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Effects of high glucose on mesenchymal stem cell proliferation and differentiation

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Abstract

High glucose (HG) concentrations impair cellular functions and induce apoptosis. Exposition of mesenchymal stem cells (MSC) to HG was reported to reduce colony forming activity and induce premature senescence. We characterized the effects of HG on human MSC *in vitro* using telomerase-immortalized MSC (hMSC-TERT) and primary MSC (hMSC). HG (25 mM) enhanced hMSC-TERT proliferation in long-term studies in contrast to hMSC where proliferation was unchanged. Thioredoxin-interacting protein, which is involved in apoptosis regulation, was stimulated by glucose in hMSC-TERT. However, apoptosis was not influenced by HG in both cell types. MSC treatment with HG favored osteogenic differentiation.

MSC are resistant to HG toxicity, depending on the stemness of MSC. Proliferation and osteogenic differentiation are stimulated by HG. Effects of HG on the transient amplifying compartment of MSC may differ from those in mature cells. Further research is needed to unravel the molecular mechanisms of HG resistance of MSC.

Keywords: Mesenchymal stem cells; Glucose; Thioredoxin-interacting protein; Osteogenic differentiation

MSC are distributed all over the organism as a source of tissue formation and regeneration. MSC are rarely proliferating cells, situated in stem cell niches, which can give rise to a transient amplifying cell compartment after asymmetric cell division. In bone several populations of probably variable signature can be obtained, e.g. from bone marrow, from trabeculae and periosteum. For cell-based regenerative therapies MSC are expanded *in vitro* and differentiated towards different cell entities like osteoblasts and chondrocytes [1] by applying culture media of different compositions. The components of the applied media can influence cell proliferation, viability including replicative senescence, and apoptosis [2]. Common media contain varying concentrations of glucose ranging from 1 to 4.5 g/l (5.6–25 mM).

Glucose is a central source of energy for all cells. Glucose uptake is mediated by specific glucose transporters of two families, the Na^+-coupled glucose transporters (SGLT) and glucose transporter facilitators (GLUT) [3]. Elevated glucose concentrations may impair cellular functions and finally induce apoptosis. This affects pancreatic beta cells in the first line but is also relevant for clinical features of uncontrolled diabetes like neuropathy, renal insufficiency, and arteriosclerosis [4]. In MSC obtained from rats high glucose induced cellular senescence, while reduction of glucose enhanced proliferation, decreased apoptosis, and increased the number of colony forming units [5].

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The glucose-responsive gene thioredoxin-interacting protein (Txnip, VDUP-1) has 46 kDa with homology to arrestins [6], which represent structural adaptor proteins involved in endocytotic trafficking, tyrosine kinase signaling, and cell-cycle regulation, migration, and inflammation [7]. Txnip has repeatedly been described to be upregulated in several cell types and most dramatically in pancreatic beta cells [8]. It is also induced by Vitamin D [9,10] while it is downregulated by NO [11]. Txnip knockout mice show insulin hypersecretion and hypoglycemia, demonstrating that Txnip is involved in the regulation of beta cell function [12]. In addition, Txnip was identified to be involved in glucose uptake in adipocytes and muscle cells. Employing patch-clamp techniques it was demonstrated that overexpression of Txnip inhibited glucose uptake while its knockdown acted vice versa [13]. Txnip is part of the thioredoxin reductase (TrxR)/thioredoxin (Trx) system of interacting proteins and suppresses the expression and activity of Trx [14]. This system is involved in neutralization of reactive oxygen species, reduction of many substrates by Trx, the main substrate of the TrxRs, and it modulates many different functions including beta cell regulation, gluconeogenesis, and glycolysis as well as induction of apoptosis [12,15,16].

Apoptosis can be detected on mRNA levels by marker gene amplification e.g. the enzyme transglutaminase (TGM), which catalyses the irreversible cross-linking of cytoplasmatic proteins to form an insoluble, stable protein scaffold. Another marker gene is the forkhead transcription factor FOXO3a (forkhead box, class O3A), which activates Bim, a proapoptotic relative of Bcl-2. It was reported that TGM and FOXO3a expression can be used as markers for detection and quantification of apoptosis [17,18].

