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5-Azacytidine Improves the Osteogenic Differentiation Potential of Aged Human Adipose-Derived Mesenchymal Stem Cells by DNA Demethylation

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Abstract

The therapeutic value of adipose-derived mesenchymal stem cells (Ad-MSCs) for bone regeneration is critically discussed. A possible reason for reduced osteogenic potential may be an age-related deterioration of the Ad-MSCs. In long term in vitro culture, epigenomic changes in DNA methylation are known to cause gene silencing, affecting stem cell growth as well as the differentiation potential. In this study, we observed an age-related decline in proliferation of primary human Ad-MSCs. Decreased Nanog, Oct4 and Lin28A and increased Sox2 gene-expression was accompanied by an impaired osteogenic differentiation potential of Ad-MSCs isolated from old donors (>60 a) as compared to Ad-MSCs isolated from younger donors (<45 a). 5-hydroxymethylcytosine (5 hmC) and 5-methylcytosine (5 mC) distribution as well as TET gene expression were evaluated to assess the evidence of active DNA demethylation. We observed a decrease of 5 hmC in Ad-MSCs from older donors. Incubation of these cells with 5-Azacytidine induced proliferation and improved the osteogenic differentiation potential in these cells. The increase in AP activity and matrix mineralization was associated with an increased presence of 5 hmC as well as with an increased TET2 and TET3 gene expression. Our data show, for the first time, a decrease of DNA hydroxymethylation in Ad-MSCs which correlates with donor-age and that treatment with 5-Azacytidine provides an approach which could be used to rejuvenate Ad-MSCs from aged donors.

Introduction

Extensive bone loss after trauma or diseases often results in delayed or impaired bone healing [1]. Deteriorated bone regeneration and repair is observed with increasing age of patients [1,2]. The use of mesenchymal stem cells (MSCs) in tissue engineering has great potential for a novel approach in acute bone and cartilage repair, e.g., some pioneer clinical study have already entered the phase III stage [3].

MSCs are one kind of adult stem cells. In the mechanism of osteogenesis, MSCs are considered to be one type of progenitor cells, which are able to proliferate and later on differentiate into osteogenic cells [4]. Hence, the process of bone regeneration requires the recruitment, expansion and differentiation of MSCs [5]. In vitro experiments demonstrated an active self-renewal capacity and multi-lineage differentiation potential of MSCs [6,7]. MSCs can be isolated from various tissues; most frequently from adipose tissue and bone marrow. Due to their easy access, low immune rejection as well as a low risk of tumorigenesis [8,9], MSCs derived from adipose tissue (Ad-MSCs) could be an ideal source for patient-specific cell therapy. However, the osteogenic differentiation potential of these Ad-MSCs has been critically discussed. Furthermore, it has been reported many times that adult stem cells, including MSCs, suffer from a decline in stem cell function with increasing age during long term culture of the cells [10,11]. The decline observed in the self-renewal capacity, resulted in an incomplete differentiation into the committed cell lineage [12].

Epigenetic modification of the genome is considered to be one of the most important regulatory pathways affecting stem cell aging. These dynamic changes mainly appear in DNA methylation and/or chromatin remodeling [13]. Although selected histone-deacetylase inhibitors could improve osteogenic function in differentiated MSCs, they are not suitable for use because of their negative effects on cell proliferation due to DNA damage and cell-cycle inhibition [14]. Efforts have been made to investigate a
milder epigenetic modification approach. It has been demonstrated that DNA demethylation can be induced by 5-Azacytidine, a DNA methyltransferase (DNMT) inhibitor [15]. In our hands 5-Azacytidine treatment improved the hepatic differentiation capability of Ad-MSCs, in correlation to the DNA demethylation [16]. Just recently an active DNA-demethylation mechanism was described in embryonic stem cells. In these cells induction of TET gene expression promoted the conversion of nuclear 5-methylcytosine (5 mC) to 5-hydroxymethylcytosine (5 hmC) and thus DNA demethylation, in order to maintain the self-renewal capacity and the pluripotency state [17].

