

The effect of cachexia-inducing lung tumor-conditioned medium on the therapeutic potential of human mesoangioblasts

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Abstract

Half of all lung cancer patients develop cachexia, characterized by severe muscle wasting. This results in direct and indirect mortality by lowering anti-cancer therapeutic efficacy. There is no effective therapy for cachexia. Mesoangioblasts are vessel-associated progenitor cells that can be isolated from muscle biopsies, expanded *ex vivo* and migrate through the bloodstream into damaged muscle where they differentiate into muscle fibers. These stem cells have shown pre-clinical efficacy and clinical promise in treating muscular dystrophies and myopathies and have the potential to inhibit or reverse cachexia. We investigated if this potential could be affected by chemotherapeutics, administered to patients, and/or by factors secreted by the tumor itself. First, we assessed the effects of commonly used chemotherapeutics on mesoangioblast survival. Compared to lung cancer epithelial cells, the survival of mesoangioblasts was affected by cisplatin, but not by etoposide. Secondly, we investigated the effect of several tumor-conditioned media from (cachexia inducing-) lung tumor cell lines on mesoangioblast proliferation, transendothelial migration and differentiation. The tumor conditioned media had no effect on proliferation and migration but tumor-conditioned medium from a severe cachexia-inducing lung tumor cell line halted mesoangioblast differentiation, in combination with lower RNA levels of myogenin and myosin heavy chains 1 and 7. After removing this tumor-conditioned medium halfway (day 5) through the differentiation process, the myogenic index resumed to normal. Our data demonstrates that mesoangioblasts have the potential to combat lung cancer cachexia muscle wasting, but that in some cases the block on muscle differentiation needs to be overcome.

1. Introduction

Cachexia is a complex condition with physical, behavioral and metabolic clinical manifestations, which arises from an underlying disease [1, 2]. Most notable manifestations are loss of skeletal muscle mass [3] and adipose tissue [4, 5]. Cancer is a common cause and roughly half of all lung cancer patients develop cachexia [6, 7]. In advanced stage lung cancer these numbers increase to 70–80% [6, 7]. Direct mortality rate of cachexia in lung cancer patients is estimated at 20–30% [8]. Additionally, cachectic muscle wasting is indirectly linked to increased mortality in lung cancer due to lowering the efficiency of anti-cancer treatment [9, 10]. Underlying pathological mechanisms involve changes in both metabolism and tissue-damage responses. These include increased protein degradation and limited effects of caloric intake [1, 11]. Although a causal relationship has been established with chronic inflammation, it is unclear exactly how these underlying mechanisms develop into muscle wasting [1, 4, 12]. There is no cure for cachexia. Current cachexia therapies focus on nutrition, exercise and/or anti-inflammatory drugs [13].

As a novel therapeutic strategy to combat cachexia in lung cancer, we propose administration of patient-derived muscle stem cells called mesoangioblasts, which can induce systemic muscle regeneration. Mesoangioblasts are vessel-derived cells, which 1) have the capacity to extensively proliferate *ex vivo*, 2) migrate towards the affected muscles after intra-arterial injection, and 3) have a high myogenic potential [14–16]. This therapy has been preclinically tested in mice- and dog- models with muscular dystrophy

[17–21] and in clinical trials of patients with Duchenne muscular dystrophy [22] and with mitochondrial myopathy due to the m.3243A > G mutation in the mtDNA [23, 24]. Before proceeding to autologous transplantation of mesoangioblasts as treatment for cachexia, we needed to determine if the therapeutic potential is affected by the chemotherapy used and/or by tumor-derived factors. Therefore, we first investigated the effect of standard care lung cancer chemotherapy on the survival of human mesoangioblasts. We tested both etoposide and cisplatin, which are commonly administered intravenously after diagnosis of stage III non-small cell lung cancer (NSCLC) [25], often given at a dose for six times every three weeks. Next, we investigated the effect of the tumor factors, present in tumor-conditioned media, on proliferation, migration, and differentiation of human mesoangioblasts. Tumor conditioned media (CM) was produced using human lung epithelial cell lines that have been shown to cause non-, a mild or a severe cachectic phenotype when injected subcutaneously in mice [26]. Our results provide a first indication of the potential of mesoangioblast administration as a therapy to combat muscle wasting in lung cancer cachexia.

2. Methods

Cell culture

Human primary mesoangioblasts were cultured as described before [27]. Donors (50/50 F/M, n = 6) were an average age of 45.8 (range 23–74) years at the time of biopsy. Ethical approval was given by the Ethical Committee of Maastricht University Medical Center (clinicaltrial.gov NCT06077734, NCT05199740). In short, mesoangioblasts were isolated from the *m. vastus lateralis* and cultured in IMDM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate and 1x non-essential amino acids, 1x insulin-transferrin-selenium, 2-mercaptoethanol, 260 pM FGF-2 and 0.1% gentamycin. Cells were kept at 37 °C, at 4% O₂ and 5% CO₂.

Four human lung cancer epithelial cells were used [26]. H1650 (*EGFR/TP53*-mutant) and H1944 (*KRAS/STK11*-mutant) were obtained via Cyton, and BEAS-2B and A549 (*KRAS/STK11/TP53*-mutant) were kindly provided by the department of Respiratory Medicine, Maastricht University, NL. Cells were cultured in RPMI 1640 (ThermoScientific) supplemented with 10% FBS and 1% penicillin/streptomycin (p/s) at kept at 37 °C at 5% CO₂. Endothelial cells EA.hy926 cells (ATCC) were cultured in DMEM (ThermoScientific), supplemented with 10% FBS and 1% penicillin/streptomycin at 37C on 1.5% gelatin (Fluka Analytical)-coated flasks.

Inhibitory concentration curve (IC₅₀)

Mesoangioblasts, A549 and BEAS-2B were exposed to standard care chemotherapies etoposide and cisplatin for a period of 72 hours. Concentrations ranged from 0 uM – 1 mM. Cell-by-cell confluency was used to determine the inhibitory effect using Incucyte S3 Live Cell Analysis Instrument (Sartorius).