We show here that proliferation of telomerase-immortalized hMSC-TERT cells is dose-dependently enhanced by high glucose concentrations during short-term experiments and long-term exposure. In contrast, varying glucose concentrations have no influence on the proliferation rate of primary hMSC. In hMSC-TERT the apoptosis rate is not affected by high glucose concentrations but decreased in hMSC by short-term exposure. Txnip expression is enhanced by glucose in hMSC-TERT and may thereby contribute to the relative resistance to glucose toxicity.

Materials and methods

Cell culture. Cell culture media and fetal calf serum were obtained from PAA Laboratories (Linz, Austria). hMSC-TERT cells were produced and cultured as described [19]. Primary hMSC were isolated from bone marrow as described previously [1,2]. Cells were stimulated with variable glucose concentrations as indicated.

Osteogenic differentiation. For osteogenic differentiation, hMSC were cultivated in 6-well plates until confluence and incubated in stem cell medium (SCM, 17.6 mM glucose) or in DMEM high glucose (HG, 25 mM glucose) containing ascorbate, β-glycerophosphate and dexamethasone (Sigma–Aldrich GmbH, Schnelldorf, Germany) for 2 and 4 weeks. Mineralized extracellular matrix was detected by Alizarin red staining as described previously [20,21].

Cell proliferation and apoptosis assays. For long-time glucose incubation hMSC-TERT and hMSC were pre-cultivated up to 4 weeks in medium containing the different glucose concentrations prior to seeding on 96-well plates. For short-term stimulation cells were directly seeded on 96-well plates (1000 cells/well) in medium containing 5.6, 11, and 25 mM glucose. After 4 days of cultivation, proliferation and apoptosis rates were measured by the CellTiter-Glo Luminescent Cell Viability Assay (ATP assay) and the Caspase-Glo 3/7 Assay (apoptosis) (both Promega GmbH, Mannheim, Germany) according to the manufacturer's instructions. Luminescence was measured with an Orion II Luminometer (Berthold Detection Systems, Pforzheim, Germany).

TrxR assay. TrxR activity was measured as described previously [2,22]. All chemicals were obtained from Sigma–Aldrich GmbH. Protein content of cell extracts was determined using Rotiquant Protein Assay (Carl Roth GmbH, Karlsruhe, Germany). The activity of TrxR was expressed as milliunits per milligram of protein.

RT-PCR. Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. RNA was reverse-transcribed with MMLV reverse transcriptase (Promega GmbH) and cDNA was amplified with Taq DNA polymerase from Peqlab GmbH (Erlangen, Germany) [Primers: TrxR2: sense 5′-ATGCGCAGGTGATGCGGACCGTG-3′, antisense 5′-GTCG TCATCATCTGGCACCAGGAG-3′ (381 bp); Foxo3a: sense 5′-AACC CAGGGCGCTCTTGGTG-3′, antisense 5′-ATGAGTTCACTACGGA TAATGGA-3′ (281 bp); TGM: sense 5′-CTGGGTGGAGTCGTGG ATGA-3′, antisense 5′-GATATCCTCCCGCTCGTCTC-3′ (309 bp); EF1α: sense 5′-AGGTGATTATCCTGAACCATCC-3′, antisense 5′-AA AGGTGGATAGTCTGAGAAGC-3′ (234 bp)].

Real-time PCR. For monitoring TrxR1 mRNA expression real-time PCR was performed with the DNA Engine Opticon system (MJ Research, Waltham, MA) using SYBR Green (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) as fluorescent dye. [Primers: TrxR1 sense: 5′-TCGCTTTGGAGTGCGCTGGA-3′, antisense 5′-GATTGCAACTGG GGTGAGCT-3′ (439 bp)]. For quantification and statistical analyses, TrxR1 mRNA expression was normalized to the expression levels of the housekeeping gene EF1α using the relative expression software tool (REST) [23]. Specificity of amplicons was confirmed by melting curve analyses.

Results

Effects of high glucose on cell proliferation and apoptosis

HMSC-TERT cells were exposed to various concentrations of glucose, where high glucose (25 mM) caused significant increase of proliferation. hMSC-TERT cells, which are usually cultured in low glucose media containing 5.6 mM glucose, showed a dose dependent stimulation of proliferation after short-term (4 day) and long-term (4 weeks) exposure (Fig. 1A and B, black bars). High glucose did not cause significant changes in apoptosis rates (Fig. 1A and B, gray bars).

