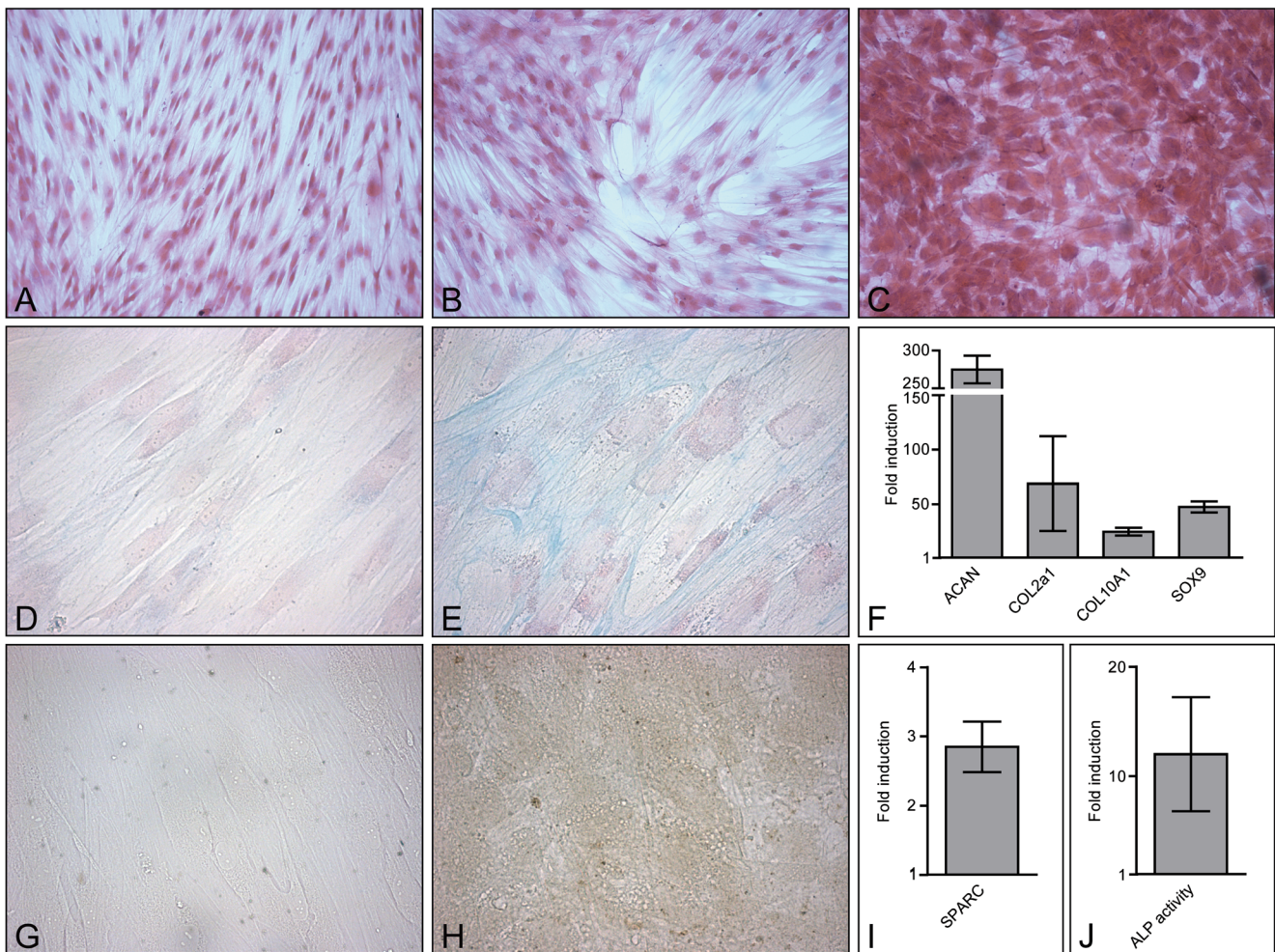
Fig. 1 Transdifferentiation of fibroblasts. HE staining of FBs (**a**), ICs (**b**) and IOs (**c**). Alcian blue staining of FBs (**d**) and ICs (**e**), glycosaminoglycans are stained blue. Fold change in mRNA expression of chondrogenic markers in ICs (**f**), expression levels in FBs are set at 1. Von Kossa staining of FBs (**g**) and IOs (**h**), deposited calcium is stained brown. Fold change in mRNA expression of the osteogenic marker osteonectin (*SPARC*) in IOs (**i**), expression levels in FBs are set at 1. Fold change in ALP activity in IOs (**j**), all values are mean ± standard deviation of 3 healthy control cell lines in independent experiments, activity in FBs is set at 1. Gene expression data: all values are mean ± standard deviation of replicates in one representative experiment in a control cell line (expression levels in fibroblasts were detectable but often below limit of quantification, therefore no standard deviation could be calculated between different experiments). All analyses were repeated at least once in independent cell cultures in another control cell line and MPS I cell line, with similar results

