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# Mesenchymal stem cells preserve their stem cell traits after exposure to antimetabolite chemotherapy

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ABSTRACT

*Background:* Mesenchymal stem cells (MSCs) participate in the regeneration of tissue lesions induced by antimetabolite chemotherapy; however, the influence of this class of anti-cancer compounds on the stem cells remains largely unknown.
*Methods:* The survival of MSCs after exposure to 5-fluorouracil (5-FU) and gemcitabine was measured by viability and clonogenic assays. MSC morphology, surface marker expression, adhesion potential, cellular velocity and differentiation potential were determined after antimetabolite treatment. Cell cycle distribution and apoptosis were assessed using flow cytometry, and senescence induction was evaluated by beta-galactosidase staining. Gene expression arrays were used to analyze the expression of enzymes involved in DNA metabolism and multidrug resistance.
*Results:* Here, we show that human primary bone marrow MSCs are relatively resistant to treatment with the widely used antimetabolite drugs 5-FU and gemcitabine. The stem cells were able to largely retain their functional abilities and defining stem cell traits after antimetabolite exposure. MSCs surface markers were found stably expressed, and the characteristic multi-lineage differentiation potential was maintained irrespective of 5-FU or gemcitabine treatment. High expression levels of enzymes involved in DNA metabolism and multidrug resistance transporters may contribute to the resistance to antimetabolite chemotherapy.
*Discussion:* The observed resistance and functional integrity may form the basis for further investigations of MSCs as a potential therapy for antimetabolite-induced tissue damage.

## 1. Introduction

Antimetabolite compounds comprise a large group of substances that inhibit components of the cellular metabolism and are widely used for the treatment of cancers, benign proliferative diseases or autoimmune diseases (Peters et al., 2000; Cipriani et al., 2014; Batista et al., 2010; Green et al., 2014). Many antimetabolite cancer agents exhibit structural similarities to the purine or pyrimidine bases of DNA and act by competitively inhibiting the synthesis of these molecules or their incorporation into nascent DNA strands, thereby blocking DNA replication (Kinsella et al., 1997; Hatse et al., 1999). Due to their efficiency against many cancer types, cytostatic antimetabolites form the largest group of anticancer agents currently in clinical use (Johnston et al., 1996).

The prototypical antimetabolite cancer drug, 5-fluorouracil (5-FU) was developed as a pyrimidine analogue and received approval for clinical utilization in the early 1960s; 5-FU has since been introduced intro treatment protocols for breast, skin, head-and-neck, pancreatic,

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esophageal, gastric, colorectal and anal cancers (O'Connell et al., 1994; Jacobs et al., 1992; Berlin et al., 2002; Cunningham et al., 2006; Al-Batran et al., 2016). While the drug's exact mechanism of action is yet to be completely understood, it involves blocking of the enzyme thymidylate synthase, resulting in a lack of phosphorylated deoxythymidine and a toxic accumulation of deoxyuridine (Hatse et al., 1999). Gemcitabine is a newer antimetabolite drug and was approved for clinical use in 1995; it is phosphorylated intracellularly and can then be incorporated into DNA instead of cytidine nucleotides. As it does not lead to DNA strand breaks, its incorporation is masked for physiological DNA repair mechanisms, thus creating commonly irreparable DNA damage (Plunkett et al., 1995). Gemcitabine is used against pancreatic, bladder, non-small cell lung, ovarian and breast cancers (Berlin et al., 2002; Messing et al., 2018; Cardenal et al., 1999). Both 5-FU and gemcitabine have well-known myelosuppressive effects that may result in life-threatening leukopenia or thrombopenia (Okusaka et al., 2006).

Mesenchymal stem cells (MSCs) were first isolated from human bone marrow where they participate in the maintenance and regulation of the hematopoietic stem cell homeostasis (Sugrue et al., 2012; Friedenstein et al., 1974). Unlike their hematopoietic counterparts, MSCs form a heterogeneous group of multipotent stromal cells that require a combination of functional and molecular markers in order to be adequately characterized, including their fibroblast-like spindle shape, their ability to adhere to plastic surfaces, their differentiation potential along the osteogenic, chondrogenic and adipogenic lineages and a comprehensive set of positive and negative surface markers (Dominici et al., 2006).

MSCs have shown regenerative effects in vitro and in animal models, mainly by the creation of a protective microenvironment and to a lesser extent by differentiation into organ-specific functional cell types (Nicolay et al., 2015a; Usunier et al., 2014). Secretion of cytokines, growth factors and microvesicles by MSCs are known to play an important role in the repair of injured tissues (Ulivi et al., 2014; Liu and Hwang, 2005; Lopatina et al., 2014). Benefits of MSC-based therapies have been demonstrated for the attenuation of mucosal damage induced by antimetabolite chemotherapy, but further data regarding the stem cells' protective effects have not yet been published (Zhang et al., 2012; Ruhle et al., 2018a; Ruhle et al., 2019). Similarly, the effects of antimetabolite compounds on MSCs themselves are unknown.

Here, we analyzed the effects of the clinically used antimetabolite compounds, 5-FU and gemcitabine on the survival, proliferation and cellular functions of human MSCs. Additionally, we characterized the influence of the antimetabolite drugs on the defining stem cell properties and molecular markers of these stem cells.

## 2. Materials and methods

### 2.1. Cells and culture

Human MSCs were isolated from the iliac crest bone marrow of healthy voluntary donors and isolated as published previously (Nicolay et al., 2016a) before cell culture using Mesenchymal Stem Cell Growth Medium (Lonza, Basel, Switzerland). As functional and regenerative abilities are known to partly depend on the donor's age, MSCs derived from three different donors were used in this study (MSC1: male donor (34 years old), MSC2: female donor (41 years old), MSC3: male donor (29 years old)) (Kornicka et al., 2017). HS68 cells were purchased from the ATCC (Manassas, USA) and were grown in Dulbecco's Modified Eagle Medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum. All cells were maintained at 37 °C in a humidified incubator with 5% $CO_2$, and medium was changed twice a week. Written consent was obtained prior to bone marrow aspiration and this investigation was approved by the Heidelberg University ethics committee (#S-384/2004).

### 2.2. Drug treatment

Stock solutions of 5-FU and gemcitabine were obtained from the Heidelberg University Hospital central pharmacy and stored at 4 °C for a maximum of 7 days. Drugs were diluted in cell culture medium to the required concentrations immediately before each experiment. All experimental setups were protected from light after addition of the drugs.

### 2.3. Viability assays

Cellular viability after antimetabolite treatment was assessed by the MTS assay. $2 \times 10^3$ cells were plated in each well of a 96-well plate 24 h prior to drug treatment, and afterwards, cells were allowed to proliferate for 96 h. Following incubation with 20 µL of 1.9 mg/mL MTS solution (Promega, Madison, USA) at 37 °C, light absorbance was measured at 492 nm using a microplate reader (Tecan, Crailsheim, Germany).

### 2.4. Clonogenic survival assays

$1 \times 10^3$ cells were plated in T25 flasks and treated 24 h later. After drug treatment for 4 h, cells were allowed to grow for 14 days, and colonies were fixed using 25% acetic acid in methanol and stained with crystal violet solution. Colonies containing > 50 cells were counted by using an inverted microscope, and the survival fraction was calculated according to the following formula: $(\#\text{colonies}/\#\text{plated cells})_{\text{treated}}/(\#\text{colonies}/\#\text{plated cells})_{\text{untreated}}$. All clonogenic survival assays were repeated thrice.

### 2.5. Cell adhesion measurements

Cells were grown in T75 flasks to a confluence of 70% and exposed to 5-FU or gemcitabine for 4 h. $1 \times 10^2$ cells per well of a 96-well plate were seeded and number of attached cells were counted at different time points. The ratio between attached and seeded cells was calculated to determine the adhesion rate.

### 2.6. Cellular motility assays

The average velocity of MSCs and HS68 fibroblasts was measured by time-lapse microscopy as a surrogate for the cells' migratory ability. $4 \times 10^3$ cells were plated on glass cover slips in 24-well plates and treated with 5-FU or gemcitabine. Time-lapse microscopy was conducted on an IX70 inverted microscope fitted with an incubator box (Olympus, Hamburg, Germany) at 37 °C and 5% $CO_2$. Manual single-cell tracking with ImageJ software (National Institutes of Health, Bethesda, USA) was used to quantify cellular velocity, and at least 10 cells from three randomly chosen fields of views in each well were tracked.