Conditioned media production

H1650, A549 and H1944 cell lines were switched to 0.13 ml/cm² mesoangioblast differentiation medium (DM), mesoangioblast growth medium (GM) or epithelial growth medium (RPMI) 24 hours after seeding. Media was collected after 72 hours and centrifuged at 1200 RPM for 5 minutes. FGF-2 was supplemented after mesoangioblast GM production to avoid activation of RAS/MAPK pathways in the epithelial cell lines. Seeding densities for the production of CM were based on the differences in relative proliferation rate (**Supplementary Fig. 1**) of H1650 (15,000 cells/cm²), A549 (8000 cells/cm²) and H1944 (24,000 cells/cm²).

Proliferation assay

Relative proliferation rate was determined during 96 hours via an Incucyte S3 Live Cell Analysis Instrument. Both growth rates of mesoangioblasts and lung epithelial cell lines were assessed following seeding of 5000 cells/cm² with various medium compositions. Control condition consisted of 100% mesoangioblast GM. Tumor CM and RPMI spike-in were both RPMI-based media diluted 1:1 with mesoangioblast GM, with RPMI spike-in acting as a negative control. Classical confluency was determined using Incucyte 2022 analysis software. Growth was defined as cell confluence over time.

Migration assay

EA.hy926 cells were seeded at a density of 20.000 cells/cm² onto 1.5% gelatin-coated 8 µm pore-size transwell inserts (ThermoScientific) according to Giannotta *et al.* [28]. A Dextran permeability assay ensured functional integrity of the monolayer. 250µg/ml 70 kDa FITC-dextran (Merck Life Science) was added to the upper compartment of the transwell and passively diffused into the lower compartment for over 4 hours. Diffusion was measured over multiple timepoints (15 min – 4 hours). Absorbance was measured at 490nm using the CLARIOstar plus Plate Reader (BMG Labtech). Apparent permeability index (*Papp*) was calculated using Eq. 1.

$$(1) Papp (cms^{-1}) = (1/AC_0) \cdot (\Delta Q/\Delta t) \text{ (Eq. 1)}$$

in which A = membrane surface area (cm²), C₀ = concentration Dextran in upper compartment t = 0 (nmol/ml), ΔQ/Δt = concentration Dextran bottom compartment as a function of time (nmol/s).

Mesoangioblasts were pre-starved 24 hours prior to migration in IMDM medium with 2% FBS. Mesoangioblasts were stained using 5 µM CMFDA cell tracker dye (ThermoScientific), diluted in serum-free culture medium for 30 minutes according to manufacturer's protocol. Medium in the upper compartment of each transwell was replaced with 2% FBS (low serum; control) DMEM supplemented with 1% penicillin G streptomycin (p/s), the lower compartment was filled with 10% supplemented DMEM. CM consisted of low serum medium with tumor factors. 30,000 mesoangioblasts were added to the upper compartment of each transwell to migrate across the endothelial layer for 24 hours. After migration, mesoangioblasts were quantified in the bottom chamber via measuring fluorescence (510 nm) using the CLARIOstar plus Plate Reader.

Myogenic Index

Mesoangioblasts were plated on Geltrex-coated glass slides with cell seeding density of 28,500 cells/cm² and switched to differentiation medium (DM; control) or DM-based CM compositions consisting of DMEM supplemented with 2% FBS and 1% p/s upon reaching full confluency. Mesoangioblasts were differentiated for 8 days and stained overnight for myosin II (Myosin 4 MF20 clone, eBioScience 14-6503-82) according to previously described [24]. MF20 positive nuclei were calculated using QuPath (v0.5.1). Myogenic Index was calculated via Eq. 2.

$$(2) \text{ Myogenic Index (MI)} = n \text{ MF20 positive nuclei} / n \text{ total nuclei (Eq. 2)}$$

mRNA isolations and quantitative polymerase chain reaction

Epithelial lung (tumor) cells and mesoangioblasts were lysed in TRIzol reagent (ThermoScientific). RNA extraction was performed using the Direct-Zol RNA Miniprep kit (ZYMO Research) according to the manufacturer's instructions. cDNA was synthesized using the qScriptTM cDNA Supermix kit (QuantaBio). Expression levels of cell cycle genes tumor protein p53 (*TP53*), cyclin dependent kinase inhibitor 1a (*CDKN1A*), Inhibitor of DNA-binding/differentiation 3 (*ID3*) and Nuclear factor 1A (*NF1A*) and myogenic markers Myoblast determination protein 1 (*MYOD*), Myogenin (*MYOG*), Myosin heavy chains (*MYH*) *MYH1* and *MYH7* were determined using a LightCycler 480 Instrument (Roche). Primers and settings can be found in **Additional** Table 1. Relative expression was calculated after normalization to *TBP* expression using the $2^{-\Delta\Delta C_t}$ method.

Statistics

Before each statistical test, homoscedasticity and normality was checked. All statistical significance was tested using One-way ANOVA with Tukey correction unless stated otherwise. Two-tailed testing was applied. All statistics were performed via GraphPad Software Inc., v 8.4.3. Ct-value differences > 0.5 within technical replicates were excluded from further analysis during qPCR data analysis. Statistical significance was considered as P-value ≤ 0.05 .

3. Results

Mesoangioblasts are less sensitive to etoposide than lung tumor epithelial cells

Mesoangioblasts of two donors were exposed to cisplatin and etoposide at various concentrations for a period of 72 hours and compared to lung tumor cells A549 and BEAS2B (Fig. 1A, 1B). Plateaus with an area under the curve (AUC) of 0.684 (0.039 SE) and 0.712 (0.062 SE) were observed in Donor 1 and Donor 2, respectively (Table 1). Both had significantly larger AUCs compared to A549 and BEAS2B, suggesting that the mesoangioblasts were less sensitive for cytostatic effects of topoisomerase II inhibitor etoposide than the tumor line A549 and immortalized control BEAS2B. While IC₅₀ values of cisplatin were significantly different between groups, both mesoangioblast lines treated with alkylating

agent cisplatin remain equally sensitive to the compound compared to A549 cells and BEAS2B, which is reflected in the AUC.