detected between genistein-treated and untreated FBs (results not shown).

Levels of phosphorylated tyrosine

To confirm that genistein inhibited tyrosine kinase activity, we determined global phosphorylated tyrosin (pTyr) levels in cells treated with genistein. Protein levels of pTyr differed between cell lines, however, in all cell types, pTyr levels were clearly decreased in genistein treated cells compared to their untreated controls (Fig. 3). When data from the different cell lines were grouped and mean pTyr levels were calculated, a significant decrease in pTyr levels in MPS I ICs treated with genistein was observed, as compared to untreated controls. In MPS I IOs and healthy control FBs, a trend towards lower pTyr levels ($p=0.08$) was observed when data from different cell lines were grouped (results not shown). These results indicate that genistein inhibited tyrosine phosphorylation in these cell culture models.

Gene expression of enzymes involved in HS synthesis

The influence of genistein on gene expression of enzymes involved in HS synthesis was measured using RT-qPCR. Significant differences in gene expression of ≥2 fold gene expression were considered biologically relevant and significant differences of ≥1.5 fold were considered as a trend towards higher or lower gene expression. In control ICs treated with genistein, there was a significant increase (Fig. 4a) in *NDST2* (2.4 fold, $p<0.05$) and *HS2ST1* (2.3 fold, $p<0.05$). In