In hMSC no consistent effect was seen on proliferation in short-term exposure (4 day) to 25 mM glucose. In long-term cultures (4 weeks) of individual donors variable trends were seen and the overall analysis showed a slight but significant decrease of proliferation after 25 mM glucose treatment (Fig. 1C and D, black bars). Especially the apoptosis rates were very variable and did only change slightly with glucose concentrations in short-term and long-term cultures (Fig. 1C and D, gray bars).

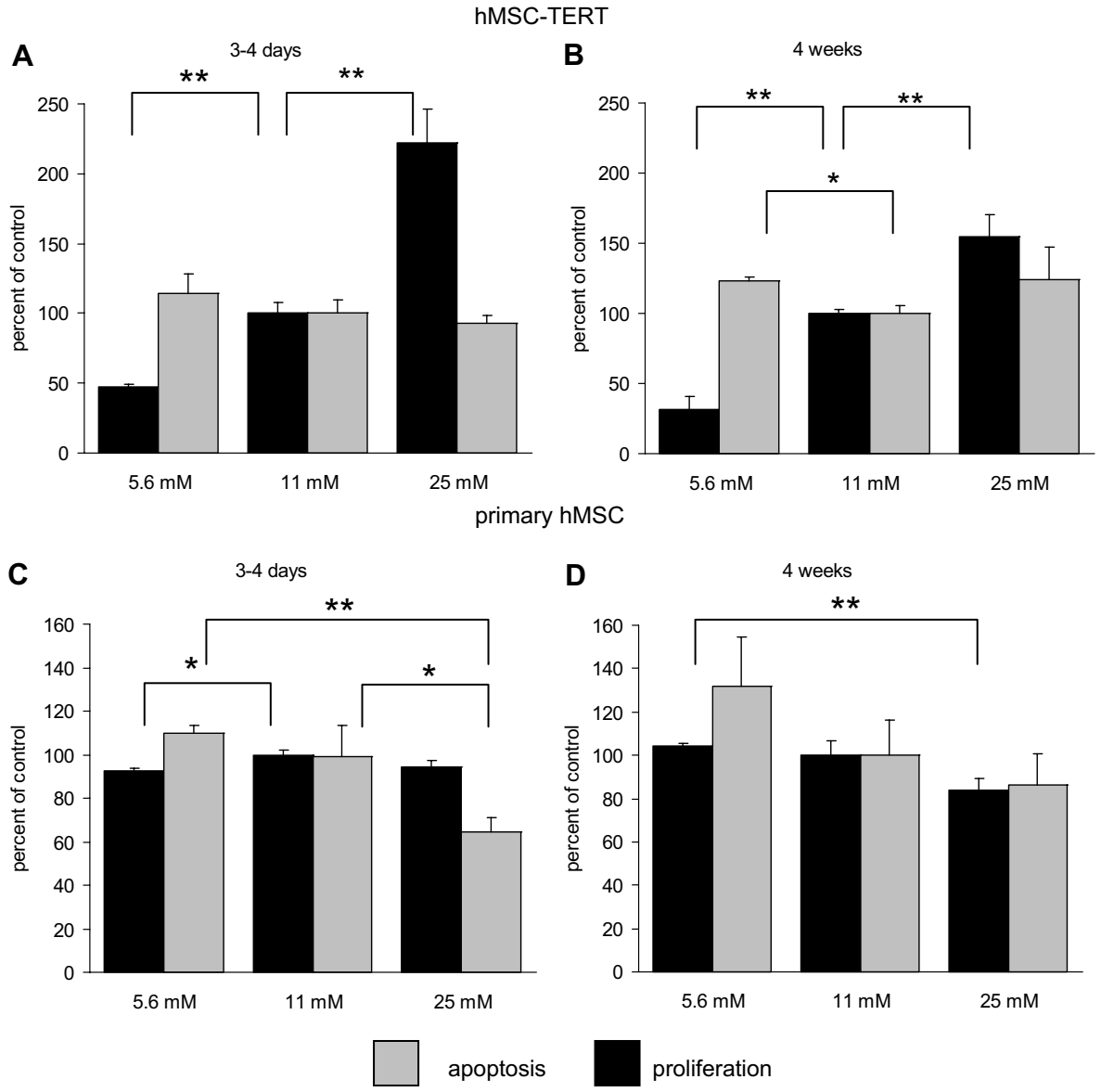

Fig. 1. Proliferation and apoptosis rates of hMSC-TERT (A,B) and primary hMSC (C,D). Cells were cultivated for 3–4 days (A,C) and for 4 weeks (B,D) with 5.6, 11, and 25 mM glucose and the proliferation rate (black bars) and apoptosis rate (gray bars) were determined. Data are expressed as means of six different measure points of three independent experiments as percent of controls (control 11 mM glucose) ± SEM. Significances were calculated by Student's *t*-test ($^{*}p < 0.05$; $^{**}p < 0.001$).

Txnip is glucose-regulated in hMSC-TERT

Txnip mRNA levels increased upon rising glucose concentrations from 5.6 mM to 25 mM in hMSC-TERT cells. This was consistently and reproducibly seen in cultures of short-term and long-term exposure (Fig. 2A). In hMSC the expression of Txnip was not influenced by different glucose concentrations (data not shown).