Table 1
IC50 and Area under the curve (AUC) values of etoposide and cisplatin on human mesoangioblasts compared to adenocarcinoma and lung epithelial cells.
IC50: Non-linear fit, Sum-of-squares F test. ☒ = statistically significant between all groups (P-value ≤ 0.0001). AUC: ** = statistically significant compared to A549 (P-value ≤ 0.01); # = statistically significant compared to BEAS2B (P-value ≤ 0.05).

Cell line	Etoposide		Cisplatin	
	IC50 [uM]	AUC ± SE	IC50 [uM] (☒)	AUC ± SE
Donor 1	1.35	0.684 ± 0.039 (**/#)	7.57	0.686 ± 0.017
Donor 2	1.23	0.712 ± 0.062 (**/#)	27.35	0.661 ± 0.030
A549	1.09	0.469 ± 0.033	3.62	0.652 ± 0.056
BEAS2B	0.07	0.510 ± 0.038	1.45	0.605 ± 0.033

Mesoangioblast proliferation capacity remains unchanged by tumor conditioned medium

Conditioned media (CM) was produced to expose mesoangioblasts to tumor factors of three cell lines H1650, A549 and H1944, which cause, respectively, a non-, mild or severe cachectic phenotype when injected subcutaneously in mice, to study its effects on proliferation, migration and differentiation over a course of 96 hours (Fig. 2A). No significant differences in proliferation rate were detected between the experimental conditions (CM) or vehicle condition (RPMI) compared to control (GM). All groups reached cell confluency between 30–50% after 96 hours with no significant differences (Fig. 2B, 2C). Additionally, expression levels of proliferation genes, including NFIA, TP53, ID3 and CDKN1A (P21), were measured via quantitative PCR (Fig. 2D). mRNA expression levels of most genes remained unchanged in all groups after 48 hours incubation. Only muscle differentiation-inhibitor ID3 was 1.6-fold increased in A549-exposed mesoangioblasts compared to control GM media (0.70 (0.15) SD vs 1.125 (0.05), P = 0.047).

Transendothelial migratory capacity of mesoangioblasts was not affected by exposure to tumor conditioned media

The capacity of mesoangioblasts to migrate through an endothelial monolayer after treating them with control and tumor conditioned media was determined in a transwell system. This system was optimized and leakage was functionally tested with Dextran. Gelatin coated inserts with an intact cell monolayer of EA.hy926 cells showed minimal Dextran leakage (**Additional Fig. 2**). Mesoangioblasts cultured for 72 hours in control differentiation medium (DM) or conditioned medium of the 3 tumor cell lines were introduced in the top-well of the optimized system (Fig. 3A). Around 51–55% migrated across the endothelial layer within 24 hours. No significant differences were observed between mesoangioblasts cultured under different conditions (Fig. 3B), indicating that the tumor-conditioned media tested did not interfere with mesoangioblast transendothelial migration.

Early mesoangioblast differentiation is disrupted after exposure to conditioned media from cachexia-inducing H1944-cell line

Mesoangioblasts were differentiated into myotubes over a span of 8 days in the presence of DM-based CM (Fig. 4A). When exposed to H1944-CM, Myosin 4 (MF20) revealed a significant 30.2% decrease in differentiation capacity, compared to control media and the other tumor-conditioned media (Fig. 4B). This is also reflected in the significantly 10-fold decrease of MF20 positive area per nucleus (Fig. 4C), indicating impaired myogenic differentiation or the activation of muscular atrophy pathways. Gene expression markers revealed a significant decrease of almost half (0.42 fold (0.39)) in early differentiation marker Myogenin (*MYOG*) only when exposed to H1944, which was continued by decreased late myogenic markers *MYH1* and *MYH7* at both day 3 and day 8 of differentiation (Fig. 4D). To confirm myogenic commitment, which requires cell cycle exit, levels of cell cycle regulatory gene expression in differentiated mesoangioblasts were tested with or without H1944-CM compared to proliferative mesoangioblasts in growth medium (Control-GM) and differentiating mesoangioblasts (Control-DM). Proliferative marker *PCNA* showed comparable expression levels between both exposures at day 3 and 8 (Fig. 4E). *CDKN1A* expression level after H1944 exposure at day 8, however, remains low when compared to Control-DM on day 8 (3-fold vs 6-fold compared to day 0, respectively). Additionally, there was no difference in the number of nuclei observed after differentiation, thus loss of cells is not expected to be a cause for the discrepancies (Fig. 4F).

Disruption of mesoangioblast differentiation by cachexia-inducing H1944-conditioned medium is reversible

A recovery experiment was performed to determine the reversibility of myogenesis repression by H1944-CM. To this end, mesoangioblasts were differentiated for 8 days while exposed to either normal DM or H1944-CM, or 4 days of H1944-CM followed by 4 days of normal DM (recovery condition). Although to a lesser extent than controls, recovered mesoangioblasts were able to produce more myofibers as visually confirmed by MF20 expression compared to 8-day H1944-exposed mesoangioblasts (Fig. 5A). MI and MF20 area of H1944-CM mesoangioblasts are both decreased as expected after 8-day exposure (0.015% (0.01) and $0.009 \mu\text{m}^2$ (0.005); $P < 0.005$) and show a trend for recovery when switched back to DM after 4 days (0.08% (0.05) and $0.039 \mu\text{m}^2$ (0.014), respectively) (Fig. 5B, 5C). Upregulation of *MYOD* indicated that initiation of myogenic differentiation of mesoangioblasts was possible, though expression of late myogenic markers *MYH1* and *MYH7* at day 8 remained unchanged compared to H1944-CM (Fig. 5D).

