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Class I and II Histone Deacetylase Inhibitor LBH589 Promotes Endocrine Differentiation in Bone Marrow Derived Human Mesenchymal Stem Cells and Suppresses Uncontrolled Proliferation

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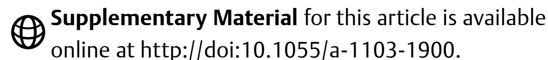

ABSTRACT

Mesenchymal stem cells are useful tools employed in clinical and preclinical medicine. Their beneficial potential in especially degenerative as well as autoimmune diseases is a constant focus of research. Regarding diabetes mellitus, transplantation of stem cells is seen as a possible therapeutic approach to overcome the loss of endocrine pancreatic cells. It was reported that co-transplantation of mesenchymal stem cells with pancreatic islet cells improves function and survival of the graft. However, these multipotent progenitors may be able to form tumors, especially under immunosuppressed conditions. Histone deacetylase inhibitors might offer the potential to overcome this issue. These small molecules can induce cell differentiation and control proliferation. Their potential to control lineage development of stem cells has been distinctly demonstrated in the treatment of cancer, mainly in hematopoietic neoplasias.

In this study, we demonstrate that human bone marrow-derived mesenchymal stem cells exhibit low carcinogenic potential in an immunosuppressed condition *in vivo*. Further, the effect of histone deacetylase inhibitors LBH589, MS-275, and MGCD0103 was examined after normalizing histone deacetylase activities in culture. Interestingly, transcripts of insulin gene enhancer protein and paired-box-gene 6, two markers of pancreatic endocrine differentiation were constitutively expressed in the cell line. The broad spectrum inhibitor of class I and class II histone deacetylases LBH589 upregulated the expression of these transcription factors in a significant way, whereas addition of selective class I histone deacetylase inhibitors MS-275 and MGCD0103 did not result in significant changes in gene expression.

In conclusion, we deliver evidence that a combined class I and II histone deacetylase inhibition is able to modulate the transcripts of differentiation markers of mesenchymal stem cells. The treatment holds the capability to facilitate endocrine differentiation in future approaches to replace endocrine cells by stem cell therapy.

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Introduction

Mesenchymal stem cells are multipotent precursor cells characterized by the ability to differentiate into adipocytes, chondrocytes and osteoblasts [1]. Furthermore, transformation into cells originating from other germ layers has been described [2–4], such as neuronal cells and insulin- or glucagon-expressing cells [5, 6].

Combined with their immunomodulatory properties, mesenchymal stem cells (MSC) offer great potential for the use in transplantation and regenerative medicine. Recently, it has been shown

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that the co-transplantation of MSC improves the function of transplanted pancreatic islet cells in diabetic mice [7–10]. They facilitated beta-cell proliferation and improved blood glucose levels compared to recipients of islet cell transplants only [9].

Histone deacetylase inhibitors (HDACi) have been reported to induce cell differentiation, inhibit cell growth, and enhance apoptosis in stem cells via epigenetic changes to DNA structure [11, 12]. Various epigenetic DNA modifications are known, such as methylation, phosphorylation, ubiquitylation or acetylation. By removing acetyl groups from the lysine moieties of histones, histone deacetylases (HDACs) tighten the DNA structure and lead to repressed gene expression. In humans, four different classes of HDACs are distinguished. Class I HDACs (HDAC 1, 2, 3, 8) are mainly located in the nucleus and are expressed ubiquitously. Class II HDACS (II a: HDAC 4, 5, 7, 9; II b: HDAC 6, 9) are expressed in a tissue-specific manner and shuttle between the nucleus and the cytoplasm. Class III HDACs function via a zinc ion in the catalytic domain and class IV of the HDAC family consists of only HDAC 11, [13, 14].

By inhibiting these enzymes, HDACi preserve an open DNA structure, leading to increased gene expression [11–13, 15]. Panobinostat (LBH-589) is a broad-spectrum inhibitor, which inhibits HDACs of class I as well as class II. Entinostat (MS-275) and mocetinostat (MGCD0103) are specific for class I HDACs [16–18].