### 2.7. MSC surface marker expression

For each treatment condition, MSCs were grown in T75 flasks to a confluence of 70% and treated with 20 µM 5-FU or 120 nM gemcitabine for 4 h. At 48 h after antimetabolite treatment, cells were harvested, and MSC surface marker expression was analyzed using the MSC Phenotyping Kit (MiltenyiBiotec, Bergisch-Gladbach, Germany) following the manufacturer's instructions. Surface marker expression was determined on a FACSCanto flow cytometer (BD, Heidelberg, Germany), and data analysis was performed with FlowJo 7.6.5 software (FlowJo LLC, Ashland, USA). Autofluorescence and isotype controls were included in each measurement.

### 2.8. MSC differentiation analyses

For adipogenic differentiation, $3 \times 10^4$ cells were plated on glass

cover slips in 24-well plates before drug treatment with 5-FU or gemcitabine. To induce adipogenic differentiation, cell culture medium was replaced by STEMPRO® adipogenic differentiation medium (Gibco, Grand Island, NY, USA). After 21 days, specimens were stained using 1 μg/mL BODIPY (493/503) (Life Technologies, Darmstadt, Germany) for 30 min, and nuclei were counterstained with 2 μM Hoechst33342 (Sigma, Steinheim, Germany).

For osteogenic differentiation, $2.5 \times 10^4$ cells were plated on glass cover slides in 24-well plates and exposed to 5-FU or gemcitabine. STEMPRO® Osteogenesis Differentiation medium (Gibco) was used to induce osteogenic differentiation. In order to quantify osteogenic differentiation, cells were incubated with OsteoImage™ Staining Reagent (Lonza) which specifically binds to hxdroxyapatite. Staining was performed according to the manufacturer's instructions, and nuclei were counterstained with 2 mM Hoechst33342.

Chondrogenic differentiation was performed in 96-well plates using the STEMPRO® Chondrogenesis Differentiation Kit (Gibco). Cells were treated with 5-FU or gemcitabine, and $1 \times 10^5$ cells were subsequently plated in each well of a 96-well plate in order to induce spheroids. After 21 days, spheroids were fixed with 4% paraformaldehyde in PBS solution for 30 min, frozen at −20 °C and sectioned on a cryomicrotome. For permeabilization and blocking of nonspecific binding sites, pellet sections were incubated with 0.3% Triton X-100, 1% BSA and 10% normal donkey serum in PBS for 60 min at room temperature. The sections were then stained with a goat antibody against human aggrecan (1:10; R&D Systems, Minneapolis, MN, USA) for 60 min at room temperature before they were incubated with an Alexa488-coupled secondary antibody (1:200, Donkey Anti-Goat; Abcam, Cambridge, UK). After staining with 2 μM Hoechst33342, quantification of aggrecan staining was performed with ImageJ. Fluorescence images of all differentiation experiments were taken using a Keyence BioRevo9000 microscope (Keyence, Neu-Isenburg, Germany). For all differentiation analyses, staining intensities were normalized to cell number.

### 2.9. Cell cycle and apoptosis measurements

Cells were treated with 20 μM 5-FU or 120 nM gemcitabine for 4 h and harvested 24, 48 or 96 h later before fixation with 3% paraformaldehyde in PBS for 10 min. After permeabilization of cells using ice-cold 70% ethanol, cells were washed thrice with 0.5% bovine serum albumin (BSA) in PBS. To assess apoptosis, cells were incubated with an antibody against activated caspase-3 (1:20; BD Pharmingen) dissolved in 3% BSA in PBS for 1 h. After centrifugation, cells were resuspended in 1 μg/mL 4′-6-diamidino-2-phenylindole (DAPI) staining reagent. Cell cycle distribution and apoptosis rates were assessed using a LSR II flow cytometer (BD), and analysis was carried out using FlowJo 7.6.5 software.

### 2.10. Senescence analyses

$2 \times 10^3$ cells were seeded on glass cover slips in a 24-well plate and 24 h later, cells were treated with 5-FU or gemcitabine. Cells were fixed at 96 h after treatment, and β-galactosidase activity was determined using the senescence-associated β-galactosidase Staining Kit (Cell Signaling Technology, Leiden, Netherlands) following the manufacturer's instructions. After nuclear staining with 2 μM Hoechst33342 for 5 min, images were obtained on a Keyence BioRevo9000 microscope, and assessment of cell numbers and β-galactosidase-positive cells was performed using ImageJ software.

### 2.11. Gene expression analyses

RNA was extracted from log-phase MSCs and HS68 fibroblasts using an RNeasy Mini Kit (Qiagen, Hilden, Germany); whole human genome microarrays 4 × 44 k (Agilent Technologies, Böblingen, Germany) were then used to quantify the gene expression patterns. Gene expression data were extracted and analyzed using the Agilent feature extraction software 9.1 (Agilent Technologies).

### 2.12. Statistics

At least three experimental replicates were carried out to calculate mean values and standard deviation. GraphPad Prism 8.0.1 software (Graph Pad Software, San Diego, CA, USA) was used for statistical analyses. Comparison between control and treatment groups was performed using unpaired, two-sided Student's *t*-tests. For clonogenic survival and viability assays, groups were compared using one-way ANOVA with post-hoc Tukey tests. *P*-values < .05 were assumed significant for all experiments.

## 3. Results

### 3.1. MSCs and differentiated fibroblasts show similar sensitivities to antimetabolite treatment

Sensitivity of human primary MSCs and differentiated fibroblasts to antimetabolite treatment was measured by viability and clonogenic survival assays; chosen treatment doses mimicked measured plasma concentrations of patients receiving antimetabolite chemotherapy (Casale et al., 2004; Ciccolini et al., 2016). Viability of MSCs was found generally higher than HS68 differentiated fibroblasts both after treatment with 5-FU ($P < .001$, one-way ANOVA with post-hoc Tukey test) or gemcitabine ($P < .001$ for MSC1, $P < .01$ for MSC2) (Fig. 1A).

Clonogenic survival assays as a measure of proliferative activity demonstrated comparable resistance of MSCs and differentiated fibroblasts to antimetabolite treatment with no significant difference for any of the tested doses (Fig. 1B). While MSC3 was found more resistant to 5-FU exposure compared to MSC1 and MSC2 in the viability assays ($P < .01$ for MSC3 vs. MSC1, $P < .001$ for MSC3 vs. MSC2), clonogenic survival assays showed a comparable 5-FU sensitivity between all MSC samples. In contrast, MSC3 demonstrated reduced viability levels after gemcitabine treatment in comparison with MSC1 and MSC2 ($P < .001$ for MSC1 vs. MSC3, $P < .01$ for MSC2 vs. MSC3), while clonogenic survival assays revealed no significant difference between different MSC preparations.

### 3.2. Antimetabolite treatment does not impede MSC adherence or motility

The ability for adherence to plastic surfaces is a defining trait of MSCs; cell adhesion was measured over 24 h after antimetabolite exposure for 4 h. The ability for adherence seemed not influenced by antimetabolite treatment, and no treatment-induced reduction or delay in cellular adhesion could be observed for any of the tested MSC samples following treatment with 5-FU (Fig. 2A) or gemcitabine (Fig. 2B). Similarly, no measurable effect of antimetabolite exposure was found on the adhesion of differentiated HS68 fibroblasts.

Motility of MSCs and adult fibroblasts was assessed by time-lapse microscopy over an observation period of 35 h. Treatment with 20 or 80 μM of 5-FU for 4 h did not result in a reduction of the average cellular velocity for all tested MSC samples and differentiated fibroblasts, and no dose-dependent effect on the cellular motility could be observed (Fig. 2C). In contrast, treatment with 120 nM gemcitabine led to a reduction in the cellular velocity in MSC1 ($P < .01$, Student's *t*-test) and MSC3 ($P < .01$) samples and in HS68 fibroblasts ($P < .05$), while lower doses of 60 nM only reduced motility in one MSC preparation (MSC3: $P < .01$) and HS68 fibroblasts ($P < .05$). In contrast, MSC2 cells showed no changes in their cellular velocity after antimetabolite treatment demonstrating the heterogeneity between MSCs derived from different donors.