Fig. 2 GAG levels and sulfate incorporation. GAG levels measured with HPLC-MS/MS in ICs (**a**) and IOs (**b**) after 7 days of treatment with 50 μM genistein. GAG levels in FBs after 2 days (**c**), 4 days (**d**), 7 days of treatment (**e**). The results were expressed as milligrams GAG per gram of protein. Sulfate incorporation in FBs treated for 4 days (**f**). The results are expressed as radioactive counts per μg of protein. All values are mean ± standard deviation of three healthy control cell lines and five MPS I cell lines in figure **a** and **b**, one healthy control cell line and three MPS I cell lines in figure **c**–**e** and three healthy control cell lines and three MPS I cell lines in figure **f**. Each sample was analysed in duplicate, *$p<0.05$, **$p<0.01$
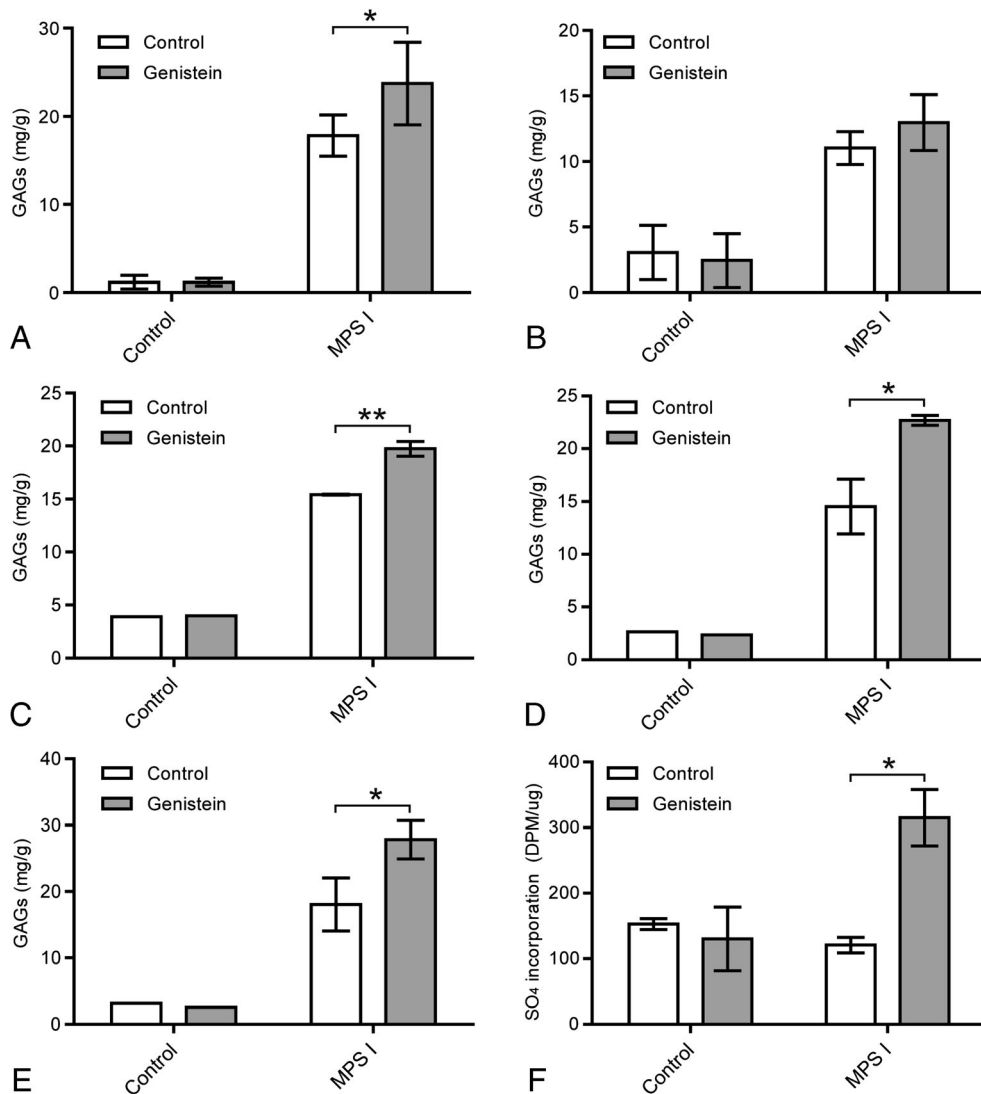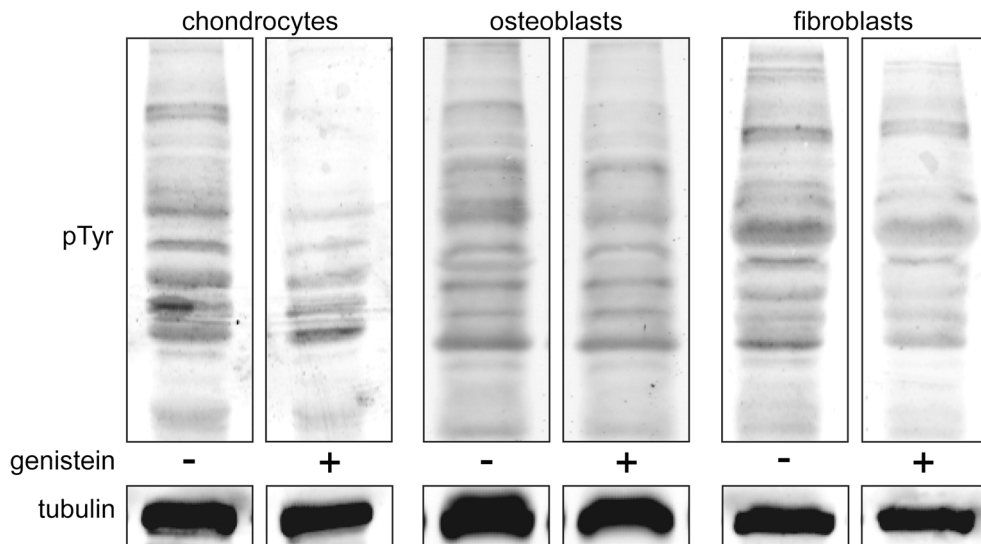

Fig. 3 Levels of phosphorylated tyrosine. pTyr levels in ICs, IOs and FBs either untreated or treated with 50 μM genistein for 7 days. Western blot analysis was performed in three different control and MPS I cell lines, only blots of 1 MPS I cell line are depicted. All analyses were repeated at least once in an independent experiment with similar results

MPS I ICs, a 2.3 fold increase ($p<0.01$) in *HS6ST1* was observed, as compared to untreated ICs. There was a trend towards higher expression of most of the other HS synthesis genes in genistein treated ICs (Fig. 4a). As compared to untreated ICs, *EXT2* was 1.5 fold ($p<0.05$) increased in control ICs and *NDST1*, *NDST2* and *HS2ST1* were 1.5 fold ($p<0.05$), 1.5 fold ($p<0.01$) and 1.9 fold ($p<0.001$) increased, respectively, in MPS I ICs. There were no (biologically relevant) differences detected in FBs or IOs treated with genistein, as compared to untreated cells (Fig. 4b, c).