Currently, HDACi are employed as an approach for the treatment of cancer, especially in hematological diseases [19, 20]. LBH589 is already in medical practice for therapy of multiple myeloma [21]. Other inhibitors offer promising effects in preclinical trials for a variety of neoplastic diseases like myelodysplastic syndromes, cutaneous T-cell lymphoma, breast cancer or B-cell lymphoma. They have been shown to be safe for application in patients [22–24]. Furthermore, as enhancers of epigenetic signals, HDACi have been applied to modulate cell differentiation *in vitro* [25–28]. In this work, we examined the effects of LBH589 and class I-specific HDACi MS-275 and MGCD103 on gene transcription in human mesenchymal bone-marrow derived stem cells. To analyze changes in cell fate, the transcription factors paired box 6 (Pax6) and insulin gene enhancer binding protein (Isl1), two important factors required for endocrine lineage development, as well as markers of multipotency, i. e. cell surface antigen CD90 (Thy-1) and stem cell factor (SCF) were selected as targets [1, 29–31]. The induction of therapeutic effects by agents modifying cell differentiation was demonstrated in a series of clinical trials [22, 32–34]. We hypothesized that HDACi might enhance the utilization of MSC in pancreatic islet cell transplantation. Therefore, MSC were cultivated in the presence of HDACi to examine a gene profile characteristic of pancreatic beta-cells. In this regard, another aim of this study was to examine the safety of MSC as it was reported that MSC induce tumor formation in experiments with immunocompromised mice [35–37].

Materials and Methods

Cell culture conditions, verification and s.c. injection of human MSC into nude mice

A human bone marrow-derived MSC line, immortalized with telomerase (hMSC -TERT), was provided by Prof. Dr. Peter Czermak (THM, Giessen, Germany). To verify their mesenchymal origin, we analyzed constitutive surface antigens using flow cytometry (FACS Canto™). Cells were washed with PBS and incubated with antibodies against CD73, CD90, and CD105 (positive cocktail, BD Stemflow™ hMSC Analysis Kit, BD Biosciences, USA) as well as antibodies against CD11b, CD19, CD34, CD45, and HLA-DR (negative cocktail, BD Biosciences).

For subcutaneous transplantation into 8–10 weeks old male NMRI nude mice (Janvier laboratory, France), hMSC TERT were grown at a density of 5×10^4 cells/ cm^2 in RPMI (Roswell Park Memorial Institute) media with 10 % FBS (foetal bovine serum), 1 % L-glutamine, and 1 % penicillin-streptomycin. As control, PANC1 cells, a human pancreatic tumor cell line, were grown in DMEM media with 10 % FBS and 1 % penicillin-streptomycin at 37 °C with 5 % CO_2 in incubator. Cells were harvested after 70–80 % confluency. Then, 0.5×10^6 PANC1 and hMSC-TERT were resuspended in 100 µL of Minimum Essential Medium (MEM) without supplements and transplanted subcutaneously in the flank of NMRI nude mice, respectively. Tumor size was measured with a Vernier calliper thrice a week up to 42 days. The mouse experiment was approved by the local Animal Welfare Committee under the code 31/2017 and conducted according to German animal welfare law.

For exposure to histone deacetylase inhibitors, hMSC TERT were cultured in MEM (Life Technologies, Darmstadt, Germany) with 11 % fetal calf serum, 1 % L-glutamine, and 1 % penicillin/streptomycin. After three passages, cells were stimulated with 20 nM LBH589, 1.5 µM MS-275 and 1.7 µM MGCD0103 for five days, each experiment was performed six times.

HDAC activity assay

hMSC-TERT cells were incubated with HDAC inhibitors *in vivo* over a period of up to 20 days, and were lysated at different timepoints (5, 15, 30, and 60 min; 6, 12, 24, and 48 h; 5, 10, 15, and 20 days) before starting activity assay. Concentrations of HDAC inhibitors were chosen based on IC50 values obtained from manufacturers' information (Selleck Chemicals, Houston, TX, USA). hMSC-TERT cell lysates were labelled with Boc-Lys(Ac)-AMC (I-1875, BACHEM, Bubendorf, Schweiz) to detect deacetylation activity. Deacetylated Boc-Lys-AMC is cleaved by trypsin to give fluorescent 7-amino-4-methylcoumarin (AMC). Using black 96-well microplate (Greiner, Frickenhausen, Germany) 150 µM Boc-Lys(Ac)-AMC substrate solution was added to 20 µg lysate and diluted with distilled water to a final volume of 100 µl/well. Wells were incubated for 35 min at 37 °C and 50 µl reaction solution was added consisting of trypsin (1.5 mg/ml, trypsin-EDTA 1X, 0.05 % Life technologies, Darmstadt), NP-40 buffer (10 v/v, from USB, Swampscott, USA), and doubly-distilled water (90 v/v). The incubation time was ten hours at 37 °C. Fluorescence was detected at 360/460 nm with a microplate reader (Mithras LB940). Experiments were performed three times for each inhibitor.