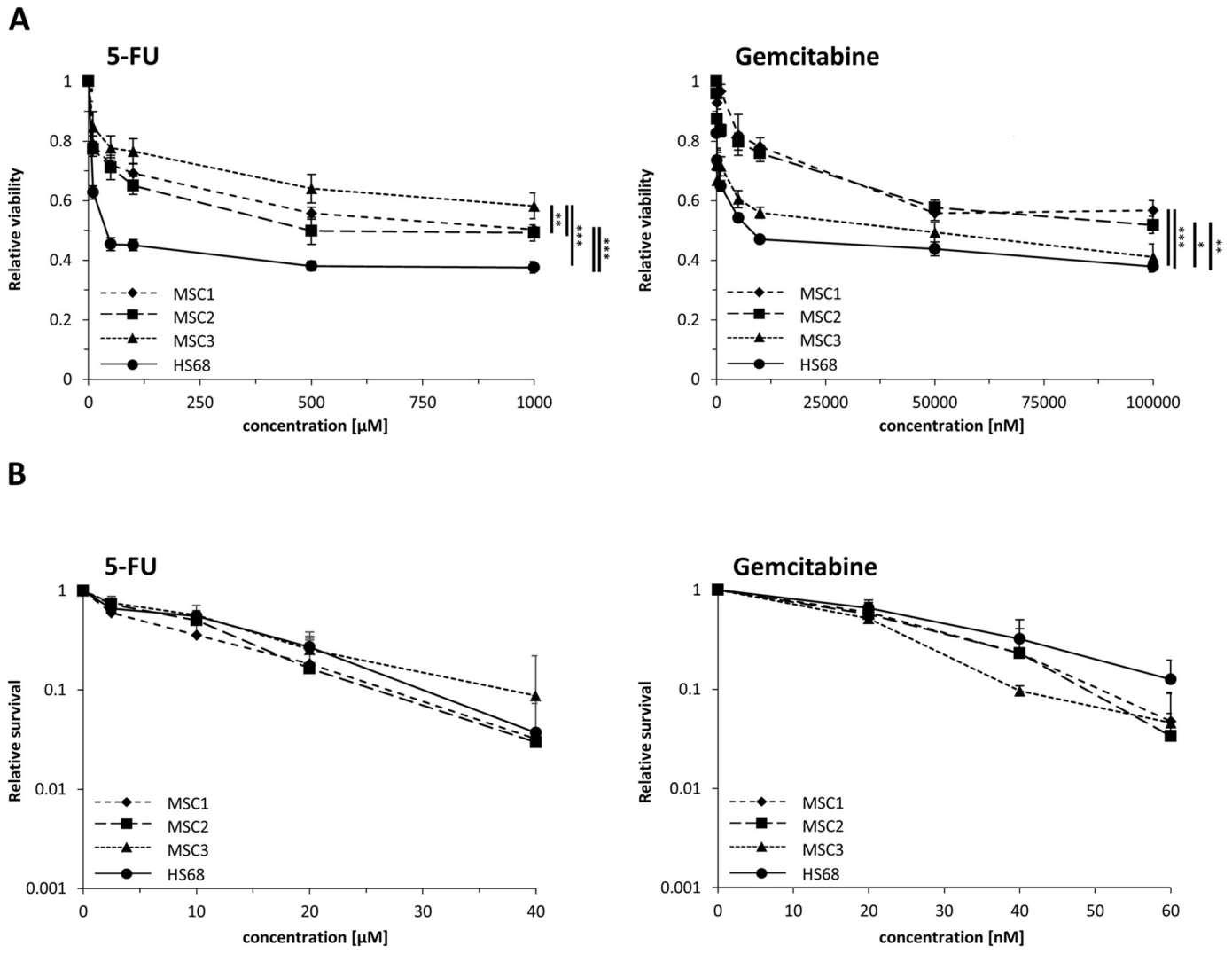


**Fig. 1.** Cellular viability and clonogenic survival of human MSCs after treatment with 5-FU and gemcitabine is comparable to adult fibroblasts. (A) MTS assays for MSCs and HS68 fibroblasts demonstrating cellular viability at 96 h after 4-h treatment with 5-FU (left panel) or gemcitabine (right panel). (B) Clonogenic survival assays for MSCs and HS68 fibroblasts after 4-h exposure to 5-FU (left panel) or gemcitabine (right panel). *$P < .05$, **$P < .01$, ***$P < .001$ (one-way ANOVA with post-hoc Tukey test).

### *3.3. Antimetabolite treatment does not influence MSC morphology or surface marker expression*

Cellular morphology of primary MSCs and differentiated fibroblasts appeared largely unaltered after exposure to 20 or 80 μM of 5-FU or 60 and 120 nM of gemcitabine, and no morphological marks of increased apoptosis could be observed by light microscopy at 24 h following antimetabolite treatment (Fig. 3A).

Established surface markers of human MSCs were measured by FACS analysis at 48 h following antimetabolite exposure. Expression levels of positive surface markers CD90 and CD105 were found not altered or reduced in all three MSC specimens after treatment with high doses of 5-FU or gemcitabine (Fig. 3B). Compared to CD105 expression, reduced expression levels of CD90 were observed, especially for MSC1 and MSC3 (Table 1). CD105 expression ranged between 97.5% after treatment with 120 nM gemcitabine and 100.0% after exposure to 20 μM 5-FU. Likewise, exposure to 5-FU or gemcitabine did not influence the absent expression of the "negative" MSC markers CD14, CD20, CD34 or CD45.

### *3.4. The multi-lineage differentiation potential of MSCs is not abrogated by antimetabolite treatment*

The ability of MSCs to undergo induced differentiation along the adipogenic, osteogenic and chondrogenic lineages is a defining characteristic of these stem cells. Immunocytochemical analyses were carried out to quantify potential effects of antimetabolite treatment on the differentiation potential of MSCs.

The ability for adipogenic, osteogenic and chondrogenic differentiation was found intact in all three tested MSC specimens even after treatment with high doses of 5-FU or gemcitabine. 5-FU exposure resulted in no reduction of induced adipogenic differentiation in any MSC sample, and there was a trend towards an increased differentiation potential in MSC1 ($P < .05$) and MSC3 ($P < .01$) for lower doses of 20 μM 5-FU (Fig. 4A). Osteogenic differentiation capacity was found significantly increased in MSC1 and MSC3 after 80 μM 5-FU ($P < .05$ for MSC1, $P < .001$ for MSC3), while there was a non-significant trend towards elevated osteogenic differentiation levels for MSC2 ($P = .08$) (Fig. 4B). The ability to undergo induced chondrogenic differentiation was not significantly influenced by 5-FU treatment in any of the analyzed MSC samples (Fig. 4C).