Thus, aim of the present study was to investigate donor-age-related changes in the self-renewal of Ad-MSCs as well as their osteogenic differentiation potential and which role DNA methylation plays in this process. Furthermore, we want to investigate if these donor-age-related changes can be reversed by epigenetic modulation. We mainly focused on osteogenic differentiation potential and which role DNA methylation plays in this process. Furthermore, we want to investigate if these donor-age-related changes can be reversed by epigenetic modifications of the DNA. The stem cell capacity will be assessed by measuring the expression levels of transcription factors characteristic for embryonic and induced pluripotent stem cells, namely, Sox2, Lin28A, Oct4 and Nanog. Cell proliferation will be determined by the amount of cells stained positive for Ki67. The osteogenic differentiation potential will be defined by measuring the AP-Activity and matrix mineralization after differentiation, as well as by measuring the expression levels of the osteogenic marker gene osteocalcin and the osteogenic transcription factors Runx2 (early) and osterix (late) [18].

Materials and Methods

Cell culture plastics, collagenase II, phosphate buffered saline (PBS), fetal calf serum (FCS), DMEM medium and cell culture supplements were purchased from PAA Laboratories GmbH (Pasching, Austria). GenJET RNA Purification Kit, DNase I (RNase-free) and First Strand cDNA Synthesis Kit were purchased from Fermentas (Ontario, Canada). Oct-4A (C30A3) Rabbit mAB, Sox2 (D6D9) Rabbit mAB, Nanog (D73G4) Rabbit mAB, Lin28A (D84C11) Rabbit mAB and the corresponding secondary anti-rabbit IgG, HRP linked Antibody were purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Chemicals as well as the Anti-TET2, Anti-TET3 and Anti-Oct4A (C30A3) Rabbit mAB, Sox2 (D6D9) Rabbit mAB, Nanog (D73G4) Rabbit mAB, Lin28A (D84C11) Rabbit mAB and the corresponding secondary anti-rabbit IgG, HRP linked Antibody were purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Chemicals as well as the Anti-TET2, Anti-TET3 and Anti-Oct4A (C30A3) Rabbit mAB, Sox2 (D6D9) Rabbit mAB, Nanog (D73G4) Rabbit mAB, Lin28A (D84C11) Rabbit mAB and the corresponding secondary anti-rabbit IgG, HRP linked Antibody were purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Chemicals as well as the Anti-TET2, Anti-TET3 and Anti-Oct4A (C30A3) Rabbit mAB, Sox2 (D6D9) Rabbit mAB, Nanog (D73G4) Rabbit mAB, Lin28A (D84C11) Rabbit mAB and the corresponding secondary anti-rabbit IgG, HRP linked Antibody were purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Chemicals as well as the Anti-TET2, Anti-TET3 and Anti-Oct4A (C30A3) Rabbit mAB, Sox2 (D6D9) Rabbit mAB, Nanog (D73G4) Rabbit mAB, Lin28A (D84C11) Rabbit mAB and the corresponding secondary anti-rabbit IgG, HRP linked Antibody were purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Chemicals as well as the Anti-TET2, Anti-TET3 and Anti-Oct4A (C30A3) Rabbit mAB, Sox2 (D6D9) Rabbit mAB, Nanog (D73G4) Rabbit mAB, Lin28A (D84C11) Rabbit mAB and the corresponding secondary anti-rabbit IgG, HRP linked Antibody were purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany).