RT-PCR

Total RNA from untreated hMSC-TERT cells was isolated with an RNeasy Plus Micro Kit (Qiagen, Düsseldorf, Germany) and complementary DNA was produced with superscript III reverse transcriptase (Invitrogen, Darmstadt, Germany). DNA derived from the PCR reactions was sequenced using 1.75 % agarose gel electrophoresis. The following primers were used:

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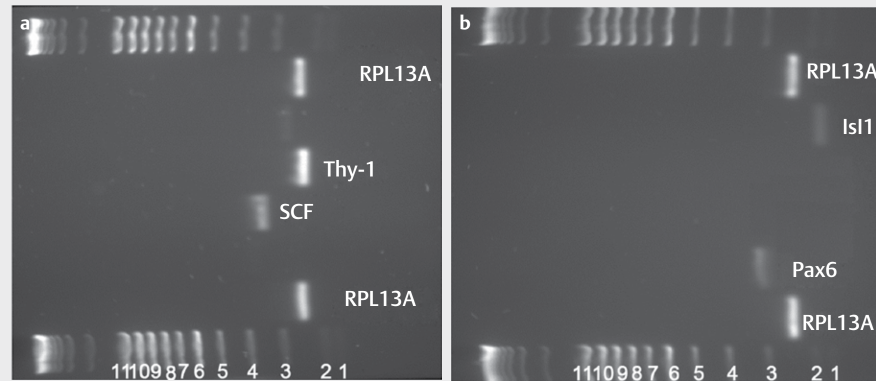
▶ **Table 1** Primers used for PCR. RPL13A = ribosomal protein L13a, Isl1 = insulin-gene-enhancer binding Protein, Pax6 = Paired box 6, Thy-1 = thymocyte antigen 1, SCF = stem cell factor.

gene	accession	size/bp	forward primer	reverse primer
RPL13A	NM_012423	126	cctggaggagaagaggaaagaga	ttgaggacctctgtgtatttgtcaa
Isl1	NM_002202.2	75	caactggtcaatttttcagaagga	ttgagaggacattgatgctacttcac
Pax6	NM_000280.4	187	ttgcagcctacattccctga	gggtctccaaagtctctgct
Thy-1	NM_001311160	112	atgaaggtcctctacttatccgc	gcactgtgacgttctggga
SCF	M59964.1	222	ggtggcaaatcttccaaaa	tctttcacgcactccacaag



▶ **Fig. 1** Agarose gel electrophoresis. **a** stem cell markers *Thy-1* (112 bp) and SCF (222 bp). **b** transcription factors *Isl1* (75 bp) und *Pax6* (187 bp). *RPL13A* (126 bp) was used as a housekeeping gene. DNA ladder: 1 = 80 bp, 2 = 100 bp, 3 = 200 bp, 4 = 300 bp, 5 = 400 bp, 6 = 500 bp, 7 = 600 bp, 8 = 700 bp, 9 = 800 bp, 10 = 900 bp, 11 = 1031 bp. bp = base pairs.

Total RNA from hMSC-TERT cells after 6 h, 24 h, 48 h and 120 h of treatment with LBH589 (20 nM), MS-275 (1.5 µM), or MGCD0103 (1.66 µM) was isolated according to the manufacturer's instructions (RNeasy Plus Micro Kit, Qiagen, Düsseldorf, Germany). The primers used for real-time PCR are shown in ▶ **Table 1**. Real-time PCR analysis was performed with a StepOnePlus PCR system (Applied Biosystems, Darmstadt, Germany) six times for each inhibitor. SYBRGreen (Applied Biosystems, Darmstadt, Germany) was used for the detection of fluorescent signals. The samples were run in triplicate, and quantification was performed by the ΔΔCT method with normalisation to *RPL13A*.