Gemcitabine treatment did not significantly influence the

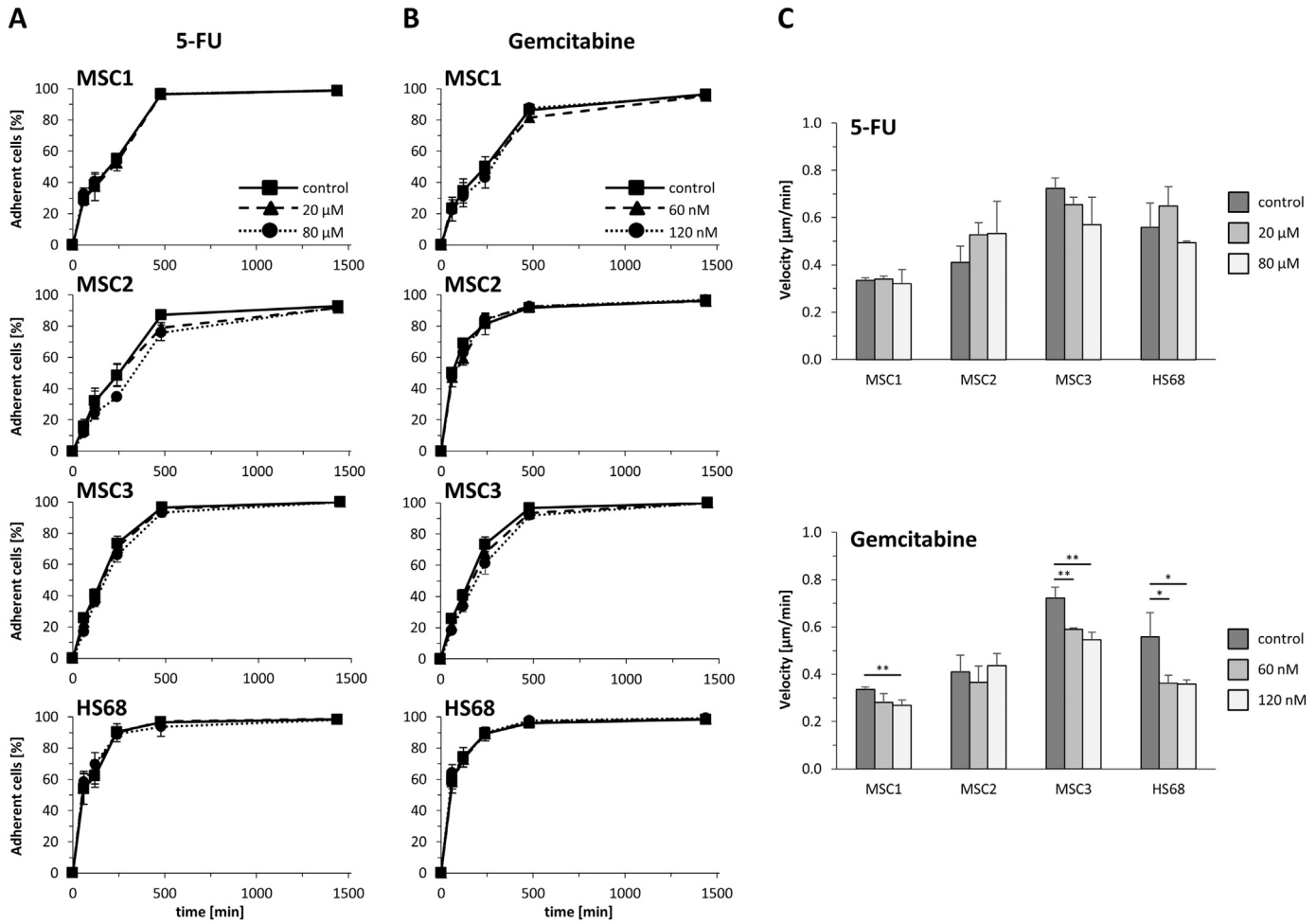


**Fig. 2.** MSC adhesion and velocity are unaffected after treatment with 5-FU and gemcitabine. (A and B) Relative adhesion rate of MSCs and adult fibroblasts after 4-h drug treatment with 5-FU (A) or gemcitabine (B). (C) Average velocity of MSCs and HS68 fibroblasts after exposure to 5-FU or gemcitabine. *$P < .05$, **$P < .01$ (Student's unpaired two-sided *t*-test).

adipogenic differentiation potential of any analyzed MSC preparation. Similar to the results of the osteogenic differentiation capacity after 5-FU exposure, high doses of gemcitabine (200 nM) resulted in an increased osteogenic differentiation ability in all tested MSC preparations ($P < .05$). In contrast, the chondrogenic differentiation ability was found significantly reduced to varying degrees in two of the tested MSC samples, when treatment was performed with very high doses of 200 nM gemcitabine ($P < .01$ for MSC1 and MSC2), while MSC3 did not exhibit any alterations in induced chondrogenic differentiation upon gemcitabine treatment.

### 3.5. Antimetabolite treatment does not induce apoptosis in MSCs

The influence of antimetabolite treatment on MSCs and differentiated fibroblasts was assessed by FACS analyses. Treatment with 5-FU for 4 h entailed only minor cell cycle effects with no clear accumulation of stem cells or HS68 fibroblasts in any phase of the cell cycle (Fig. 5A). In contrast, gemcitabine treatment resulted in significant increases of the S phase population for MSC1 and MSC2 that persisted up to 96 h ($P < .05$) (Fig. 5B). A similar S phase accumulation was observed in differentiated fibroblasts at 96 h after exposure to gemcitabine ($P < .01$).

Apoptosis induced by antimetabolite chemotherapeutics was measured by the cellular sub-G1 population and the activation of caspase-3. All analyzed MSC samples did not undergo apoptosis after treatment with 5-FU or gemcitabine, and levels of caspase-3-positive stem cells ranged below 2% for both agents and all tested time points, while sub-G1 cell populations were even below 1% (Fig. 6A, supplementary Fig. 1). While differentiated fibroblasts exhibited a small time-dependent increase of apoptotic cells after exposure to 5-FU with up to 5% of cells activating caspase-3, gemcitabine treatment resulted in apoptosis induction in 13% of HS68 fibroblasts after 96 h ($P < .001$).

As it has been reported that MSCs respond to cellular stress by inducing senescence rather than apoptosis, antimetabolite-mediated senescence levels were assessed by β-galactosidase staining. Only MSC1 exhibited a dose-dependent induction of senescence after exposure to 20 or 80 µM 5-FU ($P < .05$ for 20 µM, $P < .001$ for 80 µM), while MSC2 and MSC3 did not exhibit any increased β-galactosidase signal (Fig. 6B). Gemcitabine treatment induced senescence in two of the three MSC samples with a 2.1-fold increase after 200 nM in MSC1 ($P < .05$) and a 2.5-fold increase in MSC2 ($P < .001$). In contrast, higher doses of both compounds caused significant senescence levels in HS68 fibroblasts ($P < .001$ for 5-FU, $P < .01$ for gemcitabine).

### 3.6. MSCs and differentiated fibroblasts exhibit differential expression of genes involved in antimetabolite metabolism

The cellular sensitivity to antimetabolite treatments has been linked to the presence and activity of various enzymes involved in nucleotide metabolism as well as different transmembrane transporters. Gene array data were obtained from both MSC1 and MSC2 specimens and HS68 fibroblasts and demonstrated a differential expression between