TrxR activity and expression of TrxR and apoptosis markers

To clarify if other components of the TrxR/Trx/Txnip system could potentially contribute to create a proapoptotic situation under high glucose conditions, expression levels and TrxR enzyme activity was determined in hMSC-TERT. The activity of TrxR in hMSC-TERT (12–15 mU/mg protein) was not influenced by glucose supplementation (Table 1). TrxR1 and 2 mRNA expression was also unchanged by applying different glucose concentration, which was detected by RT-PCR (TrxR2, Fig. 2B) and real-time PCR (TrxR1, Table 1). The expression levels of apoptosis markers like TGM and Foxo3a were also unchanged in hMSC-TERT after long-term glucose treatment (Fig. 2B).

Effects of putatively toxic glucose concentrations (40 mM) on primary hMSC

To determine the effect of putatively toxic glucose concentrations on hMSC, the cells were cultivated in 40 mM

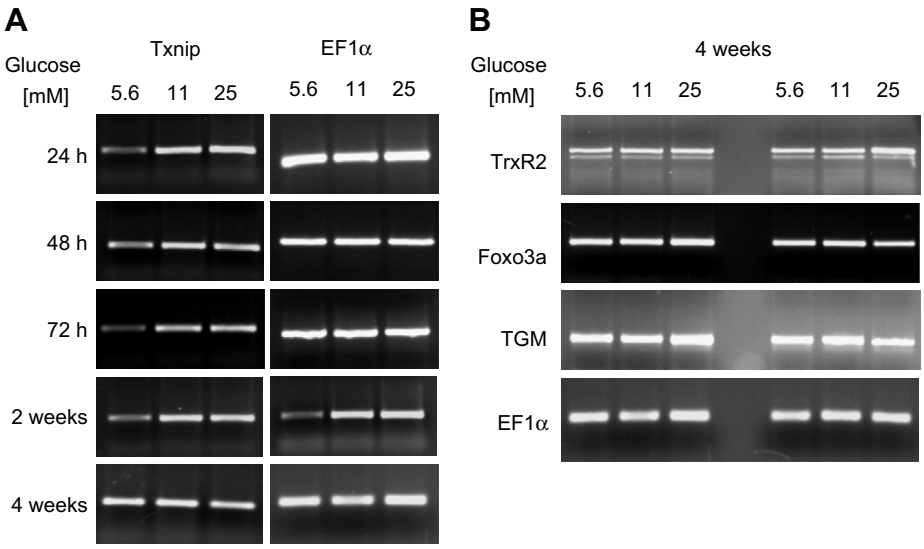

Fig. 2. Txnip expression and expression of apoptosis markers and TrxR2 in hMSC-TERT. Txnip expression was determined by RT-PCR in hMSC-TERT after 1–3 days, 2 and 4 weeks of stimulation with different glucose concentrations (A). The expression of the apoptosis markers transglutaminase (TGM) and Foxo3a and the expression of TrxR2 were determined by RT-PCR after 4 weeks of stimulation with different glucose concentrations (B). EF1α was amplified as a housekeeping gene. Two independent experiments are shown.