Significance was tested by the Kruskal-Wallis test and Dunn's multiple comparison test as well as the Kolmogorov-Smirnov test. Values of $p < 0.05$ were considered as statistically significant. Data are presented as mean ± SEM.

Results

The hMSC-TERT cell line is a multipotent stem cell of mesenchymal origin

Referring to the International Society for Cellular Therapy, the expression of the surface antigens CD73, CD90, and CD105 is mandatory for mesenchymal stem cells, while markers of hematopoietic origin must not be expressed [1]. Using flow cytometry, we confirmed the mesenchymal origin of the hMSC-TERT cell line. 99.1 % of the cell population expressed CD73, 99 % were positive for CD90, and 79.1 % expressed CD105. Typical markers of hematopoietic precursor cells were found in fewer than 2 % of the tested cell population.To assess the potency of the hMSC-TERT cells, RT-PCR for typical markers of multipotency was performed. Cells expressed Thy-1 and SCF, confirming multipotent stem cell capacity (▶ **Fig. 1a**). Interestingly, we also found constitutive expression of Isl1 and Pax6, two important transcription factors in endocrine and neuronal lineage (▶ **Fig. 1b**).

The hMSC-TERT cell line holds no tumorigenic potential

The multipotent nature of MSC raises concerns about a carcinogenic potential in the immunosuppressed recipient. Therefore, we investigated the tumor-forming ability of the hMSC-TERT cell line upon subcutaneous transplantation to nude mice. During the observation period of 42 days following transplantation of 0.5×10^6 hMSC-TERT cells, recipient mice exhibited no manifestation of tumor growth at the transplantation site. By contrast, a considerable tumescence formed 15 days after the transplantation of PANC1 cells and increased in volume up to the end of the observation time as shown in ▶ **Fig. 2a, b**. No significant differences were observed in body weight between the two transplanted groups (▶ **Fig. 2c**).

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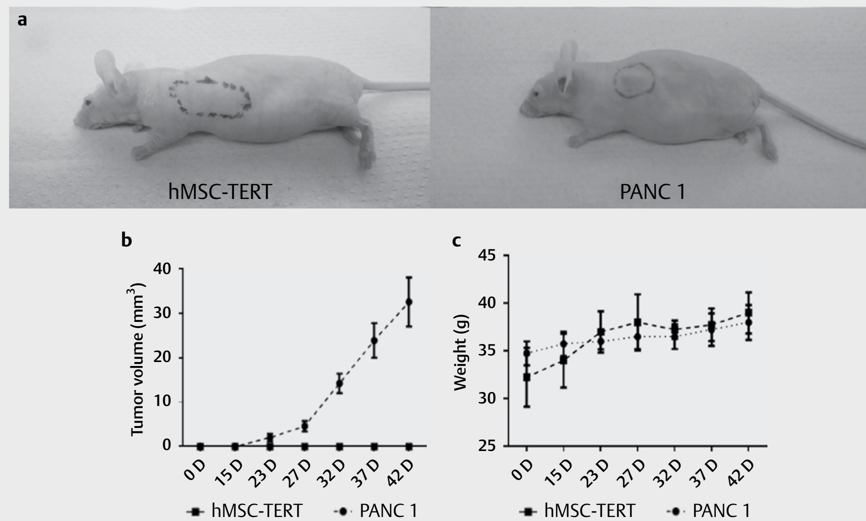

▶ **Fig. 2** Tumor formation in the NMRI nude mouse model **a** Sample images of tumor formation after 42 days; hMSC-TERT is a mesenchymal stem cell line and PANC 1 is a cell line derived from adenocarcinoma of the pancreas, **b** and **c** represent tumor volume and body weight. n = 7.

Class I and II HDACi inhibit HDAC enzymes and reduce proliferation of hMSC-TERT cells

LBH589, MS-275 and MGCD0103 were examined for inhibition of HDAC enzymes in hMSC-TERT by quantitative HDAC activity assay. Cells were treated *in vivo* with inhibitors in different concentrations. High fluorescence signals correlated with a strong activity of HDAC enzymes, suppressed signals indicated HDAC inhibition (▶ **Fig. 3a**).

