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Human Muscle Precursor Cells Overexpressing PGC-1α Enhance Early Skeletal Muscle Tissue Formation

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Muscle precursor cells (MPCs) are activated satellite cells capable of muscle fiber reconstruction. Therefore, autologous MPC transplantation is envisioned for the treatment of muscle diseases. However, the density of MPCs, as well as their proliferation and differentiation potential, gradually declines with age. The goals of this research were to genetically modify human MPCs (hMPCs) to overexpress the peroxisome proliferator-activated receptor γ coactivator (PGC-1α), a key regulator of exercise-mediated adaptation, and thereby to enhance early skeletal muscle formation and quality. We were able to confirm the sustained myogenic phenotype of the genetically modified hMPCs. While maintaining their viability and proliferation potential, PGC-1α-modified hMPCs showed an enhanced myofiber formation capacity in vitro. Engineered muscle tissues were harvested 1, 2, and 4 weeks after subcutaneous injection of cell–collagen suspensions, and histological analysis confirmed the earlier myotube formation in PGC-1α-modified samples, predominantly of slow-twitch myofibers. Increased contractile protein levels were detected by Western blot. In summary, by genetically modifying hMPCs to overexpress PGC-1α, we were able to promote early muscle fiber formation in vitro and in vivo, with an initial switch to slow-type myofibers. Therefore, overexpressing PGC-1α is a novel strategy to further enhance skeletal muscle tissue engineering.

Key words: Human muscle precursor cells (hMPCs); Differentiation; Skeletal muscle tissue engineering; Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α)

INTRODUCTION

Damage or loss of skeletal muscles is a major concern in many diseases. Autologous stem cell therapy is on the doorstep to successful clinical application and represents a novel treatment option for various muscle-related pathologies, including urinary incontinence[1], vocal cord dysfunction[2], and vesicoureteral reflux[3]. Satellite cells are quiescent cells, residing underneath the basal lamina of skeletal muscle fibers. They get activated after muscle tissue injury, turning into proliferating myoblasts or muscle precursor cells (MPCs). MPCs grant sufficient progeny for tissue repair[4,5]. Because of their potential to form new contractile myotubes, these cells are being investigated for muscle tissue engineering and reconstruction in the treatment of a variety of muscle disorders[6–9].

Despite recent progress in the field of muscle tissue bioengineering, a decreased proliferative capacity of MPCs due to donor age remains the main shortcoming of this approach[10–12]. This challenge for autologous cell therapy may be addressed by exercise and/or therapeutic regulation of gene expression, which enhances the ability of MPCs to restore muscle fibers. The transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a key player in neuromuscular activity of skeletal muscle and regulates important exercise-mediated adaptations[13,14]. The PGC-1α expression in a muscle is proportional to the amount of exercise and protects skeletal muscle cells from atrophy, thereby being beneficial for cell survival[15]. It serves as a transcriptional coactivator of nuclear receptors and transcription factors, which play essential roles in the regulation of cellular differentiation, development, and metabolism (carbohydrate, lipid, and protein) of higher organisms[15,16]. Hence, PGC-1α is regulating the mitochondrial biogenesis and is adapting the

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oxidative state in muscles[14]. Increase in PGC-1α levels goes along with a shift in the myofiber composition toward high-endurance muscle fibers that are capable of sustaining long-term contractions[17]. Slow-twitch myofibers mostly use oxygen as an electron acceptor to generate energy [i.e., adenosine triphosphate (ATP)], which is gradually released for sustained, long contractions, resistant to fatigue[18]. Additionally, this high-endurance muscle phenotype is characterized with pronounced tissue vascularization, increased mitochondria and myoglobin levels, and enhanced import of glucose, lipids, and lactate[18]. Similar changes are observed in adaptation to exercise, a process also mediated by PGC-1α. The therapeutic potential of PGC-1α in a metabolic optimization strategy is currently being investigated and highlights the importance of novel approaches for successful muscle tissue engineering[19].