4. Discussion

Our study investigated the potential of mesoangioblasts to be used in an autologous muscle stem cell transplantation to combat lung cancer cachexia muscle wasting. To this end, it is crucial that the mesoangioblasts 1) are resistant to cancer treatment, as treatment generally starts in the first weeks of diagnosis [29]; 2) can home to the affected muscle via migration, and 3) maintain their proliferative- and

myogenic capacity in the cancer environment. This was done by assessing the effects of the commonly used chemotherapy for lung cancer, a combination of etoposide and cisplatin, on mesoangioblasts and lung (tumor) epithelial cells. Furthermore, the effects of secreted lung tumor factors on mesoangioblast proliferation, transendothelial migration and differentiation were investigated with the use of several tumor conditioned media of lung tumor cells, causing non-, mild or severe cachexia in mice models, which served as a proxy for the cancer environment.

We found that mesoangioblasts were less sensitive to etoposide, but equally sensitive to cisplatin -which are standard care chemotherapies- compared to lung epithelial cancer cells. Although IC50 values were higher for mesoangioblasts when exposed to cisplatin, there was no significant difference between AUC compared to the lung epithelial cancer cells. Whereas IC50 values are easy to interpret, AUC additionally accounts for the strength of effect [30, 31]. Our results indicated that the use of chemotherapeutics could potentially negatively impact the survival of mesoangioblasts following transplantation. While the median time from diagnosis to treatment is only 42 days [29], this implies that mesoangioblast *ex vivo* isolation and administration and would have to be combined with chemotherapy. A more extensive screening of all chemotherapeutics used in the clinic to treat lung cancer would be required to determine the cocktails which would still be effective to treat the tumor and would be suitable to combine with mesoangioblast treatment. For example, analogues of cisplatin such as carboplatin, which has been shown to induce less aspecific cytotoxicity [32], could be a starting point to replace cisplatin.

Our data showed that lung tumor epithelial H1944 conditioned medium prohibited the differentiation of mesoangioblasts into myotubes process, whereas tumor CM H1650 and A549 did not. All the cell lines used to produce tumor conditioned media were injected into non-obese diabetic severe combined immunodeficient mice to induce tumor formation [26]. Injecting H1650 tumor cells did not lead to a significant body mass loss in mice (non-cachectic), whereas injection of A549 and H1944 tumor cells resulted in the development of, respectively, a mild and severe cachectic phenotype, the latter characterized by a lean body mass loss of 20% and fat mass loss of 50% [26]. Moreover, we found reduced RNA expression of *MYOG*, *MYH1* and *MYH7* at 3 and 8 days after H1944 exposure compared to either control media or non-cachectic H1650-CM. Apparently, there is a relation between the severity of the cachexia induced by tumor factors and the inhibition of mesoangioblast differentiation. A similar study, testing the effect of tumor conditioned medium on C2C12 myotube differentiation showed a reduction in overall myosin protein content at day 4 after addition of cachexia-inducing Lewis lung carcinoma (LLC) CM (*KRAS*^{G12D}) and human lung tumor CM, but no differences were seen at day 7 anymore [33]. Although they described timing as one of the attributing factors, we argue that the specific stimuli might not have been present. Our results showcase the ability of our model to capture pathophysiological relevant tumor secretome factors and their outcomes on myotube development, similar to the work of Jackman *et al.* who showed a reduction in myotube development under the influence of cachexia-inducing colon-26 (C26) and Ehrlich carcinomas CM [34]. However, continuous release was applied via a transwell system, which honors the kinetics of tumor secretions *in vivo*; arguably a more sophisticated approach for the effect of CM on mesoangioblast differentiation.

As a follow-up we investigated at which stage mesoangioblast differentiation was halted and if this effect was reversible or not. The initial myogenic commitment is characterized by the halt of proliferation and expression of *MYOD* [35]. We found similar mRNA expression of cell cycle genes and *MYOD* in mesoangioblasts after exposure between control and H1944 conditioned medium. Moreover, we found similar numbers of nuclei between conditions during differentiation. Low expression (Fig. 4) and high expression (Fig. 5) of *MYOD* at day 8 can be attributed to the multiple functions of *MYOD*, regulating proliferation rather than differentiation. *MYOD* plays a key role in coordinating the switch from proliferating to differentiating muscle stem cells [36]. As differentiation processes have biological variations explaining the difference in myogenic switch, we conclude that early myogenic differentiation rather than the initial myogenic commitment of the mesoangioblast is affected as shown by the *MYOG* decrease in both. This is in line with to earlier findings, where following exposure to cachectic conditions, primary myoblasts demonstrated reduced RNA levels of *MYOG* and *MYH*, and satellite cells normal myogenic commitment but altered differentiation as a result of lowered *MYOG* levels [37, 38]. Importantly, we found that the effects of H1944 tumor CM on mesoangioblast differentiation were at least partially reversible when the tumor conditioned medium was exchanged by control medium, as demonstrated by the partial recovery of the myogenic index and mRNA levels of *MYOG*, *MYH1* and *MYH7*.

In spite of their negative impact on myogenic differentiation, no effects of the tumor conditioned media on mesoangioblast proliferation were found during the study. Although *ID3* mRNA levels were upregulated 1.6-fold after exposure to A549 conditioned medium, no significant differences in proliferation rates were found. This is in line with previous literature that has shown tumor factors did not inhibit satellite cell proliferation [37–40], which resemble mesoangioblasts in myogenic commitment and function [16, 41–43]. Moreover, no effects of the tumor conditioned media on transendothelial migration were found, which is needed for mesoangioblast homing after intra-arterial administration. Animal models mimicking (lung) cancer cachexia could elucidate the tumor-muscle crosstalk, survivability and migration of the intra-arterially injected cells, and consequently the therapeutic capacity of mesoangioblasts to combat cancer-associated cachexia. While full characterization of the tumor conditioned media was beyond our scope, our strategies could be refined further if the responsible secretome factor(s) and exact affected pathways were identified [44, 45]. Knowing these factors could help develop a predictive tool to anticipate differences in mesoangioblast therapeutic efficacy between cachectic patients if their serum is analyzed for the presence of these specific secretome factors. Finally, pre-loading mesoangioblasts with transcripts such as *MYOG* or a miRNA cocktail has shown to promote myogenic differentiation in mesoangioblasts and could be a promising strategy to bypass inhibition of myogenic differentiation [46].