LBH589 showed a significant decrease in fluorescence intensity from six hours of incubation up to five days in a concentration of 20 nM (▶ **Fig. 3b**). Higher concentrations (50 nM, 100 nM) lead to cell death starting between day two and five of cell culture. 2 nM and 5 nM, respectively, resulted in fluorescence signals similar to untreated cells (0 nM) suggesting insufficient inhibition of HDAC enzymes (▶ **Fig. 3a**). LBH589 was used in a concentration of 20 nM for further experiments. The same protocol was used to examine MS-275 (0.2, 0.5, 1.5, 3, and 6 µM) and MGCD0103 (0.075, 0.15, 0.29, 1.66, and 5 µM). For MS-275 a concentration of 1.5 µM lead to HDAC inhibition without cytotoxic effect over 20 days of cell culture. MGCD0103 showed these characteristics in a concentration of 1.66 µM (Suppl. ▶ **Fig. 2S**).

Concomitant with the reduced activity, the prolonged exposure to all three HDACis resulted in a significantly decreased proliferation of the hMSC-TERT cells. After five days, the untreated cells had grown to near complete confluency (▶ **Fig. 4a**), while the treated cells only presented a confluency of 50 % by inspection (▶ **Fig. 4b**).

LBH589 favors endocrine differentiation by increasing the expression of Isl1 and Pax6

To examine potential transcriptional regulation of HDACis in MSC, cells were cultured for up to five days in the presence of LBH589, MS-275, or MGCD0103. After 0, 6, 24, 48, and 120 h, the expression of *Thy-1*, *SCF*, *Isl1*, and *Pax6* was analyzed. None of the class I-specific HDACi had a significant impact on their expression (**Suppl.** ▶ **Fig. 1S**). The expression levels of *SCF* and *Thy-1* were also not modulated (▶ **Fig. 5a, b**). However, a significant increase in *Pax6* was detected after 48 and 120 h of treatment with LBH589 (▶ **Fig. 5c**). Furthermore, *Isl1* was upregulated in a significant way after 6 h and 120 h (▶ **Fig. 5d**).

Discussion

Mesenchymal stem cells offer excellent potential for the treatment of degenerative and autoimmune diseases [38]. Nevertheless, these cells have been found to form malignant neoplasia after transplantation *in vivo* [35–37].

Diabetes mellitus is hallmarked by the loss of endocrine pancreatic cells due to autoimmunity or exhaustion. So far, no feasible and widely applicable replacement therapy exists in daily clinical practice. Therefore, the utilization of stem cells for islet cell restoration is a promising strategy. However, when employed *in vivo*, their differentiation has to be purposefully controlled and uncontrolled proliferation, i. e. tumorigenesis, must not occur.

To analyze the tumor forming potential of human bone-marrow-derived mesenchymal stem cells, 0.5×10^6 hMSC were transplanted into nude mice. After 42 days, no tumor formation was observed, whereas PANC1 control cells developed a palpable tumor mass in the transplantation area. Based on this experiment, human bone marrow-derived MSC seem to be safe under immunosuppressed conditions *in vivo*, at least in the observed period of time post-transplantation. Nevertheless, there are some groups that have observed tumor formation by MSC after transplantation [35–37]. Tang et al reported on a culture protocol with insulin-producing cells from human MSC that were able to ameliorate blood glucose levels in mice after transplantation. At 40–45 days post transplantation, tumorigenesis was observed, with a palpable tumor mass around the kidneys, characteristic of malignancy [39]. Therefore, it is important to exactly define culture conditions such as type, passage and number of cells intended for use in transplantation.