**Table 1.** Sequence of primers used in qRT-PCR.

<table>
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<tr>
<th>gene bank ID</th>
<th>Forward primer (5’-&gt;3’)</th>
<th>reverse primer (5’-&gt;3’)</th>
<th>product length</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;</th>
<th># of cycles</th>
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<tr>
<td>Runx2</td>
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<td>TTCCTAGGCAGCATTTCAGGTGC</td>
<td>359 bp</td>
<td>58°C</td>
<td>30*</td>
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<td>Osterix</td>
<td>NM_152860</td>
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<td>62°C</td>
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<td>Osteocalcin</td>
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</tr>
<tr>
<td>TET3</td>
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<td>CAGAAAGCTGTAGCTGCTAT</td>
<td>263 bp</td>
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</tr>
<tr>
<td>Nanog</td>
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<tr>
<td>Oct4</td>
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<td>180 bp</td>
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<td>359 bp</td>
<td>58°C</td>
<td>30*</td>
</tr>
</tbody>
</table>

*PCR conditions were optimized with varying template concentrations to be in the quantifiable/linear range of the PCR.

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Isolation, Expansion and Characterization of Ad-MSCs

Ad-MSCs were isolated from adipose tissue obtained from surgery with the written consent of the patients. This study was specifically approved by the ethical committee of the University hospital Tübingen (Reference: 385/2012 B02) who works in accordance with national regulations and the ICH-GCP guidelines. The study was performed according to the declaration of Helsinki in its newest version. For this study adipose tissue from 23 donors was collected and isolated. Adipose tissue was minced and washed extensively with PBS before digestion with collagenase II (0.075% in PBS) at 37°C for 30 to 60 min. The digestion was stopped with culture medium (DMEM, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin). After centrifugation (600 g, 10 min) the cell pellet was collected, re-suspended and filtered through a 70 μM cell strainer. The cells were seeded in flasks which culture medium. To remove non-attached cells the Ad-MSCs were washed once with PBS 24 hours after isolation. Ad-MSCs were expanded in culture medium until passage 3 in order to minimize the number of contaminating cells in the culture. Purity of the Ad-MSCs was determined by flow cytometry as recently published by our group (CD90<sup>+</sup>, CD105<sup>+</sup>, CD14<sup>+</sup>, CD45<sup>+</sup>) [16].

Pre-treatment of Ad-MSCs with 5-Azacytidine

Ad-MSCs from young (<45 a) and old (≥60 a) donors were seeded at a cell density of 10,000 cells/cm². After cell attachment the medium was changed to freshly made culture medium containing 5-Azacytidine (5 μM and 20 μM). After 24 h the 5-Azacytidine containing culture medium was refreshed for another 24 h (total pre-treatment: 48 h).

Osteogenic Differentiation of Ad-MSCs

For osteogenic differentiation Ad-MSCs from young and old donors were incubated with osteogenic differentiation medium (DMEM, 0.5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM L-Ascorbic Acid-2-phosphate, 10 mM β-glycerol phosphate, 25 mM HEPES, 1.5 mM CaCl₂, 5 μM cholecalciferol)
Figure 1. Declined Proliferation and Osteogenic Differentiation in Ad-MSCs from old donors: Ki67 staining was performed as proliferation assay. (A) Representative image of a merged immunofluorescent staining for Ki67 (red) and nuclei (blue) from young and old Ad-MSCs. (B) Quantification of Ki67-positive Ad-MSCs (N = 3, n = 5/age group) points to a reduced proliferation in aged Ad-MSCs. After 14 days of osteogenic differentiation of young and old Ad-MSCs, osteoblasts function was assessed by measuring (C) AP activity (each group: N = 7, n = 3), (D–G) matrix mineralization and (H–J) gene expression changes. Matrix mineralization was determined by (D & F) von Kossa (each group: N = 5, n = 3) and (E & G) Alizarin red staining (each group: N = 6, n = 3). mRNA levels of the osteogenic transcription factors (H) Runx2 and (I) Osterix, as well as the osteogenic marker (J) Osteocalcin were determined by semi-quantitative RT-PCR (each group: N = 5, n = 3). GAPDH was used for normalization. *p < 0.05, **p < 0.01, ***p < 0.001 (student’s t-test).

doi:10.1371/journal.pone.0090846.g001
for 14 days. The differentiation medium was changed every 3–4 days.