Table 1
TrxR expression and activity in hMSC-TERT cells cultivated with different glucose concentrations

Glucose concentration (mM)	5.6	11	25
TrxR1 real-time PCR (mRNA expression compared to 5.6 mM)	—	n.d.	n.d.
TrxR activity ± SEM (mU/mg protein)	12.9 ± 1.26	15.4 ± 0.77	12.5 ± 0.50

TrxR1 expression was analyzed by real-time PCR. Data are obtained from three independent experiments, significances were calculated by the REST© programme [23] (n.d., not significantly different). TrxR activity was measured by the DTNB assay. Data are expressed as means of three independent experiments ± SEM.

glucose (this concentration is not achieved by using common culture media). Txnip mRNA expression was enhanced in two of three MSC preparations after 24 h treatment with 40 mM glucose (Fig. 3A). Short-term exposure to 40 mM glucose had an inhibitory effect on cell apoptosis (Fig. 3B, gray bars) and no effect on the proliferation rate. Long-term treatment had also no effect on the proliferation and no effect on the apoptosis rate (Fig. 3C).

Influence of high glucose on osteogenic differentiation of hMSC

Primary hMSC were differentiated towards the osteogenic phenotype in SCM (17.6 mM glucose) and HG media (25 mM) containing the appropriate supplements. After 2 weeks of cultivating and differentiating the cells in HG the monolayer depicted enhanced mineralization which could be detected by alizarin red staining (Fig. 4D). After four weeks the degree of mineralization was enhanced (Fig. 4F). In contrast, efficient mineralization often failed in cells differentiated in SCM (Fig. 4C and E).

Discussion

Glucose in the microenvironment markedly affects gene regulation, proliferation, and differentiation as well as apoptosis and senescence of cells. In rat MSC, reduction of glucose stimulated cell proliferation and high glucose enhanced apoptosis [5], which may be a problem in therapeutic strategies based on *ex vivo* expansion of MSC [24], and may also propagate the development of osteoporosis in diabetes [25]. Impairment of MSC regenerative proliferation and differentiation capacity can be due to a reduction of the stem cell pool, an impairment of the transient amplifying segment after asymmetric stem cell division, as well as functional defects of their offspring after maturation. In contrast to the data obtained in rats, we find the opposite in our human models of the transient amplifying segment. This may be reconciled by the hypothesis that the two different assays used describe different situations of stem cell biology, e.g., on the one hand the capacity to produce colonies and on the other hand the proliferative capacity of cells of the transient amplifying compartment [26].

The transcription of Txnip is induced by high glucose in beta cells and is involved in glucose toxicity [8,9]. Txnip is also glucose-responsive in hMSC-TERT and this effect persists over weeks of culture. Until recently, the only known function of Txnip was to bind Trx and inhibit thioredoxin-reducing activity [27]. The putative mechanism of interaction has been described to be the formation of a stable disulfide-linked complex [28], which impairs the interaction between Trx and apoptosis signal-regulating