5. Conclusion

We investigated the potential of mesoangioblast administration as a novel treatment of cachexia in lung cancer patients. The results of our studies showed that mesoangioblast proliferation and transendothelial migration were not affected by any of the tested tumor conditioned media. Only the

severe cachexia-inducing conditioned medium inhibited early myogenic differentiation, but this inhibition was reversible. Therefore, development of autologous mesoangioblast therapy can potentially reverse muscle wasting in lung cancer cachexia patients, although in some cases a block on myogenic differentiation needs to be overcome.

Declarations

Ethics approval and consent to participate: Primary human mesoangioblasts from skeletal muscle biopsies were isolated after written informed consent was obtained for participation in the study and the use of samples. Ethical approval was given by the Ethical Committee of Maastricht University Medical Center (NL81090.068.22, approval date 26-07-2023 and NL78411.068.21, approval date 24-12-2021). Lung adenocarcinoma cell lines NCI-H1650 and NCI-H1944 were obtained from Cytion (previous CLS) after signing a Non-Profit Supply Agreement.

Consent for publication: Not applicable

Availability of data and materials: All data generated or analysed during this study are included in this published article and its additional information files.

Competing interests: H.J.M.S. is CEO and shareholder of Milocron Therapeutics BV, a muscle stem cell therapy company.

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Authors' contributions: NW and NPJW equally contributed to the experimental design, all data interpretation, and writing of the manuscript. NPJW additionally produced the tumor conditioned media and executed cell experiments. NW additionally performed cell experiments regarding chemotherapies and statistical analysis of all data. FT contributed to the experimental design and provision of the human cell lines. ET performed isolations of the primary cells from skeletal muscle tissue biopsies. RL contributed towards experimental design and data interpretation. HS was involved in the experimental design, data interpretation and was a major contributor in writing of the manuscript. All authors read and approved the final manuscript.

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Figures

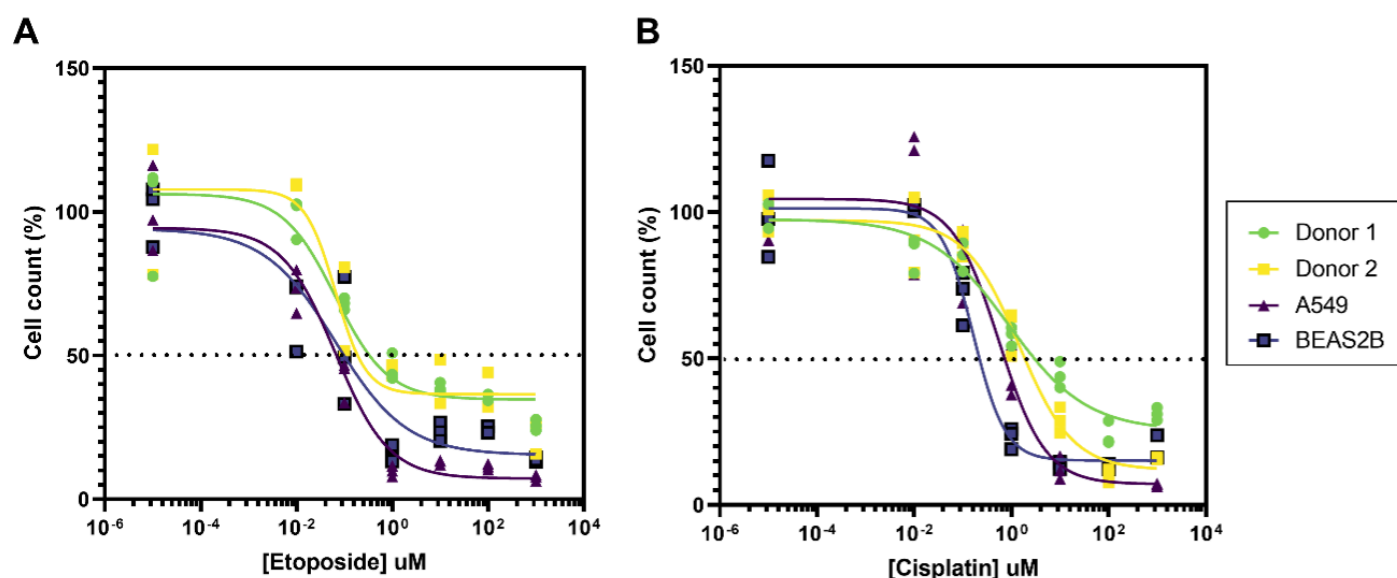


Figure 1

Effects of standard care chemotherapy on viability of human mesoangioblasts compared to lung adenocarcinoma and lung epithelial cells. IC_{50} based on donor mesoangioblasts and lung (tumor) cell count after exposure of chemotherapies etoposide (A) and cisplatin (B) for 72 hours ($n=3$).