Alkaline Phosphatase (AP) Activity

In order to determine the AP activity, the differentiated cells were incubated with substrate solution (2 mg/ml 4-Nitrophenyl phosphate disodium salt hexahydrate, 50 mM glycine, 1 mM MgCl₂, 100 mM Tris, pH = 10.5) for 120 min. The subsequent 4-Nitrophenol production in the supernatant was measured photometrically at $λ = 405$ nm with a FLUOstar Omega Plate reader (BMG Labtech, Offenburg, Germany), and normalized to relative cell numbers measured by Sulforhodamine B Staining as described before [19,20].

Matrix Mineralization

Matrix mineralization was assessed by Alizarin red and von Kossa staining. For both stainings cells were fixed with 4% paraformaldehyde solution for 1 h at −20°C. For alizarin red staining cells were incubated with 0.5% Alizarin red S solution (pH = 4.0) for 20 min, then washed extensively with tap water to remove unbound stain. For von Kossa staining cells were incubated with 3% AgNO₃ solution for 30 min, then washed extensively with tap water, exposed to 5% sodium carbonate (10% formaldehyde) solution for 2 min, followed by an incubation in 5% sodium-thiosulfate solution for 5 min in order to develop color. For both staining methods the plates were first scanned to obtain an overview image, then images with the light microscope were taken. Alizarin red staining was quantified photometrically ($λ = 562$ nm) with a FLUOstar Omega Plate reader, after resolving the stain with 10% cetylpyridinium chloride solution. The von Kossa staining was quantified densitometrically (Pixel intensity) with the Image J 1.45 s software (National Institutes of Health, USA).

Immunofluorescence Staining

Ad-MSCs were plated onto cover slips and treated according to the experimental setup. Cells were fixed with 4% paraformaldehyde solution for 15 min at RT and then washed with PBS. For permeabilization, cells were incubated with 0.5% Triton-X-100 PBS solution for 30 min at RT. To denature the DNA cells were incubated with 4 M HCl for 15 min at RT. After washing with PBS unspecific binding sites were blocked with blocking buffer (10% FCS, 0.1% Tween-20 in PBS) for 1 h at RT. Then cells were incubated with primary antibody solution anti-5 hmC rabbit polyclonal IgG (Active Motif, CA, USA), anti-5 mC mouse monoclonal IgG, (Active Motif, CA, USA) or anti-Ki67 goat polyclonal IgG (Santa Cruz Biotechnology, CA, USA) diluted 1:100 in PBS solution containing 1% FCS and 0.1% Tween-20 for 2 h at RT. After washing three times with PBS cells were incubated with secondary antibody solution (ALEXA-fluor antibodies (Invitrogen, NY, USA) diluted 1:400 in PBS solution containing 1% FCS, 0.1% Tween-20 for 1 h at RT. Nuclei were counterstained by incubation with Hoechst 33342 solution (2 μg/
ml in PBS) for 20 min at RT. After a final washing step with PBS, the stained cells were mounted with mounting medium (Fluromount G, Southern Biotech, NJ, USA). Images of the staining were taken with an EVOS fluorescence microscope (AMG, USA) under standardized condition, processed and analyzed with ImageJ 1.45 s software [21].

Quantification of Gene Expression

Total RNA from Ad-MSCs was extracted with GeneJET RNA Purification Kit. 2–3 μg of the total RNA was digested with DNase I in order to remove remaining genomic DNA. cDNA was synthesized using the First Strand cDNA Synthesis Kit. Gene expression changes were analyzed either by conventional RT-PCR or by qRT-PCR depending on the amount of target mRNA present in the sample. PCR protocols were optimized for each gene separately to be in a quantifiable (linear) range in our samples.