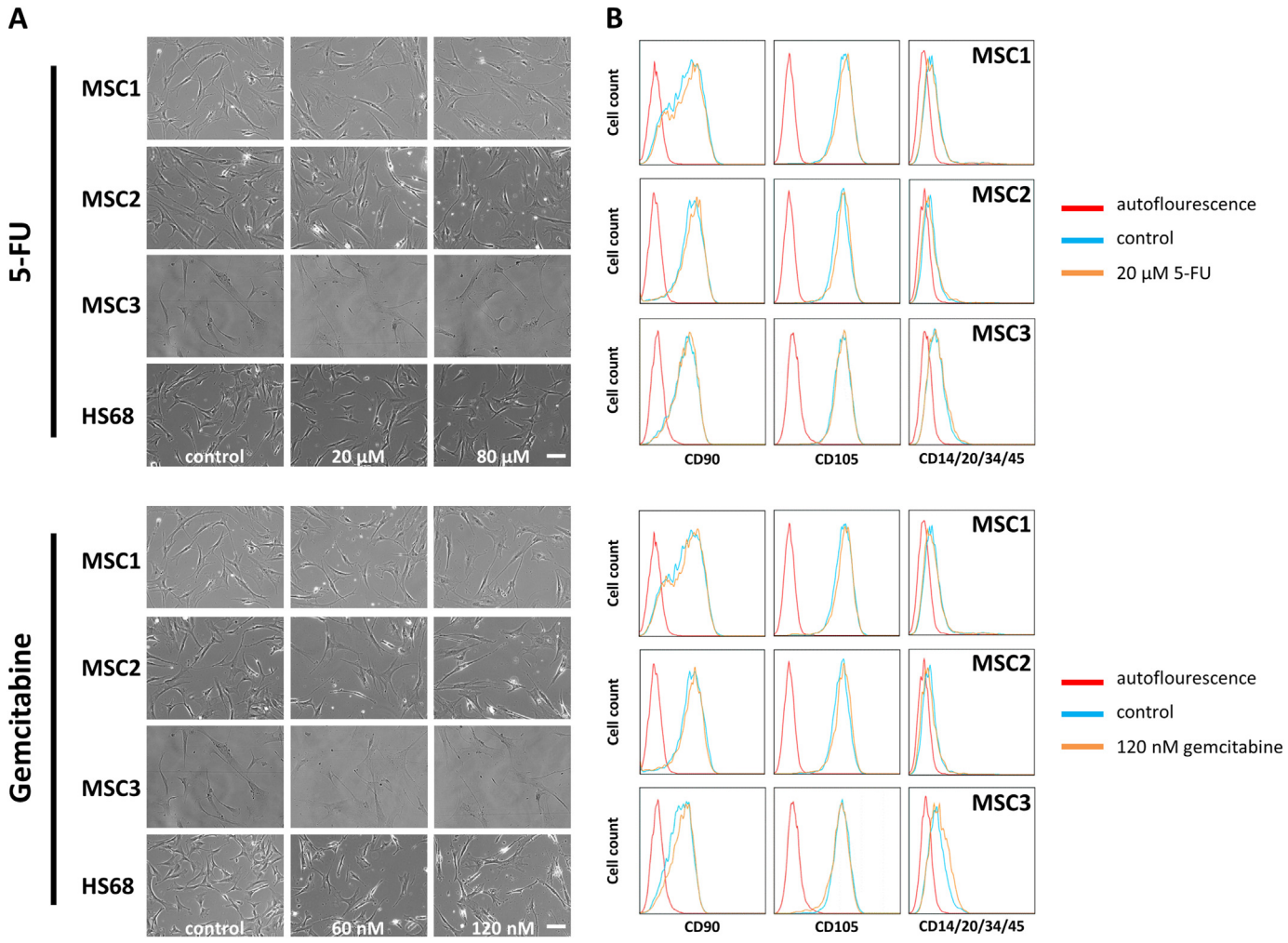


**Fig. 3.** Antimetabolite treatment does not alter MSC morphology and surface marker expression. (A) Cellular morphology of MSCs and HS68 fibroblasts at 35 h after treatment with 5-FU (upper panel) or gemcitabine (lower panel). Objective 20×, scale bar 100 µm. (B) Flow cytometry histograms of positive MSC surface markers CD90 and CD105 and negative hematopoietic markers (CD14, CD20, CD34 and CD45) at 48 h after exposure to 20 µM 5-FU (upper panel) or 120 nM gemcitabine (lower panel). Untreated MSCs were used as control group.

**Table 1**
Percentage of CD90- and CD105-positive cells. The threshold is based on isotype control signal intensity.

| | MSC1 | MSC2 | MSC3 |
|---|---|---|---|
| CD90 | | | |
| Untreated control | 73.9% | 92.7% | 81.9% |
| 20 µM 5-FU 48 h | 77.7% | 92.0% | 83.6% |
| 120 nM gemcitabine 48 h | 76.1% | 93.9% | 73.0% |
| CD105 | | | |
| Untreated control | 99.7% | 99.8% | 99.4% |
| 20 µM 5-FU 48 h | 100.0% | 99.6% | 99.3% |
| 120 nM gemcitabine 48 h | 99.7% | 99.7% | 97.5% |

stem cells and differentiated fibroblasts regarding several genes: Expression of the TYMS gene encoding the thymidylate synthase enzyme as the key target for 5-FU was found significantly higher in both MSCs than in HS68 cells ($P < .05$ for MSC1 and MSC2) (Fig. 6C, Table 2). Similarly, MSCs exhibited a higher expression of UMPS encoding uridine monophosphate synthase which catalyzes the conversion of 5-FU into active cancer metabolites ($P < .01$ for MSC1, $P < .05$ for MSC2). There was a trend for higher expression of TK1 encoding the soluble thymidine kinase 1 which aids phosphorylation of 5-fluoro-2′-deoxyuridine into the active metabolite 5-fluoro-2′-deoxyuridine monophosphate, secondary inhibiting thymidylate synthase ($P = .09$ for MSC1, $P = .10$ for MSC2). In contrast, expression of DPYD encoding dihydropyrimidine dehydrogenase, the major 5-FU-inactivating enzyme, was low both in MSCs and HS68 fibroblasts.

Both MSCs and HS68 exhibited low mRNA expression of DCK which encodes deoxycytidine kinase, catalyzing the rate-limiting step in gemcitabine activation (phosphorylation of gemcitabine into gemcitabine monophosphate). Low expression levels of CDA encoding the major gemcitabine-inactivating enzyme cytidine deaminase were found in MSCs and HS68. NT5C encoding the cytosolic 5′,3′-nucleotidase catalyzing the inactivation of gemcitabine monophosphate by dephosphorylation were found at significantly higher levels in MSCs than in fibroblasts ($P < .01$ for MSC1, $P < .05$ for MSC2). The RRM2 gene encoding a subunit of ribonucleotide-diphosphate reductase, the molecular target of gemcitabine diphosphate, was found at higher expression levels in MSCs ($P < .001$ for MSC1, $P < .05$ for MSC2). Similar expression of the genes RRM1 and RRM2B which also encode for subunits of ribonucleotide-diphosphate reductase was observed in MSCs and HS68 fibroblasts.

There was also a differential expression of genes encoding different transporter proteins regulating influx of antimetabolites into the cytosol (Fig. 6D). The ATP-binding cassette super-family G member 2 protein (ABCG2) and canalicular multispecific organic anion proteins (ABCC3, ABCC4 and ABCC5) are important transporters for 5-FU efflux. While