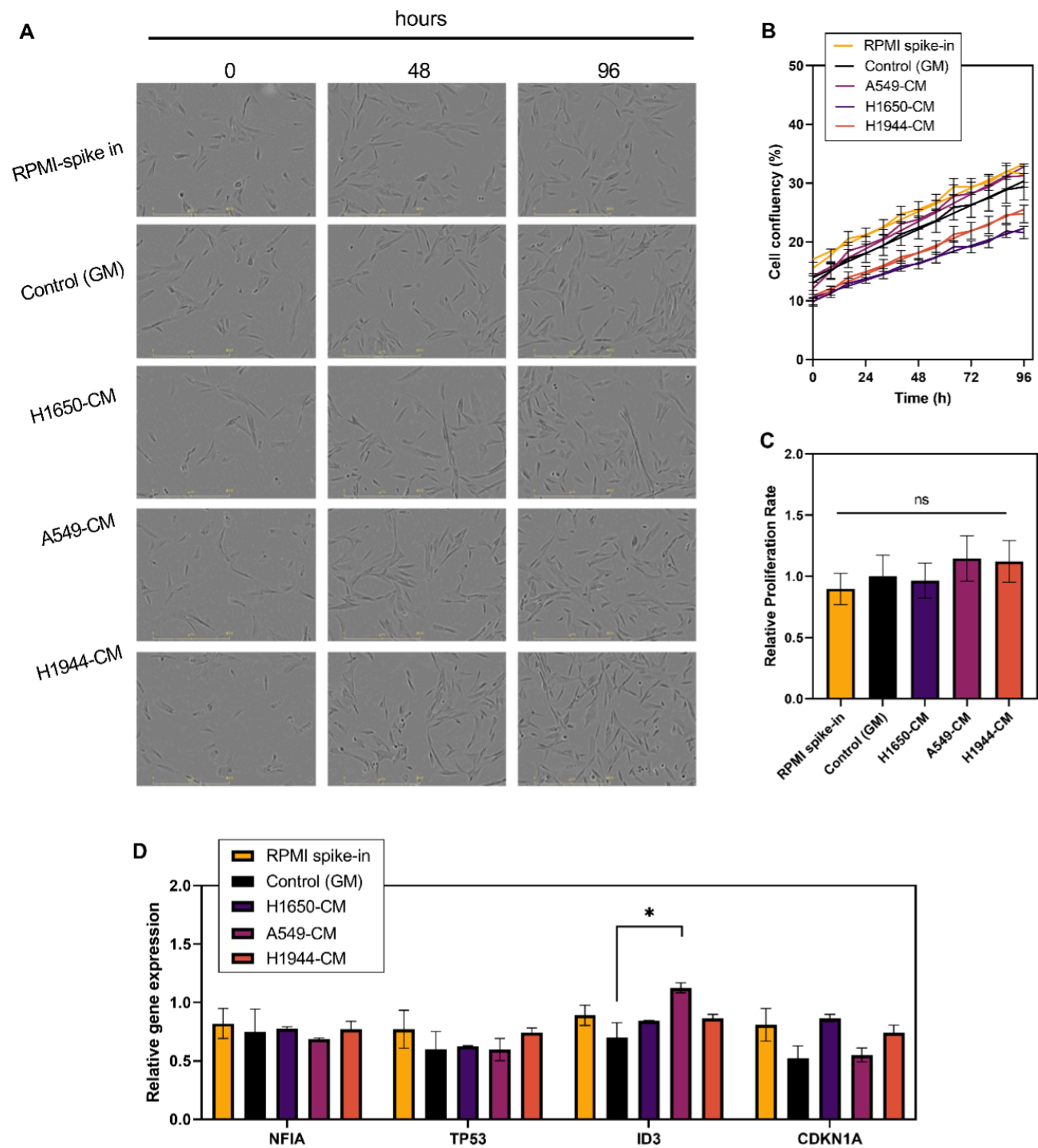


Figure 2

Assessment of proliferative capacity in mesoangioblasts in response to normal- and conditioned media. The control condition consisted of normal mesoangioblast growth medium (GM). RPMI spike-in consisted of 1:1 RPMI dilution with mesoangioblast GM. Experimental conditions consisted of RPMI-based CM diluted 1:1 with mesoangioblast GM. A: Brightfield images of mesoangioblast confluency over time for the quantification of confluency. B-C: proliferation curves and relative proliferation rate of mesoangioblasts

growing in mesoangioblast-GM, CM, or RPMI media in a 1:1 ratio with mesoangioblast-GM. D: relevant mRNA expression levels of cell cycle genes after 48h of exposure. E: Detection of 30.000 pre-starved mesoangioblasts over a 100.000-cell monolayer of EA.hy926. Experimental conditions were added in the upper compartment and migration was assessed over 24 hours. Data is presented as mean (SD). * = P-value $\leq$ 0.05.