For quantitative real-time PCR (qRT-PCR) a standardized amount of template cDNA was tested for the expression level of each target gene (primer sequences are listed in Table 1) using SYBR Green qPCR (Finnzym, Vantaa, Finland) and the Step One Plus Real-Time PCR System (Applied Biosystems, CA, USA). GAPDH was used as endogenous control. Relative changes in gene expression were calculated using the ΔΔCt Method with either untreated young cells (changes with age) or untreated age matched cells (changes due to 5-Azacytidine treatment) as reference.

For semi-quantitative (conventional) RT-PCR a standardized amount of template cDNA was tested for the expression level of each target gene (primer sequences are listed in Table 1) using KAPA Fast ready mix (Peqlab, Erlangen, Germany). Products, resolved by gel electrophoresis in a 2% (w/v) agarose gel, were visualized with ethidiumbromide. Densitometric analysis of signals was performed using ImageJ software (NIH, Bethesda, USA). Pixel Intensities of the PCR signal were normalized to GAPDH using a normalization factor (multiple of the average GAPDH signal). Relative changes in gene expression were calculated with either untreated young cells (changes with age) or untreated age matched cells (changes due to 5-Azacytidine treatment) as reference.

Western Blot

Cells were lysed in freshly prepared ice-cold RIPA buffer (50 mM TRIS, 250 mM NaCl, 2% Nonidet-P40, 2.5 mM EDTA, 0.1% SDS, 0.5% DOC, protease and phosphatase inhibitors, 100 μg/ml each). Labeled proteins were separated by electrophoresis on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked and incubated with the appropriate antibodies. Immunoreactive signals were visualized using enhanced chemiluminescence detection (Amersham). Densitometric analysis of bands was performed using ImageJ software (NIH, Bethesda, USA). Band intensities were normalized to GAPDH as an endogenous control.
Figure 4. 5-Azacytidine treatment increased TET2 and TET3 gene expression. Young and old Ad-MSCs were treated with 5 μM 5-Azacytidine for 48 h. mRNA levels of (A) TET2, (B) TET3, (E) Sox2, (F) Lin28A (2 donors from the young Ad-MSCs had to be excluded from the data set, due to artefacts in the melting curve), (G) Oct4 and (H) Nanog were determined by qRT-PCRs (N = 6, n = 3/age group). (I) Expression changes of TET2, TET3, Lin28A, Oct4, Nanog and Sox2 were confirmed on the protein level by Western blot analysis (Representative figure for N = 2/group). GAPDH was used for normalization. Data are presented as relative expression changes (log10) of 5-Azacytidine treated Ad-MSCs compared to the corresponding untreated Ad-MSCs. Distribution of 5-hydroxymethylcytosine (5 hmC) and 5-methylcytosine (5 mC) in the 5-Azacytidine treated and untreated Ad-MSCs was determined by immunofluorescent staining (N = 3, n = 5/age group). Cell nuclei were counterstained with Hoechst 33342. For quantification (D) 5 hmC and (E) 5 mC positive nuclei were counted. Data are represented as log10 (fold of control). *p<0.05, **p<0.01, ***p<0.001 as compared to untreated cells (student’s t-test).

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**Statistical Analysis**

Results are expressed as box blot (Whisker’s: min to max) or bar chart (mean ± SEM) of at least 3 independent experiments (biological replicates, N≥3) measured as triplicates or more (technical replicates, n≥3). The precise of replicates for each experiment is given in the figure legends. Individual data sets were compared by Student’s t test or one way ANOVA analysis. Significance was set as p<0.05 as the minimum level of significance.