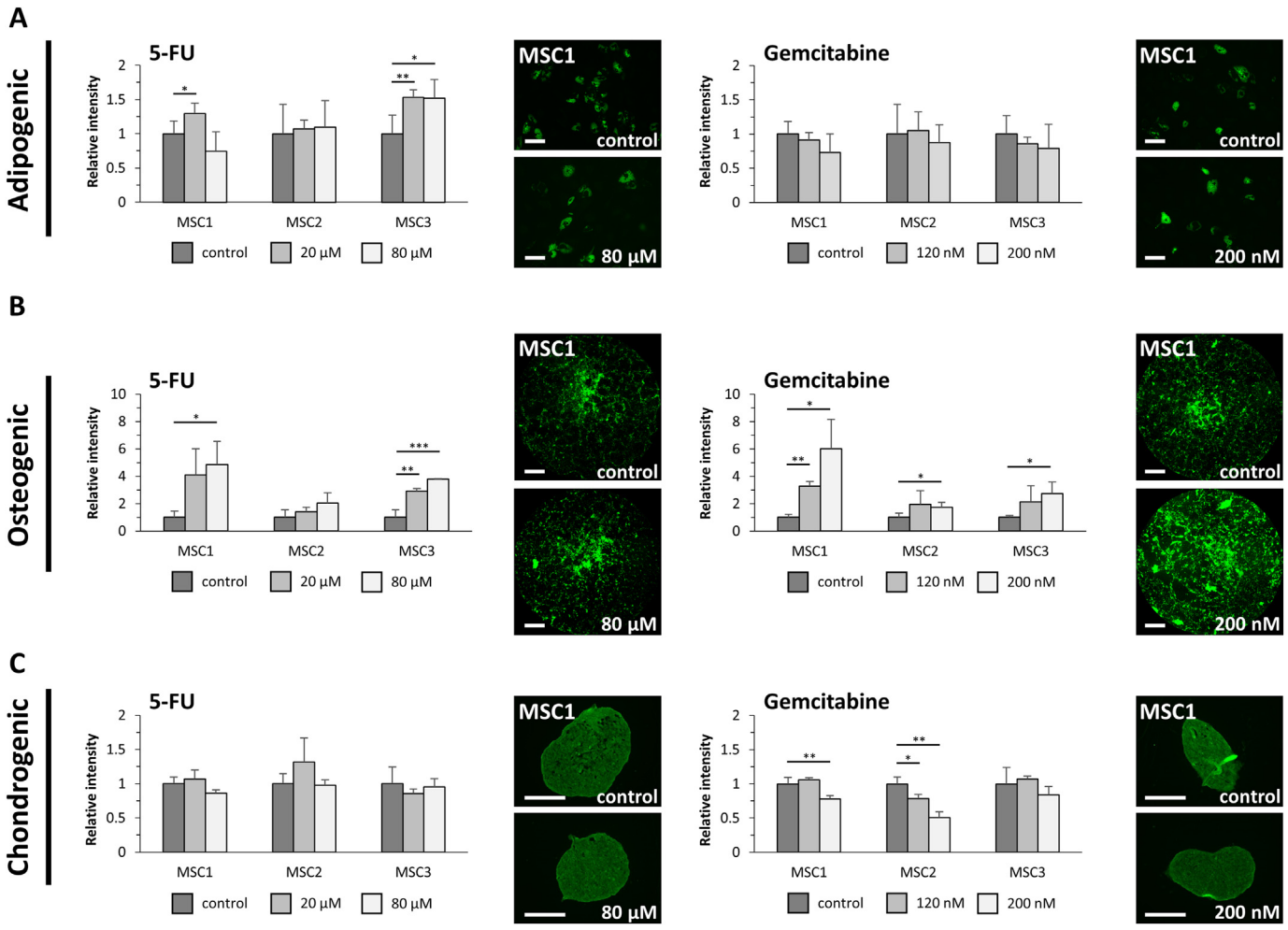


**Fig. 4.** 5-FU and gemcitabine treatment do not affect the multi-lineage differentiation potential of MSCs. (A) BODIPY (493/503) lipid droplet staining for adipogenic differentiation in MSCs after exposure to 5-FU (left panel) or gemcitabine (right panel). Objective 20×, scale bar 100 µm.(B) OsteoImage™ staining for quantification of hydroxyapatite formation after 5-FU (left panel) or gemcitabine (right panel) treatment. Objective 2×, scale bar 1000 µm. (C) Aggrecan staining for chondrogenic differentiation in MSCs after treatment with 5-FU (left panel) or gemcitabine (right panel). Objective 4×, scale bar 1000 µm. *$P < .05$, **$P < .01$, ***$P < .001$ (Student's unpaired two-sided t-test).

higher expression of ABCC3 (canalicular multispecific organic anion transporter 2) and ABCG2 was observed in differentiated fibroblasts (ABCC3: $P < .01$ for MSC1 and MSC2, ABCG2: $P < .05$ for MSC1 and MSC2), ABCC4 encoding the multidrug resistance-associated protein 4 ($P < .01$ for MSC1 and MSC2) and ABCC5 encoding the multidrug resistance-associated protein 5 ($P < .05$ for MSC1 and MSC2) were found at higher levels in both tested MSC samples.

Low expression levels were detected for the genes SLC28A1, SLC28A3, SLC29A1 and SLC29A2 encoding for nucleoside transporters involved in gemcitabine uptake.

## 4. Discussion

While MSCs have demonstrated beneficial effects regarding the attenuation of chemotherapy-induced toxicities, the effects of most of these drugs on the stem cells themselves remains incompletely understood. Here, we analyzed the influence of antimetabolite compounds on the survival and cellular functions of MSCs, as antimetabolites constitute the most widely used class of anticancer drugs. We demonstrated for the first time that MSCs were relatively resistant to the antimetabolite drugs 5-FU and gemcitabine and largely maintained their defining stem cell characteristics when exposed to clinical relevant doses that mimicked reported plasma concentrations in patients receiving antimetabolite chemotherapy (Casale et al., 2004; Ciccolini et al., 2016).

Several previous publications have reported inconsistent resistance levels of MSCs to different chemotherapeutic anti-cancer agents, including topoisomerase inhibitors, taxanes, vinca alkaloids, platinum compounds, bleomycin or ionizing radiation (Nicolay et al., 2016a; Munz et al., 2018; Nicolay et al., 2016b; Ruhle et al., 2017; Ruhle et al., 2018b; Liang et al., 2011; Nicolay et al., 2016c). In vitro, a relative resistance of MSCs against cisplatin, etoposide or vincristine has been reported, and bone marrow samples derived from cancer patients treated with these compounds have been demonstrated to contain proliferating MSCs, hence corroborating the resistance findings ex vivo (Mueller et al., 2006). However, hardly anything is known about the response of MSCs to antimetabolite treatment. An earlier report suggested that MSC proliferation was less affected by 5-FU treatment than by busulfan or doxorubicin exposure (Qi et al., 2012). In line with these findings, we observed that MSCs exhibited an increased viability compared to differentiated fibroblasts and maintained their ability for clonogenic proliferation. In another study, the influence of 5-FU on immortalized bone marrow stromal cells (HS-5) was compared with breast cancer (MCF-7) and colon cancer cells (HCT-116), and HS-5 cells were found more sensitive compared to cancer cells (Xiong et al., 2017). 5-FU treatment led to increased apoptosis rates and elevated intracellular reactive oxygen (ROS) levels in HS-5 cells resulting in impaired paracrine functions indicated by reduced secretion of hematopoietic growth

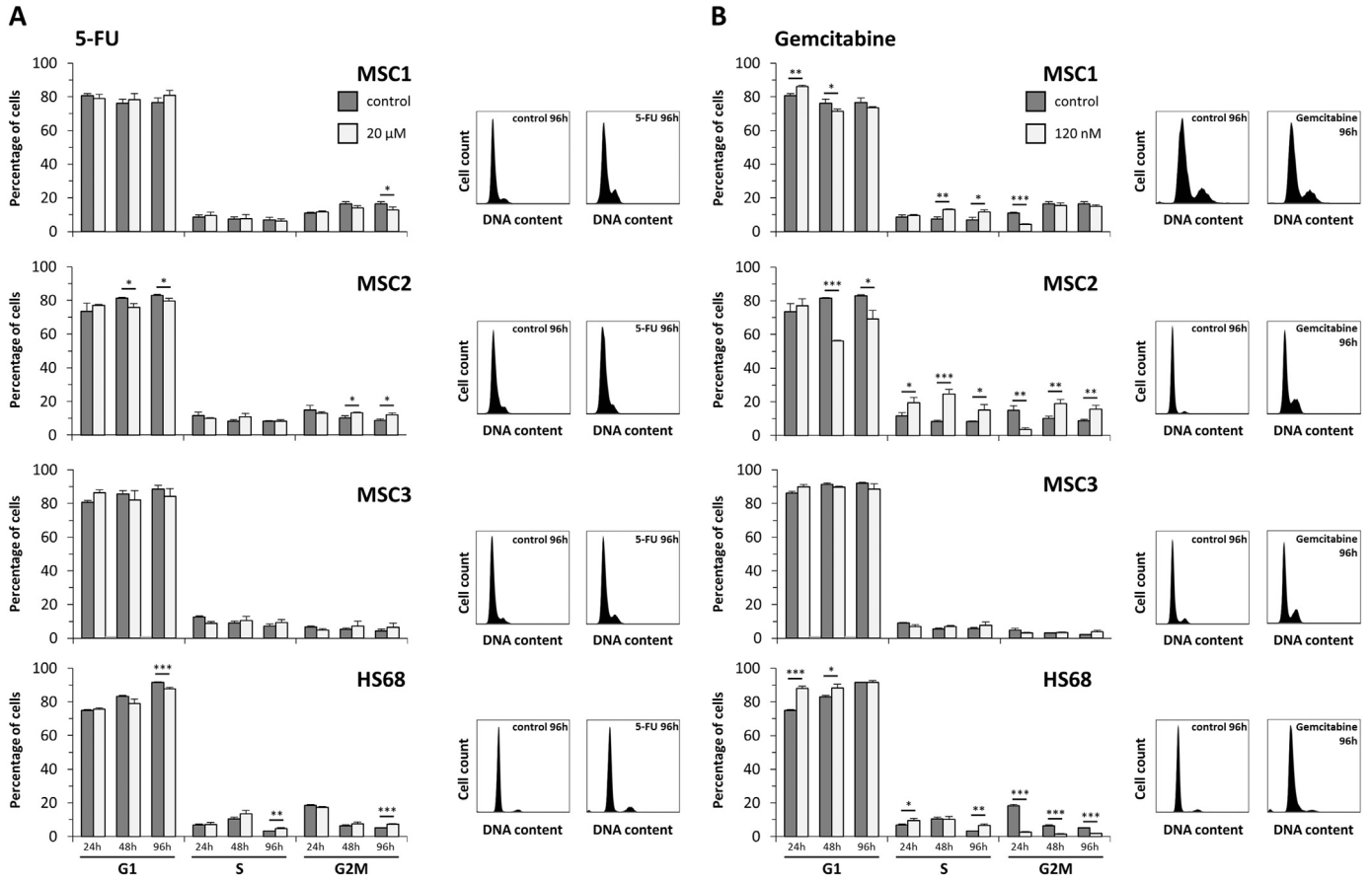


**Fig. 5.** Antimetabolite treatment does not result in cell cycle changes in MSCs. Cell cycle distribution of MSCs and HS68 fibroblasts at different time points after 4-h exposure to 20 µM 5-FU (A) or 120 nM gemcitabine (B). Histograms represent cell cycle distribution at 96 h after treatment. $*P < .05$, $**P < .01$, $***P < .001$ (Student's unpaired two-sided t-test).