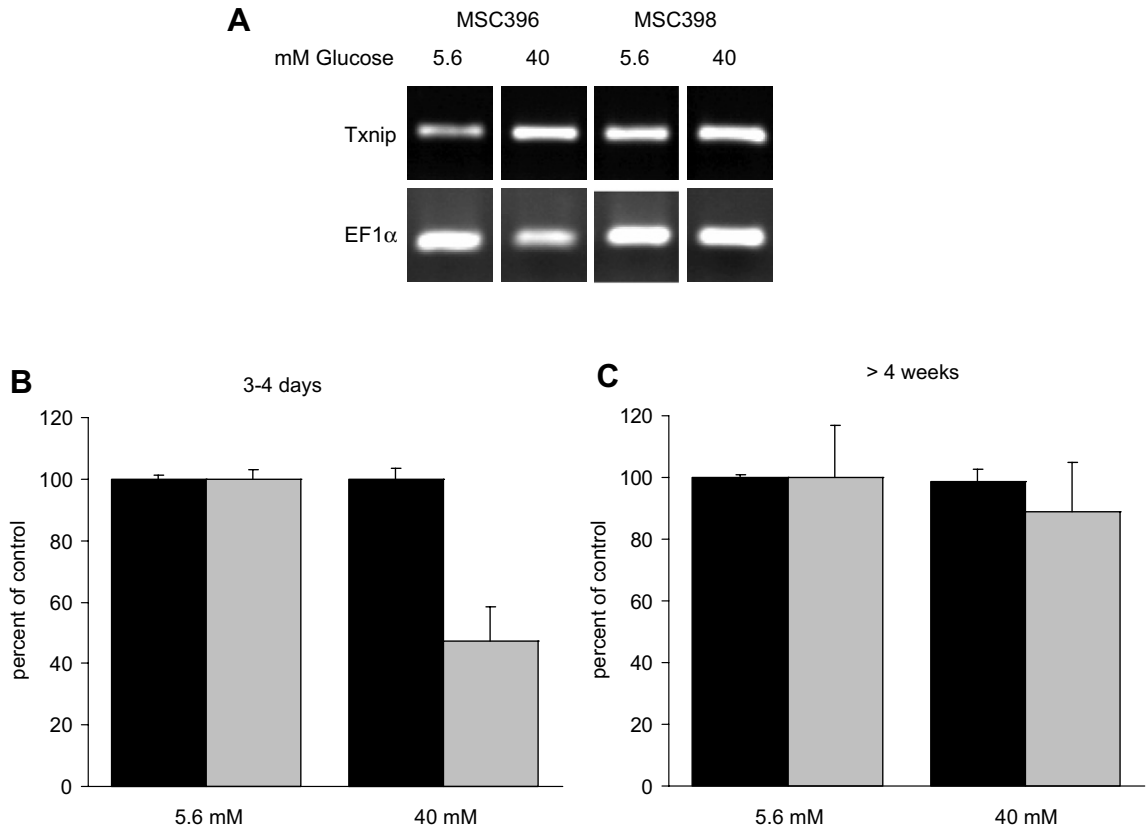

Fig. 3. Effects of putatively toxic glucose concentrations on primary hMSC. Cells were cultivated with 40 mM glucose and (A) Txnip expression was determined by RT-PCR compared to cells grown in 5.6 mM. EF1α was amplified as a housekeeping gene. After short-term (B) and long-term (C) treatment with 40 mM glucose the proliferation rate (black bars) and the apoptosis rate (gray bars) was measured. Data are expressed as means of six different measure points of three independent experiments ± SEM. Significances were calculated by using Student's *t*-test.

kinase-1 (ASK-1) in order to avoid apoptosis [29,30]. This should create a proapoptotic situation, which however does not lead to reproducibly enhanced rates of apoptosis in both hMSC-TERT and hMSC, which demonstrates that both populations are remarkably resistant to high glucose. Moreover, proliferation of hMSC-TERT is markedly enhanced and proliferation of hMSC is at least not impaired. Txnip overexpression causes beta cell apoptosis [8], while the inhibition of Txnip expression leads to the down-regulation of caspase-3 and Bax expression resulting in enhanced beta cell mass [31]. The situation in MSC is not analogous to beta cells and no enhanced apoptosis could be shown in either cell model within 4 weeks even if stimulated by putatively toxic glucose concentrations (40 mM).

The family of selenium-dependent TrxRs contains the mitochondrial TrxR2 and the cytosolic TrxR1 enzyme, the latter was characterized by our group as a vitamin D-responsive gene in human osteoblasts and monocytic cells [32,33]. Exposure to high and even putatively toxic glucose concentrations does not change the expression of components of the Trx/TrxR system, neither at the mRNA level nor at the level of enzyme activity. Trx, which is the main substrate of the TrxRs, reduces substrates (polypeptides and also small molecules) and consecutively modulates their function (e.g., DNA binding of transcription factors) and protein folding [16]. Apoptosis can be mediated by the interaction of Trx with Txnip as mentioned above or by TrxR itself. Truncated, enzymatically inactive TrxR1 polypeptides, which are expressed in selenium deficiency propagate apoptosis [15,34,35]. We have shown that selenium supplementation of MSC cultures leads to reduced DNA damage and oxidative stress [2]. Here selenium was not supplied to the cell cultures to create a situation which favors apoptosis induction but even this challenge did not influence the apoptosis rate.