**Results**

Ad-MSCs from Elderly Donors showed Impaired Proliferation and Osteogenic Differentiation Capacity

In order to assess the proliferation capacity of the cells a Ki67 staining was performed. Expression of Ki67 was significantly lower in Ad-MSCs isolated from aged donors as compared to Ad-MSCs isolated from younger donors (figure 1A & B), representing an impaired proliferation capacity of Ad-MSCs from elderly donors. In order to evaluate the differentiation potential, Ad-MSCs were differentiated for 14 days in the presence of osteogenic differentiation medium. As osteogenic markers alkaline phosphatase (AP) activity, matrix mineralization and relative gene expression of Runx2, osteon and osteocalcin were determined after the differentiation process. In comparison to Ad-MSCs from young donors, Ad-MSCs from old donors exhibited a significantly lower AP activity (figure 1C) and a significant weaker matrix mineralization, as determined by von Kossa (figure 1D & F) and Alizarin red staining (figure 1E & G). Increased expression of the early osteogenic transcription factor Runx2 and reduced expression of the late osteogenic transcription factor osteon suggest a delay in osteogenic differentiation in Ad-MSCs from aged donors. This is supported by reduced expression of osteocalcin, the most abundant non-collagenous protein in bone (figure 1H-J).

**Gene Expression of Pluripotency Markers Demonstrated Age-related Differences in Ad-MSCs**

The differentiation potential was evaluated by relative gene expression levels of the pluripotency markers Nanog, Oct4, Lin28A and Sox2 by qRT-PCR. GAPDH was used as endogenous control. Expression of Lin28A was significantly lower in Ad-MSCs from elderly donors (figure 2A). Similarly, expression of Oct4 was reduced in Ad-MSCs from aged donors (figure 2B). Furthermore, expression of Nanog was significantly lower in Ad-MSCs from elderly donors (figure 2C). Conversely, expression levels of Sox2 were significantly elevated in Ad-MSCs from aged donors (figure 2D). The observed expression changes were confirmed on protein level by Western blot (figure 2E).

**TET Gene Expression and 5 mC/5 hmC Distribution in Ad-MSCs from Young and Old Donors**

Members of the TET family (TET1-3) are reported to actively regulate DNA methylation. Therefore, we determined relative gene expression levels of TET1, TET2 and TET3 by qRT-PCR. GAPDH was used as endogenous control. Expression of TET1 was near/below the detection limit in Ad-MSCs from young and elderly donors. Therefore, no significant difference could be detected (data not shown). Expression levels of TET2 were...
Figure 5. 5-Azacytidine treatment improves osteogenic differentiation of Ad-MSCs from old donors. Ki67 staining was performed to assess the proliferation capacity of young and old Ad-MSCs after treatment with 5 μM 5-Azacytidine for 48 h. (A) Representative image of a merged immunofluorescent staining for Ki67 (red) and nuclei (blue). (B) Quantification of Ki67-positive Ad-MSCs (N = 3, n = 5/age group) points to an induced proliferation in aged Ad-MSCs after treatment with 5-Azacytidine. Young and old Ad-MSCs were pre-treated with 5 μM 5-Azacytidine for 48 h before...
significant nuclear expression of TET2 and TET3 was observed in Ad-MSCs from young donors (figure 3A). TET3 expression levels were not changed between the two age groups (figure 3B). The observed expression changes were confirmed on protein level by Western blot (figure 3C). Genomic distribution of 5-hydroxymethylcytosine (5 hmC) and 5-methylcytosine (5 mC) in Ad-MSCs from young and old donors was evaluated by immunofluorescence staining (5 hmC: red, 5 mC: green) in order to assess the DNA methylation status. Nuclei were counterstained with Hoechst 33342 (blue) (figure 3D). The 5 mC-positive fraction was significantly higher in the Ad-MSCs from old donors than in the Ad-MSCs from young donors (figure 3E). The 5 hmC-positive fraction was significantly lower in the Ad-MSCs from old donors than in the Ad-MSCs from young donors (figure 3F).