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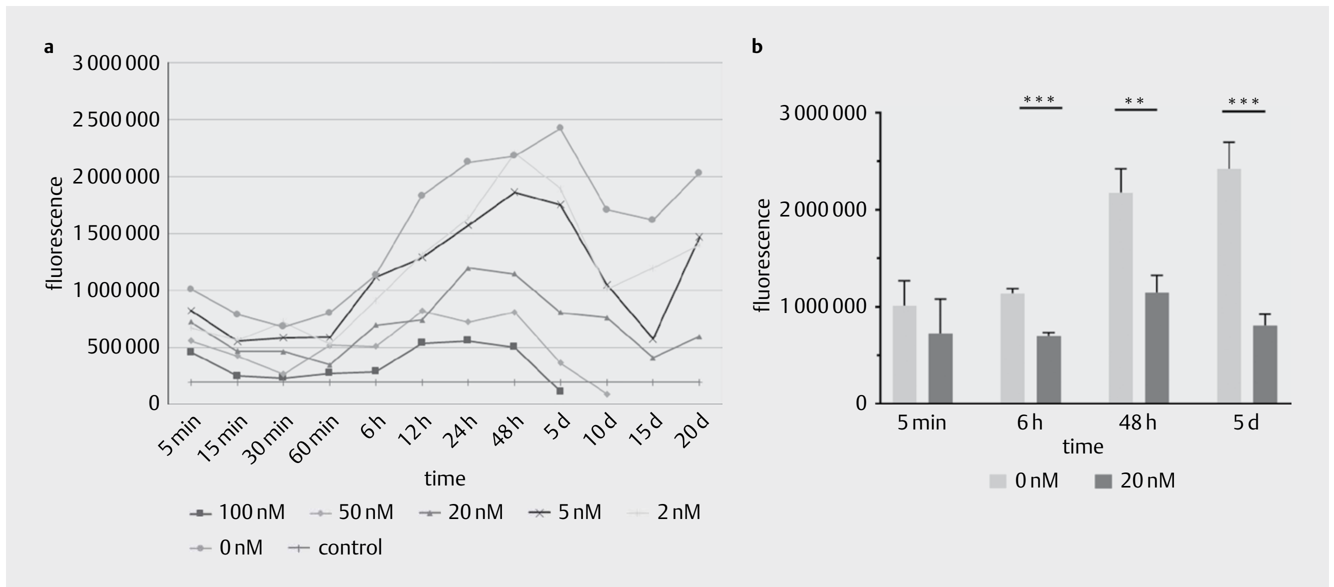
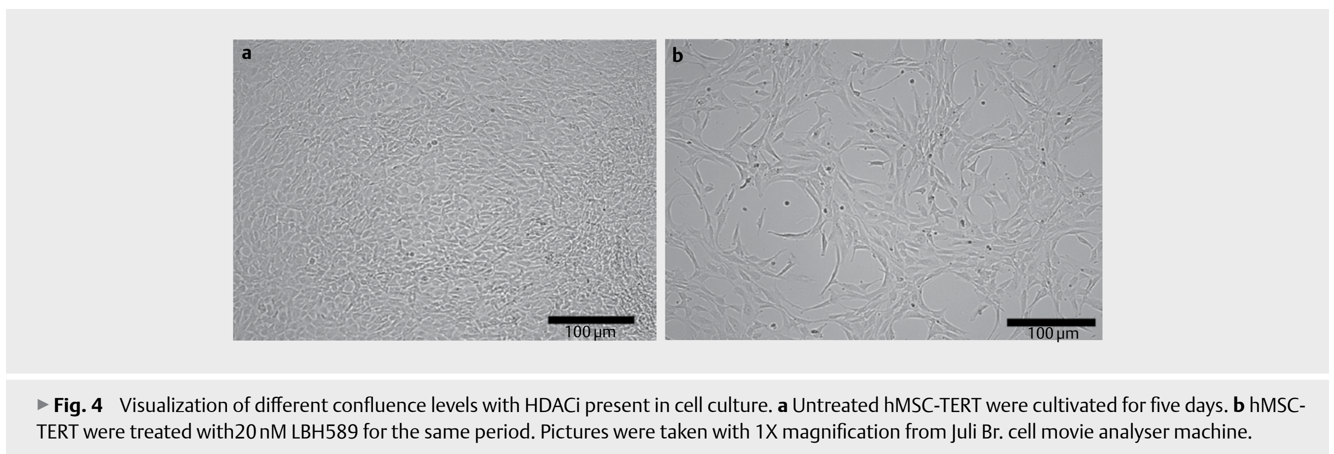