factors. However, immortalized bone marrow stromal cells transduced with the human papilloma virus E6/E7 genes (which interfere with p53 and Rb) were used in this study, making comparisons to primary human MSCs difficult (Roecklein and Torok-Storb, 1995).

In our study, MSCs did not respond with increased apoptosis levels when exposed to the antimetabolites 5-FU or gemcitabine. This avoidance of apoptosis induction has also been observed in MSCs following treatment with other DNA-damaging agents and is believed to be due to high constitutive expression levels of various anti-apoptotic factors like Bcl-2 and Bcl-xL and an apoptosis modulation by proteins of the p53 family (Rylova et al., 2012; Lu et al., 2011; Liang et al., 2010; Nicolay et al., 2015b).

Beyond a relative resistance of MSCs regarding survival, our data demonstrated that these stem cells maintained their functional characteristics and surface marker profile after antimetabolite treatment. The ability to adhere to plastic surfaces is a defining hallmark of these stem cells that is widely used to select these cells in culture (Dominici et al., 2006). Neither 5-FU nor gemcitabine significantly reduced or delayed the MSCs' adhesion potential in comparison to untreated control cells. These findings correlate well with previous reports demonstrating no effect of other anti-cancer agents on the ability of MSCs for adherence, and gene expression analyses showed a drug-induced upregulation of various genes involved in MSC adhesion (Nicolay et al., 2013; Jin et al., 2008). The influence of antimetabolites on cellular migration has not yet been examined for MSCs, but investigations in other cell types generally reported reduced migratory potentials, depending on dosage and cell type (Alcouffe et al., 2004; Seo et al., 2017). In our dataset, the ability to migrate was not affected by treatment with 5-FU, while higher doses of gemcitabine resulted in a moderately reduced MSC motility.

The multi-lineage differentiation potential has been established as a defining cellular function of MSCs and a pre-requisite for the regenerative capabilities of these stem cells (Nicolay et al., 2015a). Our data demonstrated for the first time that the MSCs' adipogenic, osteogenic and chondrogenic differentiation abilities were generally preserved even after exposure to high doses of antimetabolite compounds 5-FU or gemcitabine. While no previous reports have examined the influence of antimetabolites on the differentiation of normal tissue stem cells, it has been suggested that gemcitabine was able to promote differentiation and maturation in different tumor and normal cell types while not affecting stem cell traits (Serra et al., 2008; Pei et al., 2014; Quint et al., 2012).

Antimetabolite chemotherapeutics exert their anti-cancer effects by inhibiting the cellular DNA and RNA metabolism, either by blocking relevant enzymes or by incorporation into nascent strands. Therefore, cellular resistance to antimetabolite treatment has been attributed to differing target enzyme activities, membrane transporter expression and the ability to remove incorporated compounds from the DNA, e.g. by base excision repair (Longley et al., 2003; Mini et al., 2006; Nordlund and Reichard, 2006; Negrei et al., 2016). In our dataset, expression levels of TYMS encoding thymidylate synthase, the key target enzyme for 5-FU, were considerably higher in MSCs than in differentiated fibroblasts, and higher expression has been previously linked to increased antimetabolite resistance (Peters et al., 2002). The increased viability of 5-FU-treated MSCs compared to adult fibroblasts may at least be in part related to the increased thymidylate synthase levels. UMPS encoding for uridine monophosphate synthase was observed at higher expression levels in MSCs than in adult fibroblasts, and

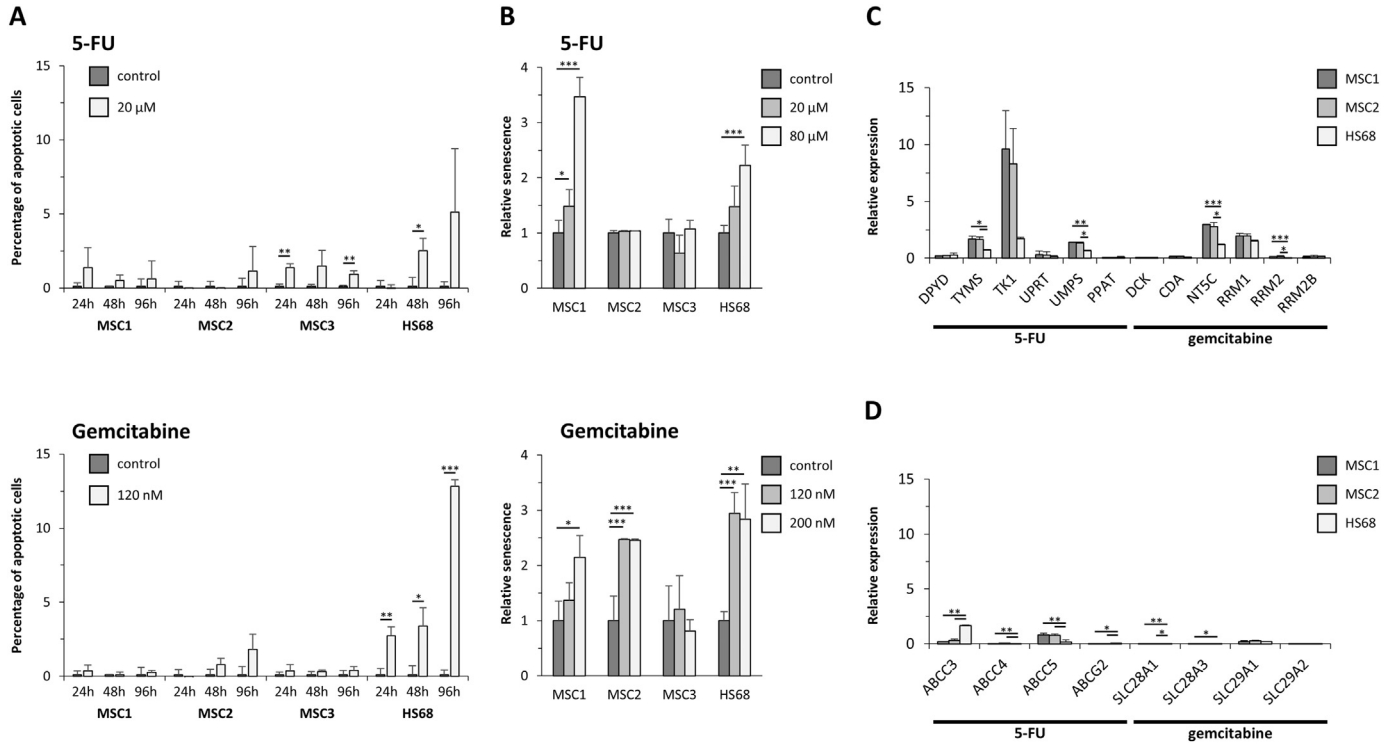


**Fig. 6.** MSCs exhibit low apoptosis levels compared to adult fibroblasts after exposure to 5-FU and gemcitabine. (A) Percentage of apoptotic cells as assessed by caspase-3 activation at various time points after antimetabolite treatment. (B) Relative β-galactosidase staining intensity of MSCs and HS68 fibroblasts at 96 h after 4-h treatment with 5-FU (upper panel) or gemcitabine (lower panel). (C, D) Relative mRNA expression for different proteins involved in antimetabolite metabolization (C) as well as in uptake and efflux (D).*$P < .05$, **$P < .01$, ***$P < .001$ (Student's unpaired two-sided t-test).