5-Azacytidine Treatment Induced Expression of TET2 and TET3 while Increasing Nuclear 5 hmC Levels

5-Azacytidine is a well characterized DNA-methyltransferase inhibitor. In order to evaluate the effect of 5-Azacytidine on the DNA methylation status, relative gene expression levels of TET1, TET2 and TET3 were determined by qRT-PCR in Ad-MSCs from young and old donors treated for 48 h with 5 μM 5-Azacytidine. GAPDH was used as endogenous control. After 5-Azacytidine treatment TET1 expression was significantly higher than the detection limit in Ad-MSCs from both young and old donors, where no significant difference could be detected (data not shown). In both age groups, TET2 expression levels were significantly increased by 5-Azacytidine treatment (figure 4A). Similarly, TET3 expression levels were increased by 5-Azacytidine treatment in both age groups, with more pronounced changes in Ad-MSCs from aged donors (figure 4B). Expression changes of TET2 and TET3 due to 5-Azacytidine treatment were confirmed by Western blot on the protein level (data not shown). Genomic distribution of 5 hmC and 5 mC in Ad-MSCs from old donors, was strongly affected by 5-Azacytidine treatment, as determined by immunofluorescence staining. The 5 hmC Positive fraction was significantly increased in Ad-MSCs from old donors after treatment with 5-Azacytidine (figure 4C). This resulted in a significant decrease in the 5 mC Positive fraction in Ad-MSCs from old donors after treatment with 5-Azacytidine (figure 4D). Consequently relative expression levels of the pluripotency markers Nanog, Oct4, Lin28A and Sox2 were changed by 5-Azacytidine treatment, as determined by qRT-PCR. Expression levels of Sox2 were significantly decreased by 5-Azacytidine treatment only in Ad-MSCs from aged donors (figure 4E). Expression of Lin28A was significantly induced by 5-Azacytidine treatment in Ad-MSCs from elderly donors (figure 4F). Expression of Oct4 was significantly induced by 5-Azacytidine treatment in Ad-MSCs from both age groups (figure 4G). In contrary to Lin28A expression of Nanog was induced by 5-Azacytidine treatment only in Ad-MSCs from young donors (figure 4H). On the protein level, pluripotency markers showed a comparable regulation to 5-Azacytidine treatment (figure 4I).

5-Azacytidine Pre-treatment Improved Proliferation and Osteogenic Differentiation of Ad-MSCs from Aged Donors

In the following experiments we wanted to analyze of the reduction in DNA methylation by 5-Azacytidine improves the proliferation and the osteogenic differentiation capacity of aged Ad-MSCs. Following the treatment of Ad-MSCs from old donors with 5 μM 5-Azacytidine for 48 h significantly more cells were Ki67 positive (1.3-fold, p<0.01), representing an improved proliferation. Conversely, no improvement of the proliferation was observed in Ad-MSCs from young donors following the treatment with 5 μM 5-Azacytidine for 48 h (figure 5A & B). After pretreatment with 5 μM 5-Azacytidine for 48 h, Ad-MSCs from aged donors were differentiated for 14 days in the presence of osteogenic differentiation medium. As osteogenic markers alkaline phosphatase (AP) activity, matrix mineralization and relative gene expression of Runx2, osteoblast and osteocalcin were determined after the differentiation process. Pre-treatment with 5-Azacytidine improved AP activity (figure 5C) only in Ad-MSCs from elderly donors. Similarly, matrix mineralization was enhanced by 5-Azacytidine treatment only in Ad-MSCs from aged donors as determined by von Kossa (figure 5D & F) and Alizarin red staining (figure 5E & G). Expression levels of the early osteogenic transcription factor Runx2 were significantly reduced by 5-Azacytidine treatment only in Ad-MSCs from aged donors (figure 5H). Conversely, expression levels of the late osteogenic markers osteoblast and osteocalcin were significantly induced by 5-Azacytidine treatment only in Ad-MSCs from elderly donors (figure 5I & J).