▶ **Fig. 3** **a** HDAC enzymatic activity assay. Fluorescence was measured in a time depended course, LBH589 was used in different concentrations (2 nM, 5 nM, 20 nM, 50 nM, 100 nM). Two controls were established; 0 nM cells were treated with dimethyl sulfoxide (DMSO) without LBH589, "control" cells were treated with a high dosage of LBH589 (3 mM) after cell culture was completed. In cells treated with 0 nM LBH589 highest fluorescence signal was expected, as HDAC enzymes were able to deacetylase boc-lys-(Ac)-AMC without inhibition, resulting in fluorescence emission. Cells treated with LBH589 after cell culture in a concentration of 3 mM (>>IC50), showed a reduced fluorescence signal. In cells treated with LBH589 2 nM and 5 nM, a fluorescence signal resembling 0 nM control was detected. A treatment with 50 nM and 100 nM lead to increased cell death in culture starting after 2 to 5 days. HDAC inhibition with LBH589 20 nM resulted in a reduced fluorescence signal over a period of 20 days. **b** Difference in fluorescence emission between cells treated with 20 nM LBH589 and 0 nM. After 6 h in cell culture, first significant reduction in fluorescence signal is detected, persisting over a period of 5 days. Data presented as mean ± SEM. Comparison was performed using unpaired t-test, test for Gauss distribution was performed using Shapiro-Wilk test. P-values < 0.05 were considered significant, P values are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Min = minutes; h = hours; d = days; nM = nanomolar. HDAC activity assay was performed in a modified version from Wegener et al. [54].



▶ **Fig. 4** Visualization of different confluence levels with HDACi present in cell culture. **a** Untreated hMSC-TERT were cultivated for five days. **b** hMSC-TERT were treated with20 nM LBH589 for the same period. Pictures were taken with 1X magnification from Juli Br. cell movie analyser machine.

Therapeutic induction of differentiation is a widely used tool in anticancer medicine, because it is associated with reduction of cell proliferation and tumor formation [20, 30–32]. HDACi are known to induce differentiation, mainly in hematooncologic diseases [22]. We suggest that HDACi treatment of MSC offers different useful features in transplantation medicine in general and in islet cell transplantation or primary transplantation of stem cells in particular. HDACi are small molecules, widespread in clinical and preclinical use for malignant haemato-oncologic diseases. They stop tumor proliferation by inducing apoptosis and cell differentiation *in vivo* [40, 41]. They are also used as supportive factors in culture to guide cell fate by the induction of gene expression [27, 28, 42–44]. In this work, we wanted to investigate the effect of HDACi LBH589, MS-275, and MGCD0103 on the differentiation of human bone-marrow derived MSC. LBH589 is a broad spectrum HDACi with inhibitory activity against nearly all HDAC enzymes of class I, II and IV. In contrast, MS-275 and MGCD0103 are highly specific for Class I HDACs [16–18].

We analyzed the gene expression of two important transcription factors of endocrine and neuronal cell fate Isl1 and Pax6, which

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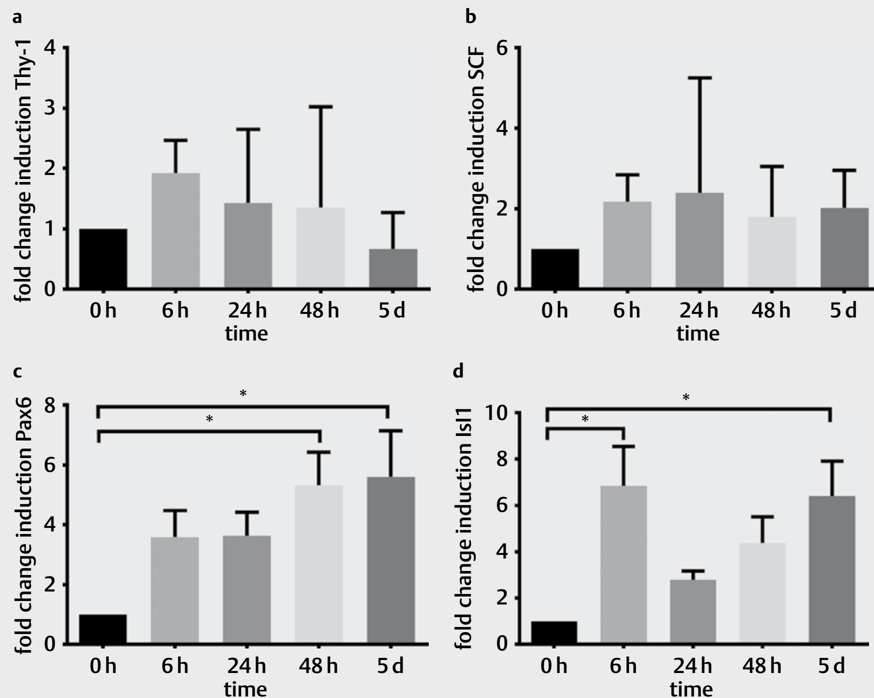