Muscle reconstruction using human MPCs (hMPCs) is a promising and feasible therapy method. However, further improvements toward engineering of larger muscles, functional myofiber formation, and increased integration into the host tissue are needed. Our project thus aimed at the generation and validation of a viral vector for ectopic expression of PGC-1α in hMPCs in order to assess the therapeutic potential of in vivo bioengineering of skeletal muscle tissue in a mouse model. We hypothesize that initial overexpression of PGC-1α would improve muscle cell survival, myofiber formation capacity, and expression of muscle-specific proteins in the hMPCs. Moreover, it would induce a myofiber-type switch into slow-type myofibers, which are highly desirable for sphincter muscle bioengineering.

MATERIALS AND METHODS

Isolation and Expansion of hMPCs

Human muscle biopsies from the M. rectus abdominis were randomly collected upon ethical approval by the local Swiss ethics institutions and after written informed consent of hospitalized patients undergoing abdominal surgery under general anesthesia. The samples were processed according to established protocols[20]. Briefly, each muscle biopsy was first minced and digested with collagenase type I 0.2% (w/v; Sigma-Aldrich, St. Louis, MO, USA) and dispase 0.4% (w/v; Gibco, Grand Island, NY, USA). The enzymatic reaction was terminated with medium containing 10% fetal bovine serum (FBS; HyClone®; Thermo Fisher Scientific, Waltham, MA, USA). Individual myofibers were then liberated by rigorous pipetting and filtered through a strainer with a pore size of 100 μm. After centrifugation, the pellet was resuspended in culture medium, and the pieces of muscle fibers and dissociated cells were transferred into 35-mm dishes coated with collagen type I (1 mg/ml; BD Biosciences, Allschwil, Switzerland) as a preplating step. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, Thermo Fisher Scientific), 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific), 18% FBS, 10 ng/ml human epidermal growth factor (hEGF; Sigma-Aldrich, Buchs, Switzerland), 1 ng/ml human basic fibroblast growth factor (hbFGF; Sigma-Aldrich), 10 μg/ml human insulin (Sigma-Aldrich), and 0.4 μg/ml dexamethasone (0.5 μM; Sigma-Aldrich)[20]. After 24 h, the supernatant containing nonadhered hMPCs was replated into dishes coated with collagen type I in order to reduce the number of contaminating fibroblasts.

Adenoviral Transduction

The AdEasy System (Agilent Technologies, Santa Clara, CA, USA) was used as a tool for recombinant adenovirus generation. N-terminal influenza hemagglutinin (HA)-tagged human PGC-1α was cloned into an adenoviral vector that codes for cytomegalovirus (CMV) promoter-driven green fluorescent protein (GFP). The expression of hPGC-1α was also under the control of a CMV promoter, thereby ensuring its robust, constitutive expression. Successful cloning was validated by sequencing, while viral transfection and expression of the fluorescent reported genes were monitored by visualizing GFP (for the PGC-1α-expressing adenoviral vector). As a control for viral transfection, a GFP adenovirus was used. The viral titer was increased through an additional amplification step and quantified by fluorescence and bright-field microscopy (Leica fluorescence microscope; CTR 6000; Leica, Heerbrugg, Switzerland). The optimal multiplicity of infection (MOI) was determined according to the manufacturer's protocol (AdEasy Viral Titer Kit). In brief, hMPCs were plated and transduced, and the fluorescent-positive cells were quantified using a fluorescence microscope. Their number was compared to the total number of cells per field and the MOI; the ratio of the number of viral particles to cells was calculated.

An optimal transduction protocol in culture media was established beforehand, showing at least 90% cell survival as determined by trypan blue (Molecular Probes, Thermo Fisher Scientific, Paisley, UK) and WST-1 (Roche, Basel, Switzerland). Finally, the transduced hMPCs were expanded for 2 days after transfection and were subcutaneously (SC) injected into nude mice following ethical approval by the local ethics committees.

Animal Experimentation

All animal experiments were approved by the Swiss animal ethics committee (Veterinäramt Zürich, Licence No. 12-2012) and performed according to the animal ethics welfare law. A total of 24 nude mice (8 weeks old; female; Charles River Laboratories, Sulzfeld, Germany) were divided in two groups (GFP and PGC-1α). For the in vivo experiments, the hMPCs were expanded to passages 3–4.