Diabetes type 1 and 2 are both associated with enhanced fracture risk, which in type 1 diabetes is associated with low bone mineral density but not correlated with blood glucose control and diabetes duration [36]. The increased fracture risk in type 2 diabetes is not strictly dependent on bone mineral density but may rather be determined by different factors [25,37]. There is also experimental and clinical evidence for negative effects of elevated glucose levels and advanced glycation end products (AGEs) on cells involved in bone formation, e.g., mesenchymal stem cells (MSC) and their osteogenic offspring, the developing osteoblasts. Moreover, AGEs may stimulate osteoclast activity via NFκB activation. Thus osteoporosis may also develop as a late feature of uncontrolled diabetes [25,36–38]. In

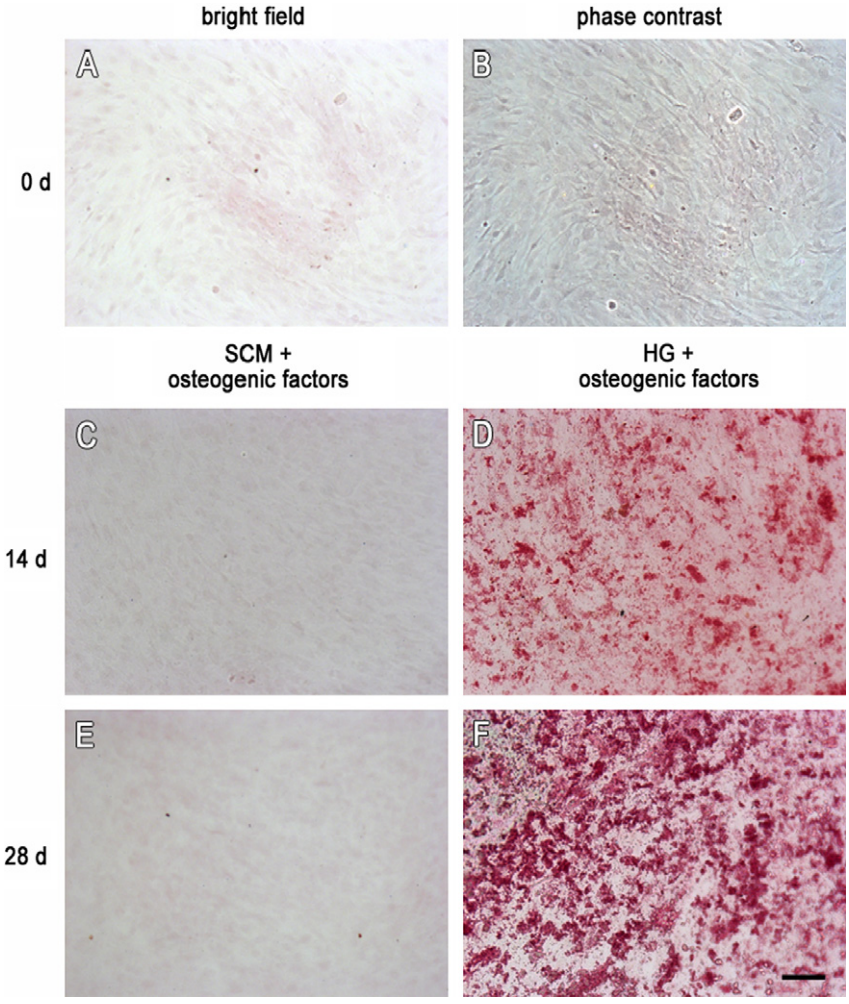

Fig. 4. Alizarin red staining of osteogenic differentiated hMSC. Cells were cultivated in SCM and high glucose medium (HG) containing osteogenic factors for 14 days (middle panel) and 28 day (lower panel) and mineralization was visualized by alizarin red staining. As a control the monolayer was stained at day 0, which was photographed in bright field and phase contrast (upper panel).

contrast it was reported that patients with early diabetes displayed a higher bone mass compared to controls [39].

In summary, we show here that high glucose stimulates Txnip in MSC and creates a proapoptotic situation, which is in theory apt to reduce the functionality and regenerative capacity of MSC and their offspring after chronic exposure. During short- and long-term exposure up to 4 weeks MSC display remarkable resistance against glucose concentrations as high as 40 mM. Glucose levels in culture, which are concordant with postprandial levels, even significantly enhance osteogenic differentiation *in vitro* when compared to fasting levels. If MSC lose regenerative capacity after months and years of weakly controlled diabetes remains to be demonstrated in *ex vivo* cultures of diabetic patients.

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