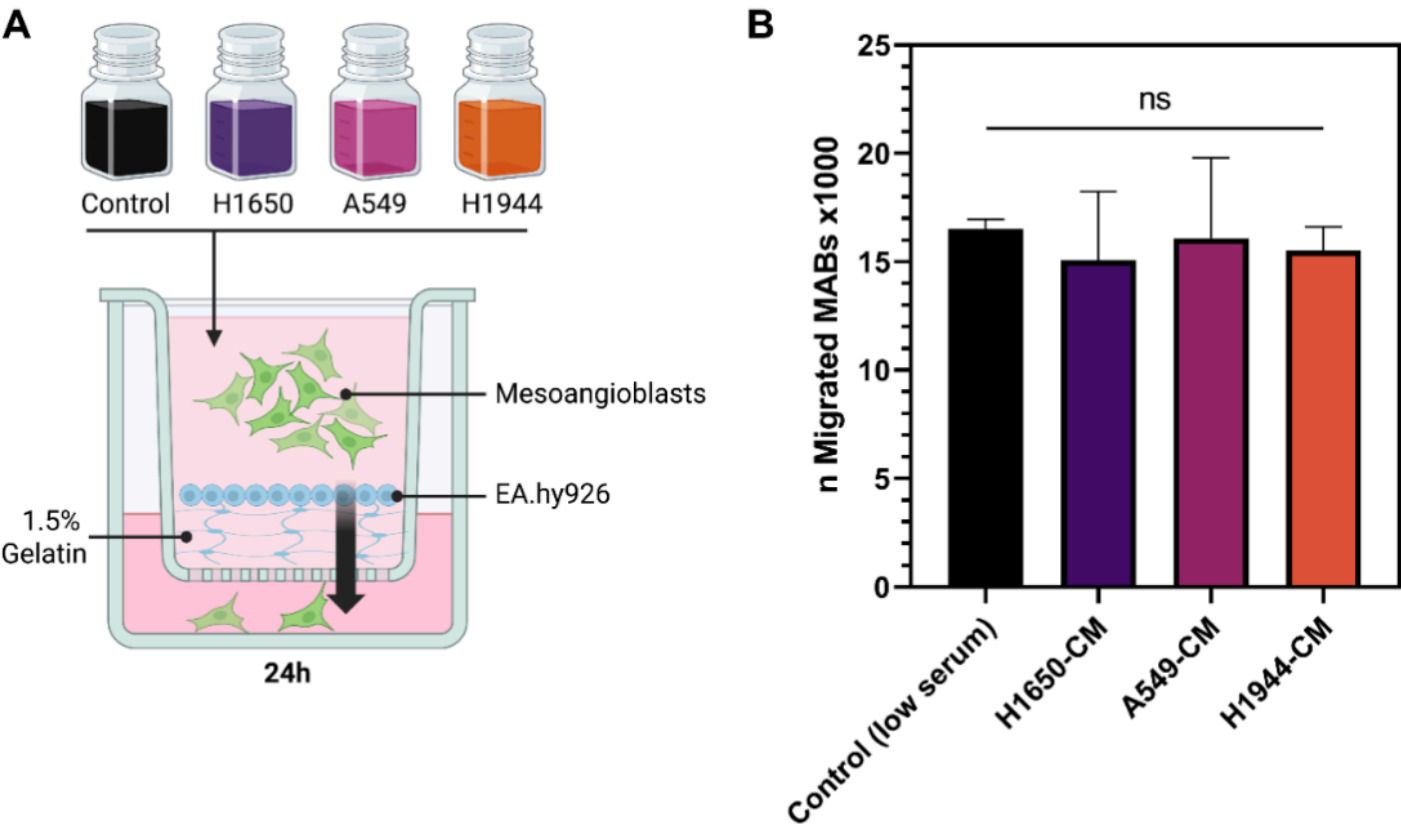


Figure 3

Transwell assay of mesoangioblasts migration through monolayer-forming endothelial cells. A: Experimental setup of migration assay. Experimental conditions were added in the upper compartment and migration was assessed over 24 hours. Figure created in BioRender, Wanders N. (2026). B: Detection of 30,000 pre-starved mesoangioblasts over a 100,000-cell monolayer of EA.hy926. Data is presented as mean (SD).