Each sample contained 30×10^6 transduced hMPCs, which were gently mixed with 500 µl of collagen type I carrier (final concentration: 2 mg/ml; BD Biosciences) and prepared for SC injection in the backs of nude mice[21]. Each animal received two bilateral SC injections, each containing 30×10^6 hMPCs, under general isoflurane (Piramal Critical Care, Bethlehem, PA, USA) anesthesia, and the engineered tissues were harvested after 1, 2, and 4 weeks.

Cell Characterization

After expansion of hMPCs to passage 3, they were transfected with the corresponding adenovirus and cultured for 2 days. The cells were characterized by fluorescence-activated cell sorting (FACS) using the following primary antibodies: anti-MyoD (1:100; BD Biosciences), anti-myosin heavy chain [MyHC; 1:1; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA], anti-desmin (1:50; Sigma-Aldrich), anti-sarcomeric α-actinin (1:200; Sigma-Aldrich), anti-CD34 (1:100; BD Biosciences), and anti-immunoglobulin G (IgG) isotype control (1:100; Santa Cruz Biotechnology, Heidelberg, Germany). Cyanine 3 (Cy3) anti-mouse IgG (1:1,000; Sigma-Aldrich) antibody was used as a secondary antibody. A total of 50,000 events were registered by BD FACSCanto flow cytometer (BD Biosciences) immediately after labeling, and the analysis was performed using FlowJo software v. 7.5 (Tree Star Inc., Ashland, OR USA). All data are expressed as percentage of maximum (% Max). All measurements were performed with at least three different human biopsies.

Additionally, the transduced hMPCs were cultured to 70%–80% confluency, fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature, permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 7 min, blocked for 30 min [5% bovine serum albumin (BSA; Sigma-Aldrich) + 0.1% Triton X-100 in phosphate-buffered saline (PBS)], and finally stained with anti-desmin (1:50), anti-sarcomeric α-actinin (1:200), and anti-CD34 (negative control; 1:100) overnight at 4°C. After washing with PBS, the cells were incubated with Cy3 anti-mouse IgG secondary antibody (1:1,000) and 4′,6-diamidino-2-phenylindole (DAPI; 1:100; Sigma-Aldrich) for 1 h at room temperature, washed again, and finally mounted (Dako, Glostrup, Denmark). Images were acquired with Leica Imager Type DM6000B (Leica), and exposures were normalized to unstained controls (secondary antibody and DAPI only).

Fiber Formation Assay

Differentiation of hMPCs into myofibers in vitro was performed as previously reported [fiber formation assay (FFA)][20]. Briefly, hMPCs were grown to 50% confluency in culture medium and afterward in differentiation medium (10% FBS) for 7–10 days until myofibers formed. The myofibers were fixed with ice-cold methanol (7 min), stained with Giemsa (45–60 min; Kantonapotheke, Zürich, Switzerland), and air dried. Five high-power fields (HPF; 10×) were obtained per condition from cell samples from four patient biopsies, and the results were expressed as number of myofibers/HPF, number of nuclei/myofiber, and number of nuclei/HPF. The fusion rate was calculated by dividing the number of nuclei/myofiber by the number of nuclei/HPF (%). A total $n=4\times5$ HPF (20×) were analyzed. The imaging software "ImageJ for Microscopy" [National Institutes of Health (NIH), Bethesda, MD, USA] was used for data assessment.

Cell Viability and Proliferation

In all cases, cell numbers and viability were confirmed by trypan blue staining after trypsinization. To evaluate proliferation and viability of the transfected cells at different time points, hMPCs were cultured for 6 days. The cell proliferation reagent WST-1 was used according to the manufacturer's protocol. For further confirmation of cell viability, hMPCs were stained with 10 µM CellTrace™ calcein acetoxymethyl AM red-orange (Life Technologies, Paisley, UK) for 30 min at 37°C. Viable cells were detected using a fluorescence microscope. All measurements were performed in duplicates of at least three different human biopsies.