▶ **Fig. 5** Real-time PCR analysis of time dependent change in gene expression under treatment with LBH589. Human bone marrow-derived mesenchymal stem cells were treated with LBH589 20 nM over five days in cell culture. Expression of stem cell markers *Thy-1* **a** and *SCF* **b** as well as expression of transcription factors *Pax6* **c** and *Isl1* **d** was measured. *Thy-1* and *SCF* did not show any significant changes over five days of treatment. *Pax6*, a neuronal/ endocrine transcription factor, was upregulated in a significant way after two and five days. Likewise, a significant upregulation was observed in the expression of *Isl1* after six hours and five days.

were found to be constitutively expressed in this MSC line [30, 32]. After 48 and 120 h, the expression of Pax6 increased in a significant way with LBH589-treatment. Also, Isl1 expression was significantly upregulated. None of the class I-specific HDACi were able to induce a change in gene expression, suggesting that the inhibition of class II HDACs might be important in the transformation of mesenchymal stem cells into the endodermal and beta-cell pancreatic lineage.

LBH589 enhanced the expression of the essential beta-cell lineage transcription factors *Isl1* and *Pax6*. Pre-treatment of MSC with HADCi before transplantation may provide beneficial results *in vivo*. Further experiments are needed to investigate the application of broad-spectrum as well as class II-specific HDACi in stem cell treatment and transplantation.

Although the transcription factors *Isl1* and *Pax6* were upregulated by LBH589, no changes in the expression of *SCF* and *Thy-1* were observed, suggesting that final differentiation did not occur, and the cells maintained an oligopotent state. The presence of HDACi was not sufficient to complete the differentiation process into pancreatic beta-cells in culture. Instead, they pushed MSC towards an intermediate level of transition with a reduced proliferation rate and thereby reduced the risk of tumor formation *in vivo*.

Other groups also have reported on the effects of class II HDACs in cell differentiation and proliferation [45]**.** For example, Lenoir et al. were able to show that HDAC 4, 5, and 9 modulate murine pancreas ontogeny. Interestingly, *HDAC5*$^{-/-}$ and *HDAC9*$^{-/-}$ mutant mice presented an increased mass of beta-cells. Furthermore, treatment with MC1568, a Class II specific HDACi, led to the upregulation of *Pax4* expression, a key transcription factor for the transition of pancreatic endocrine cells [50]. These results agree with our data, which point to the impact of class II HDACs on human pancreatic and neuronal morphogenesis. Recently, new HDACi have been developed that are highly specific for class II HDAC enzymes. By using these specific drugs, side effects might be reduced, and stronger induction of differentiation might be achieved. Further experiments on human stem cells are needed to elucidate the benefit of class II-specific HDACi [51–53].

Recently, Tanaka et al. added adipose-tissue derived mesenchymal stem cells to pancreatic islets transplanted into diabetic C57BL/6 mice. Compared to islet cells alone, the co-transplantation of MSC resulted in beta-cell proliferation in the graft and optimal blood glucose control [9]. This experiment indicates the potential of MSC in clinical pancreatic islet transplantation. Meanwhile, two more groups have confirmed the beneficial effect of MSC co-transplanted with pancreatic islets [7, 8].

Conclusion

Taken together, these results show that LBH589 increased the expression of beta-cell lineage factors *Isl1* and *Pax6* in cultivated MSC,

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which makes this compound potentially applicable in cell replacement therapy for diabetes mellitus. Furthermore, these cells are safe under immunosuppressed conditions over 42 days *in vivo*. To increase the safety of MSC in therapeutic applications, the broad-spectrum HDACi LBH589 may be preferred over inhibitors directed against specific classes of HDAC. This could drive MSC towards beta-cell transition and thereby prevent them from dedifferentiation and thus malignant tumor formation.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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