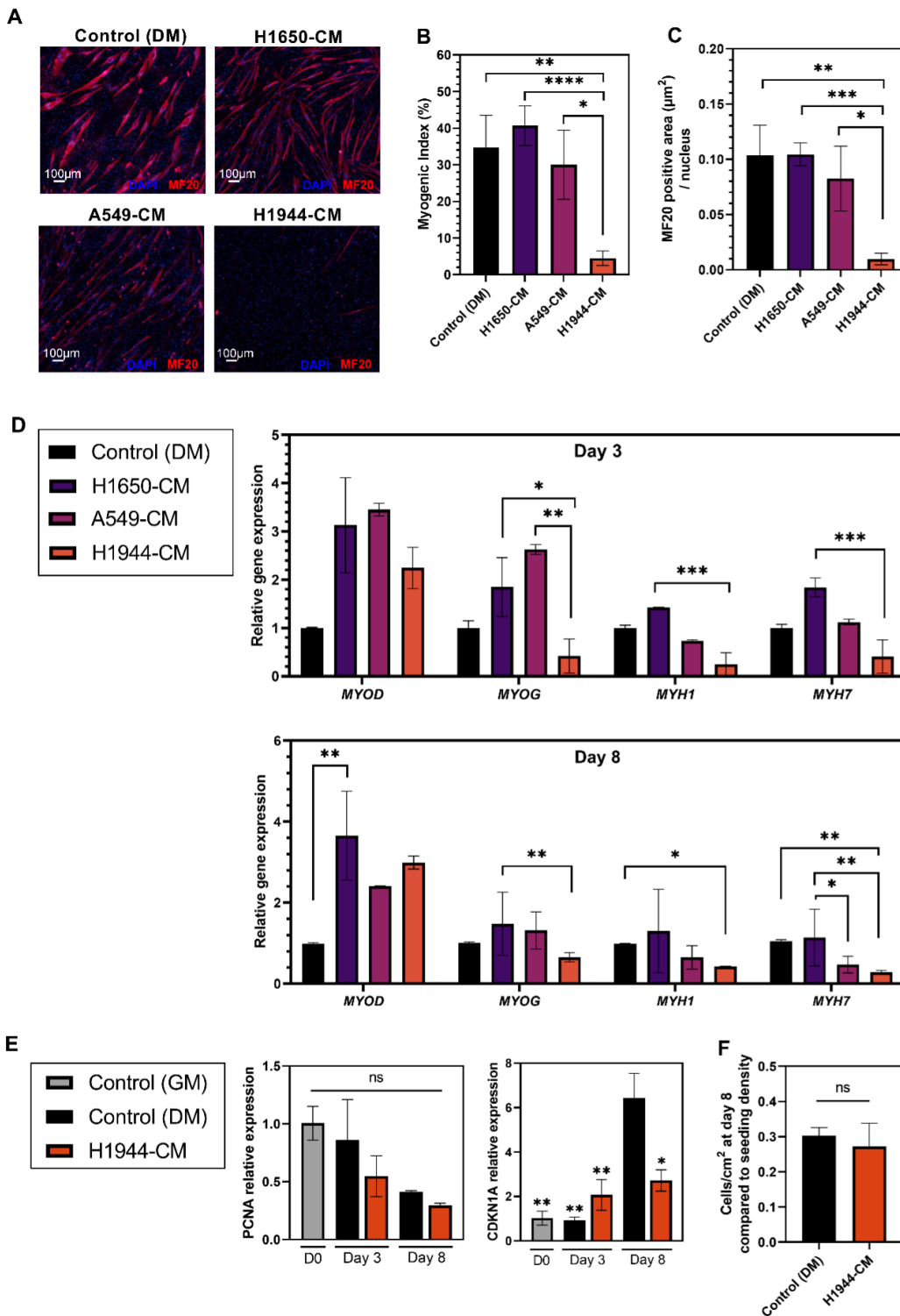


Figure 4

Skeletal muscle differentiation capacity of mesoangioblasts during conditioned medium exposure. A: Representative fluorescent images at day 8 of differentiation stained for Myosin 4 (MF20, red) and nuclei (DAPI, blue). B: Quantification of percentage of nuclei in MF20-positive cells as a measure for myogenic capacity. Kruskal-Wallis test, Dunn's correction. C: Quantification of the area of MF20-positive muscle fibers. Kruskal-Wallis test, Dunn's correction. D: Gene expression levels during mesoangioblast differentiation at

day 3 (top) and day 8 (bottom). Kruskal-Wallis test, Dunn's correction. E: gene expression levels during mesoangioblast differentiation at day 0 (D0), 3 and 8. Significance shown for comparison to H1944-CM day 8. Data is presented as mean (SD). * = $P\text{-value} \leq 0.05$, ** = $P\text{-value} \leq 0.01$, *** = $P\text{-value} \leq 0.005$.

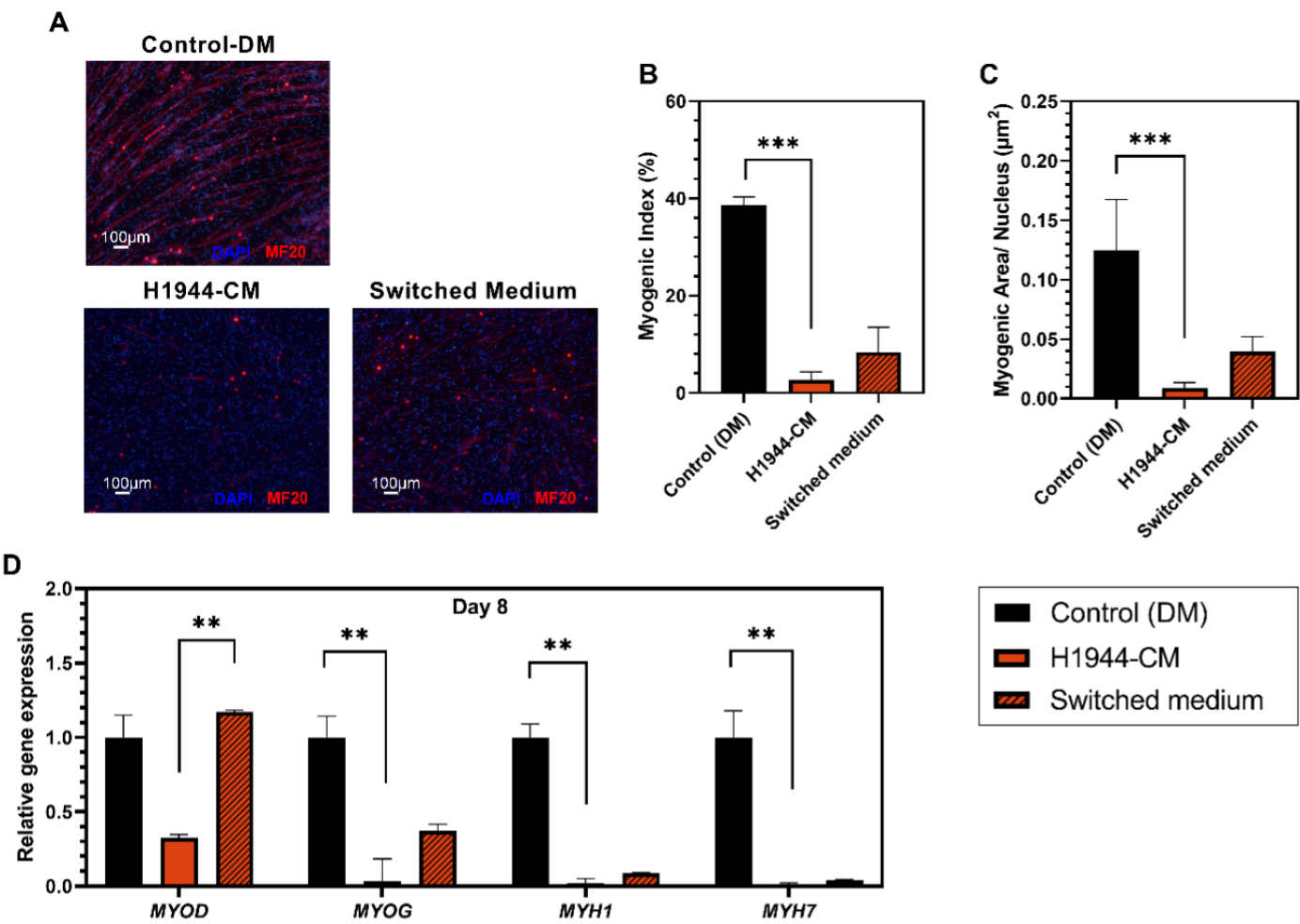


Figure 5

Skeletal muscle differentiation capacity of mesoangioblasts during a recovery experiment. A: fluorescent images at day 8 of differentiation stained for Myosin 4 (MF20, red) and nuclei (DAPI, blue). Medium was changed after 4 days to DM in the switched condition. B: Quantification of percentage of nuclei in MF20-positive cells as a measure for myogenic capacity. C: Quantification of the area of MF20-positive muscle fibers. D: Gene expression levels during mesoangioblast differentiation at day 8. B-D: Kruskal-Wallis test, Dunn's correction. ** = $P\text{-value} \leq 0.01$, *** = $P\text{-value} \leq 0.005$. Data is presented as mean (SD).

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