Immuno-/Histological Assessment

The harvested neoformed graft-derived tissues were embedded in cryopreservative optimum cutting temperature (OCT) compound (embedding medium; Cell Path; VWR, Zürich, Switzerland) immediately after isolation. Cryostat sections were prepared (10 µm) and further processed. Hematoxylin and eosin (H&E; Sigma-Aldrich) staining was performed according to the manufacturer's protocol. For immunohistological analysis, the tissues were fixed with ice-cold methanol (MeOH; 60 min), permeabilized (0.5% Triton X-100; 20 min), blocked for 30 min (5% BSA + 0.1% Triton X-100 in PBS), and finally stained with anti-MyHC (1:2) overnight at 4°C. After washing with PBS, the tissues were incubated with Cy3 anti-mouse IgG secondary antibody (1:1,000) and DAPI (1:100) for 1 h at room temperature, washed again, and finally mounted (Dako). Images were acquired with Leica Imager Type DM6000B at exposures normalized to unstained controls (secondary antibody and DAPI only).

Real-Time Polymerase Chain Reaction (RT-PCR) and Creatine Kinase (CK) Assay

For analysis of PGC-1α downstream-regulated genes (by RT-PCR) and CK levels [measured using the Cobas

c111 system (Roche Diagnostics, Basel, Switzerland) according to manufacturer's protocol], the cells were cultured for 2 days after transfection and then transferred to a differentiation medium for 9 h, or until day 6, respectively, and finally harvested for further assessments. For gene analysis of tissue, the harvested tissues were pulverized in liquid nitrogen and suspended in RNA lysis buffer. Total RNA was isolated for both, cells and tissues, using the SV Total RNA Isolation System Kit (Promega, Dubendorf, Switzerland) according to the manufacturer's protocol, which includes a DNase digestion. RNA was reverse transcribed with random primers (high-capacity cDNA reverse transcription; Life Technologies). Predesigned primers for human PPARGC1 (Hs01016719_m1), myosin heavy chain-1 (MyH1; Hs00428600_m1), desmin (Hs00157258_m1), and vascular endothelial growth factor (VEGF; Hs00900055_m1) were purchased from Life Technologies. Further primers were purchased from Microsynth (Balgach, Switzerland): human cytochrome c oxidase subunit 5 (hCox5b; forward primer: ATG GCT TCA AGG TTA CTT CGC, reverse primer: CCC TTT GGG GCC AGT ACA TT), human cytochrome c (hCycS; forward primer: CTT TGG GCG GAA GAC AGG TC, reverse primer: TTA TTG GCG GCT GTG TAA GAG), human estrogen-related receptor α (ERRα; forward primer: AGG GTT CCT CGG AGA CAG AG, reverse primer: TCA CAG GAT GCC ACA CCA TAG), human peroxisome proliferator-activated receptor γ coactivator 1α (hPGC-1α; forward primer: TCT GAG TCT GTA TGG AGT GAC AT, reverse primer: CCA AGT CGT TCA CAT CTA GTT CA), and human TATA-binding protein (hTBP; forward primer: CCC GAA ACG CCG AAT ATA ATC C, reverse primer: AAT CAG TGC CGT GGT TCG TG). 18S rRNA (4319413E) was used to normalize cDNA concentrations. For quantification, the expression of each gene was normalized to the 18S or hTBP expression in the corresponding sample. The entire experiment was repeated at least three times, and samples were analyzed in triplicate.