Discussion

Skeletal disease is one of the most prevalent and incapacitating disease manifestations of the MPSs and frequently results in the need for multiple orthopaedic surgeries (Muenzer 2011; White 2011). Disease modifying therapies for the management of MPS I (HSCT and ERT) have led to increased lifespan but have little effect on the progression of skeletal deformities (Sifuentes et al 2007; van der Linden et al 2011; Weisstein et al 2004). Therefore, therapeutic strategies targeting bone disease are urgently needed.

The isoflavone genistein has been investigated for its potential benefit in the treatment of CNS disease in MPS III, as in vitro studies showed that genistein may reduce GAG synthesis and thereby GAG accumulation (Arfi et al 2010; Jakobkiewicz-Banecka et al 2009; Kloska et al 2011; Piotrowska et al 2006). Indeed, an in vivo study revealed decreased GAG levels in the brain of MPS III mice treated with genistein (Malinowska et al 2010). As prevention of GAG accumulation in cartilage and bone might halt the progression of skeletal disease in the MPSs, we set out to study the in vitro effects of genistein on ICs and IOs, derived from MPS I FBs. However, in remarkable contrast to previously published studies on the effects of genistein in MPS FBs (Arfi et al 2010; Jakobkiewicz-Banecka et al 2009; Kloska et al 2011; Piotrowska et al 2006), GAG levels significantly increased in MPS I ICs and FBs treated with genistein and remained unchanged in IOs. The discrepancy between our results and previously published data does not appear to be due to the source of genistein, as we observed similar responses using two different sources of genistein. Also, differences in the methodology used for GAG analysis cannot explain this discrepancy as both HPLC-MS/MS analysis and sulfate incorporation measurements, which is the most frequently used test to measure GAG synthesis in in vitro studies on the effects of genistein in the MPSs, showed increased GAG synthesis or levels in MPS I FBs.

In vivo studies on the effects of genistein in MPS patients and animal models also show divergent results. When MPS III patients were treated with low dose soy extracts (containing genistein/genistin at a dose of 5–15 mg/kg/day), some open-
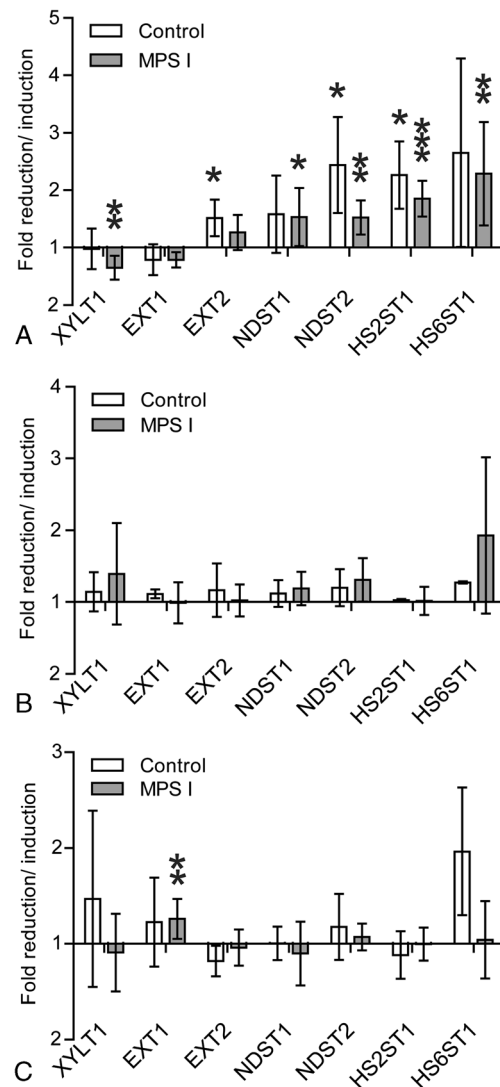