Discussion

Stem cell functions include self-renewal and multipotency or even pluripotency. In particular, self-renewal of stem cells does not only include proliferation, but also the maintenance of their differentiation potential after cell division [23]. Our results demonstrate a decreased proliferation rate of Ad-MSCs from aged donors as well as an impaired osteogenic differentiation potential, as characterized by a declined AP activity, a weaker mineralization of extracellular matrix and reduced expression of late osteogenic marker genes osteoblast and osteocalcin in the differentiated cells from elderly donors, which was accompanied by a strong DNA methylation. Increased global DNA methylation status is a well-established phenomenon that has been observed in many aged adult tissue/cell types [24].

Our preliminary research has shown that 5-Azacytidine treatment significantly reduces the global level of DNA methylation [16]. In the past, the demethylation effect of 5-Azacytidine was described as a cause of direct or indirect inhibition of the DNA methyltransferases (DNMTs) [25]. This theory resembles mainly the passive DNA demethylation process. Recently, more active mechanisms of DNA demethylation have been uncovered [26]. TET proteins mediated conversion of 5 mC to 5 hmC was shown to play a crucial role in active demethylation of DNA [27]. Here, we have presented for the first time, that Ad-MSCs display reduced levels of 5 hmC during aging. With the conversion of
5 mC to 5 hmC by TET proteins [17], further modifications can occur in order to launch the active DNA demethylation, i.e. oxidation to 5-formylcytosine (5 fC) and 5-carboxycytosine (5 caC). 5 caC eventually will be removed by a base excision pathway and thus will be replaced with unmethylated cytosine [26]. Thus, the finding of reduced 5 hmC levels in aged Ad-MSCs could account for a DNA hypermethylpation pattern in these [16] and other adult cell types [24]. This is further supported by the finding that 5 hmC has been proven to act like a poor substrate for DNMT1 recognition [28]. In this way, it is also able to take part in finding that 5 hmC has been proven to act like a poor substrate for and other adult cell types [24]. This is further supported by the work of Tsai et al, showing that with increasing time in culture, Nanog and Oct4 expression as well as osteogenic differentiation capacity decreases in MSCs [37]. Lin28A and Oct4 may play a special role in this regulatory mechanism as the treatment with 5-Azacytidine, which strongly induced the expression of Lin28A and Oct4, was able to improve proliferation and osteogenic differentiation in Ad-MSCs from elderly donors. Basal expression levels of the pluripotency factor Sox2 was very low in young Ad-MSCs, which is in line with other publications [38]. Interestingly, basal Sox2 expression levels were significantly elevated in Ad-MSCs from elderly donors and decreased with the 5-Azacytidine treatment. The decline in Sox2 expression after 5-Azacytidine treatment may be pivotal for the improved osteogenic differentiation, as there are reports showing that depletion of Sox2 promotes osteogenic differentiation in a great variety of stem cells [39,40,41,42].

In our aged Ad-MSCs 5-Azacytidine not only induced the cells proliferation but also accelerated their osteogenic differentiation. This is shown by a significant increase in AP activity and matrix mineralization. Supporting this hypothesis the early osteogenic transcription factor Runx2 is reduced in favor for a faster induction of the late osteogenic marker genes osteocalcin and osterix [18].

Conclusion
Summarizing, our data suggest that DNA methylation plays a crucial role in adult stem cell aging, as DNA methylation increases with donor age in Ad-MSCs. We could show that 5-Azacytidine, a small molecule inhibitor for DNMTs, not only passively but also actively promotes DNA demethylation in Ad-MSCs from aged donors. This balanced the expression levels of pluripotency genes and improved the osteogenic differentiation potential, thus rejuvenating aged Ad-MSCs.

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Author Contributions
Conceived and designed the experiments: XY SE JP AKN. Performed the experiments: XY AB MC CS ZW. Analyzed the data: SE LS ARS PDS JP. Wrote the paper: XY SE AB LS ZW AKN. Critical revision of the manuscript: MC CS ARS US PDS JP. Approval of final version: XY SE MC AB CS LS ZW ARS US PDS JP AKN.

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