**Table 2**
Relative mRNA expression of various proteins involved in antimetabolite metabolization as well as in uptake and efflux(Mini et al., 2006; Nordlund and Reichard, 2006; Negrei et al., 2016). Values are presented as mean [ ± standard deviation].

| Gene | Protein | MSC1 | MSC2 | HS68 | Function |
|---|---|---|---|---|---|
| DPYD | Dihydropyrimidine dehydrogenase | 0.21 [0.02] | 0.24 [0.01] | 0.27 [0.19] | Rate-limiting step in 5-FU inactivation into dihydrofluorouracil |
| TYMS | Thymidylate synthase | 1.67 [0.26] | 1.64 [0.24] | 0.72 [0.06] | Target enzyme of 5-FU monophosphate |
| TK1 | Thymidine kinase 1 | 9.60 [3.37] | 8.30 [3.10] | 1.71 [0.11] | Conversion of 5-fluoro-2′-deoxyuridine into 5-fluoro-2′-deoxyuridine monophosphate |
| UPRT | Uracil phosphoribosyltransferase | 0.30 [0.33] | 0.27 [0.30] | 0.13 [0.10] | Conversion of 5-FU into 5-FU monophosphate |
| UMPS | Uridine monophosphate synthase | 1.39 [0.02] | 1.35 [0.09] | 0.66 [0.05] | Phosphorylation of 5-FU into 5-FU monophosphate |
| PPAT | Phosphoribosyl pyrophosphate amidotransferase | 0.07 [0.00] | 0.06 [0.00] | 0.10 [0.03] | Phosphorylation of 5-FU into 5-FU monophosphate |
| DCK | Deoxycytidine kinase | 0.07 [0.00] | 0.06 [0.02] | 0.05 [0.03] | Phosphorylation of gemcitabine into its monophosphate derivate |
| CDA | Cytidine deaminase | 0.15 [0.05] | 0.16 [0.01] | 0.09 [0.03] | Inactivation of gemcitabine |
| NT5C | 5′, 3′-nucleotidase | 2.98 [0.00] | 2.77 [0.37] | 1.21 [0.04] | Inactivation of gemcitabine by dephosphorylation of gemcitabine monophosphate |
| RRM1 | Ribonucleotide reductase M1 | 1.95 [0.21] | 1.96 [0.20] | 1.49 [0.11] | Target enzyme of gemcitabine (large subunit of ribonucleotide reductase) |
| RRM2 | Ribonucleotide reductase M2 | 0.13 [0.02] | 0.14 [0.07] | 0.03 [0.00] | Target enzyme of gemcitabine (small subunit of ribonucleotide reductase) |
| RRM2B | Ribonucleotide-diphosphate reductase subunit M2 B | 0.10 [0.08] | 0.15 [0.11] | 0.16 [0.01] | Target enzyme of gemcitabine (small subunit of ribonucleotide reductase) |
| ABCC3 | Canalicular multispecific organic anion transporter 2 | 0.19 [0.01] | 0.30 [0.11] | 1.67 [0.05] | Outward transport of 5-FU |
| ABCC4 | Multidrug resistance-associated protein 4 | 0.03 [0.00] | 0.04 [0.00] | 0.01 [0.01] | Outward transport of 5-FU |
| ABCC5 | Multidrug resistance-associated protein 5 | 0.80 [0.18] | 0.78 [0.11] | 0.18 [0.19] | Outward transport of 5-FU |
| ABCG2 | ATP-binding cassette super-family G member 2 | 0.01 [0.00] | 0.02 [0.00] | 0.04 [0.01] | Outward transport of 5-FU |
| SLC28A1 | Concentrative nucleoside transporter 1 | 0.02 [0.00] | 0.02 [0.00] | 0.00 [0.00] | Uptake of gemcitabine |
| SLC28A3 | Concentrative nucleoside transporter 3 | 0.02 [0.00] | 0.00 [0.01] | 0.00 [0.00] | Uptake of gemcitabine |
| SLC29A1 | Equilibrative nucleoside transporter 1 | 0.21 [0.08] | 0.27 [0.06] | 0.20 [0.02] | Uptake of gemcitabine |
| SLC29A2 | Equilibrative nucleoside transporter 3 | 0.02 [0.00] | 0.01 [0.00] | 0.03 [0.02] | Uptake of gemcitabine |

UMPS-transfected MSCs have been shown to exhibit a 5-FU-sensitive phenotype leading to increased apoptosis levels after 5-FU treatment (Kucerova et al., 2012).

As dihydropyrimidine dehydrogenase (DPYD) is the major enzyme in 5-FU degradation and DPYD deficiency is known to increase 5-FU-related toxicity, we analyzed the expression of the DPYS gene in MSCs and differentiated fibroblasts; however, DPYD expression was found at low levels with no differences between MSCs and adult fibroblasts (Johnson and Diasio, 2001). Similarly, expression of cytidine deaminase (CDA), the main enzyme involved in gemcitabine inactivation, was similar between MSCs and differentiated fibroblasts with overall low expression levels. Considering the low expression levels of the major

antimetabolite-inactivating proteins DPYD and CDA, these enzymes are presumably not involved in preservation of stem cell traits and low apoptosis levels of MSCs after antimetabolite treatment.

Additionally, gene expression for the multidrug resistance-associated transporters 4 and 5 (ABCC4 and ABCC5) was found at significantly higher levels in MSCs, although overall expression levels for 5-FU efflux transporters were relatively low. Consistently to our results, expression of ABCG2 has been reported as similar between adipose tissue-derived MSCs and adult fibroblasts in another study (Kucerova et al., 2008).

In our dataset, MSCs derived from different donors showed heterogeneous responses to antimetabolite treatment. MSCs constitute a heterogeneous population of different subpopulations, and varying isolation and cell culture techniques may result in distinct MSC preparations in vitro. Furthermore, donor's age and sex are known to influence proliferation and differentiation ability of MSCs (Siegel et al., 2013; Choumerianou et al., 2010; Alves et al., 2012). Regarding cellular motility, differentiation capacity and senescence induction, there were significant differences regarding the effects of antimetabolite treatment on MSCs derived from different donors. However, some effects of antimetabolite treatment such as preservation of stem cell characteristics and low apoptosis rates were observed in all analyzed MSC samples.

The ability of MSCs to survive and maintain their defining functional properties after antimetabolite treatment may be of clinical importance: Antimetabolite-induced tissue toxicities are manifold and affect the bone marrow, the gastrointestinal tract, the heart as well as skin and mucosa. There have been reports that 5-FU-associated mucositis could be improved by treatment with gingiva-derived MSCs, and MSCs were shown to regenerate a 5-FU-induced disruption of the tongue epithelial lining, thereby improving mucositis-dependent weight loss (Serry et al., 2017; Zhang et al., 2011). However, further data demonstrating potential benefits of MSC-based treatments for other antimetabolite-induced organ toxicities have not yet been reported, although it is conceivable that these stem cells may also exert protective and regenerative effects in this context as reported for other classes of cytotoxic drugs (Ruhle et al., 2018a; Carrancio et al., 2011; Battiwalla and Barrett, 2014).

Therefore, the relative resistance and functional preservation of MSCs after antimetabolite treatment as shown in the present dataset warrants further research into the use of these stem cells for the attenuation of antimetabolite-induced toxicities.

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## Author contributions

N.H.N.: conception of the study, experimental design and execution of experiments, paper writing. R.L.P., F.M., D.V., A.R., T.T., S.S., B.Z. and R.S.: execution of experiments. P.W., A.L.G., J.D. and P.E.H.: critical discussion of the data and paper. All authors reviewed and approved the final manuscript.

## Statement of ethics

The study protocol has been approved by the research institute's committee on human research.

## Disclosure statement

All authors declare that they have no conflict of interest.

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