Fig. 4 mRNA expression of enzymes involved in HS synthesis. Fold change in mRNA expression of HS synthesis enzymes in ICs (**a**), IOs (**b**) and FBs (**c**), treated with 50 μM genistein for 7 days. Expression levels in untreated cells are set at 1. Each bar represents the mean ± standard deviation of two or three healthy control or five MPS I cell lines per condition. All values were normalized against the housekeeping gene *PPIB*. All measurements were performed in duplicate, *$p<0.05$, **$p<0.01$, ***$p<0.001$

label studies showed positive effects (Malinova et al 2012; Piotrowska et al 2011), while in another study no effect on both biochemical and clinical parameters was observed (Delgadillo et al 2011). In a placebo controlled trial, a small but significant reduction in plasma and urinary GAGs was observed, with no effects on clinical variables (de Ruijter et al 2012). In a study in MPS III mice, a high dose of pure genistein (160 mg/kg/day) for 9 months resulted in an impressive decrease in brain storage levels, decreased neuroinflammation and improvement of behavioural abnormalities (Malinowska et al 2010). However, no decrease of urinary GAG levels or improvement in neurocognitive or disability

scores were observed in MPS III patients who received a similar dose of 150 mg/kg/day of pure genistein for at least 1 year (Kim et al 2013).

The concentrations of genistein, used in our study, were higher or equal (50–25 μM) to concentrations used in previous in vitro studies (Arfi et al 2010; Jakobkiewicz-Banecka et al 2009; Kloska et al 2011; Piotrowska et al 2006). In MPS III patients treated with low dose soy extracts (containing genistein and its glucuronide genistin at a total dose of 10 mg/kg/day) (de Ruijter et al 2012), the total genistein/genistin concentration measured in plasma was 8.8 mM, while the concentration of the biologically active form, genistein (the aglycone form), was 48 μM (unpublished results). The concentration of genistein aglycone corresponds with the concentration used in our study.

The reduction in GAG levels, as observed in previously published in vitro studies and some of the in vivo studies, is considered a result of EGFR inhibition and subsequent downregulation of GAG synthesis (Jakobkiewicz-Banecka et al 2009). In our study, however, no downregulation of genes involved in HS synthesis was observed, in contrast, there was a trend towards upregulation of some of these genes, while the levels of phosphorylated tyrosine were lowered after genistein treatment. This indicates that genistein does not exclusively influences GAG levels through inhibition of phosphorylation of tyrosine residues of the EGFR.

Although maximal GAG synthesis requires the presence of either EGF or follicle-stimulating hormone (Tirone et al 1997), many other growth factors are also involved in the regulation of GAG production, such as insulin-like growth factor 1 (IGF1) (Blunk et al 2002), platelet derived growth factor (Hiraki et al 1988) and members of the TGF-β superfamily, including TGF-β1 (Hiraki et al 1988) and bone morphogenic proteins (Blunk et al 2003; Gooch et al 2002). The actions of these growth factors are essential for bone and cartilage metabolism (Mackie et al 2011) and genistein affects at least some of them. For instance, the IGF1 receptor is — among a large amount of other receptors — a tyrosine kinase receptor and expression levels have shown to be inhibited by genistein (Hwang et al 2013). In addition, TGF-β expression was shown to be decreased in cells treated with genistein (Yuan et al 2009), though enhancement of TGF-β secretion in the medium of cells has also been described (Kim et al 2001).

Regulation of GAG synthesis is a very complicated process and numerous feedback mechanisms are likely to be involved. Small differences in cell culture conditions (e.g. composition of the culture medium and the source of fetal bovine serum) in in vitro studies, as well as genetic heterogeneity and external factors in patients and mouse models may contribute to the differences observed between studies.

Our study underscores the complexity of the regulation of GAG synthesis which may result in seemingly conflicting

effects of isoflavones (genistein and other components of soy extracts) in in vitro and in vivo studies. We feel that our study shows that genistein should be used with caution in patients with the MPSs, as its effects are difficult to predict and may be adverse. Additional studies to elucidate the biochemical processes influenced by genistein in the MPSs, as well as placebo controlled clinical trials, are urgently needed to assess the scope of biochemical and clinical effects of genistein.

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