Gel Electrophoresis and Immunoblotting

In summary, tissues were pulverized in liquid nitrogen with a mortar/pestle and suspended in lysis buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Afterward, the samples were centrifuged for 20 min at 13,000 rpm (17,949×*g* rcf), and the supernatant was collected for protein determination. The total protein was measured with the BCA protein assay kit (Thermo Fisher Scientific, Ecublens, Switzerland), and protein lysate (30–50 µg) was loaded on a 10% or 12% gel (Bio-Rad Laboratories, Cressier, Switzerland). Western blot was performed according to the manufacturer's protocol. After the separated proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membrane (Immobilion-P; Millipore, Lucerne, Switzerland), the latter was incubated with a primary antibody at 4°C overnight in Tris-buffered saline (TBS; Münich, Germany), 0.1% Tween 20 (Sigma-Aldrich), and 5% nonfat dry milk. The primary antibodies used were anti-MyHC1 (1:5), anti-MyHC (1:1), anti-desmin (1:50), anti-PGC-1α (1:1,000; Calbiochem, Darmstadt, Germany), anti-sarcomeric α-actinin (1:2,000; Sigma-Aldrich), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2,500), and α-tubulin (1:2,000; Bioconcept, Allschwill, Switzerland). Finally, the membranes were washed in TBS with 0.1% Tween 20 for 30 min and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Pharmacia Biotech, Little Chalfont, UK) for 1 h. The signals were detected by the ECL method (ECL-Kit; Amersham Pharmacia Biotech). The data were analyzed by the Image Studio Lite (Li-Cor, Hamburg, Germany) software and represented as protein expression relative to GAPDH.

Statistics

For statistical analysis, SPSS v22 (IBM, Armonk, NY, USA) was used, and graphics were drawn with GraphPad Prism v5.04 (GraphPad Software, Inc., La Jolla, CA, USA). All data were analyzed by Student's *t*-tests for paired samples or one-way analysis of variance (ANOVA) with Bonferroni or Fisher's least significant difference (LSD) post hoc analysis ($p<0.05$ was considered significant). All presented data are expressed as means with corresponding standard error of the mean (±SEM).

RESULTS

Generation and Establishment of Genetically Modified hMPCs

Adenoviral constructs containing hPGC-1α, or GFP only, were successfully generated and amplified for further use in hMPCs. The expression of each gene was designed to be under the control of a CMV promoter in order to assure robust expression of the transgene. The transduction efficiency was confirmed by fluorescence imaging, as both constructs contain a green fluorophore, expressed under a separate CMV promoter (Fig. 1A). The constructs enabled detection of transduced cells both prior to transplantation for assessing the cell transduction efficiency and afterward on engineered muscle sections. The safety of the viral transfection was visualized by a cell viability assay (CaAM) (Fig. 1A, lower right) and further confirmed by trypan blue staining for distinction of live/dead cells (Fig. 1B), showing no significant differences between transfected [GFP (90.5±1.97, $n=6$) and PGC-1α (88.16±5.32, $n=6$)] and wild-type (WT;

89.00±3.01, $n=8$) cells. The proliferation capacity of the WT, GFP-, and PGC-1α-overexpressing hMPCs was determined by a cell proliferation assay (WST-1) over 6 days of culturing after transduction, showing no significant variations between the different groups [at 6 days: WT (2.77±0.18, $n=6$), GFP (3.12±0.19, $n=9$), and PGC-1α (2.59±0.29, $n=12$)] (Fig. 1C). Importantly, the designed adenoviruses did not affect the expression of specific muscle protein markers (sarcomeric α-actinin and desmin), as detected by immunofluorescence imaging of hMPCs (Fig. 2A) and FACS (Fig. 2B) under culturing conditions. The latter also showed expression of specific well-described markers for characterization of activated hMPCs[4], as well as a significant shift in GFP expression levels in both transgenic groups, compared to untransfected WT cells (Fig. 2C). These results confirm the successful adenoviral transduction of hMPCs and their maintained cell phenotype.

PGC-1α Overexpression Facilitates Differentiation of hMPCs Into Myotubes In Vitro

To directly assess the role of PGC-1α in myofiber formation, an in vitro hMPC differentiation experiment was performed. An FFA[22] with untransduced (WT) GFP- and PGC-1α-transfected cells revealed an increased fusion rate in PGC-1α-overexpressing myoblasts ($n=4\times5$ HPF) (Fig. 3A and B). The participation of transduced GFP$^+$ cells in the myotube formation could be visualized by fluorescence microscopy (Fig. 3A, second row, fluorescence). The facilitated initial differentiation of the PGC-1α-overexpressing cells was further confirmed by Western blot (Fig. 3C), where elevated desmin levels were

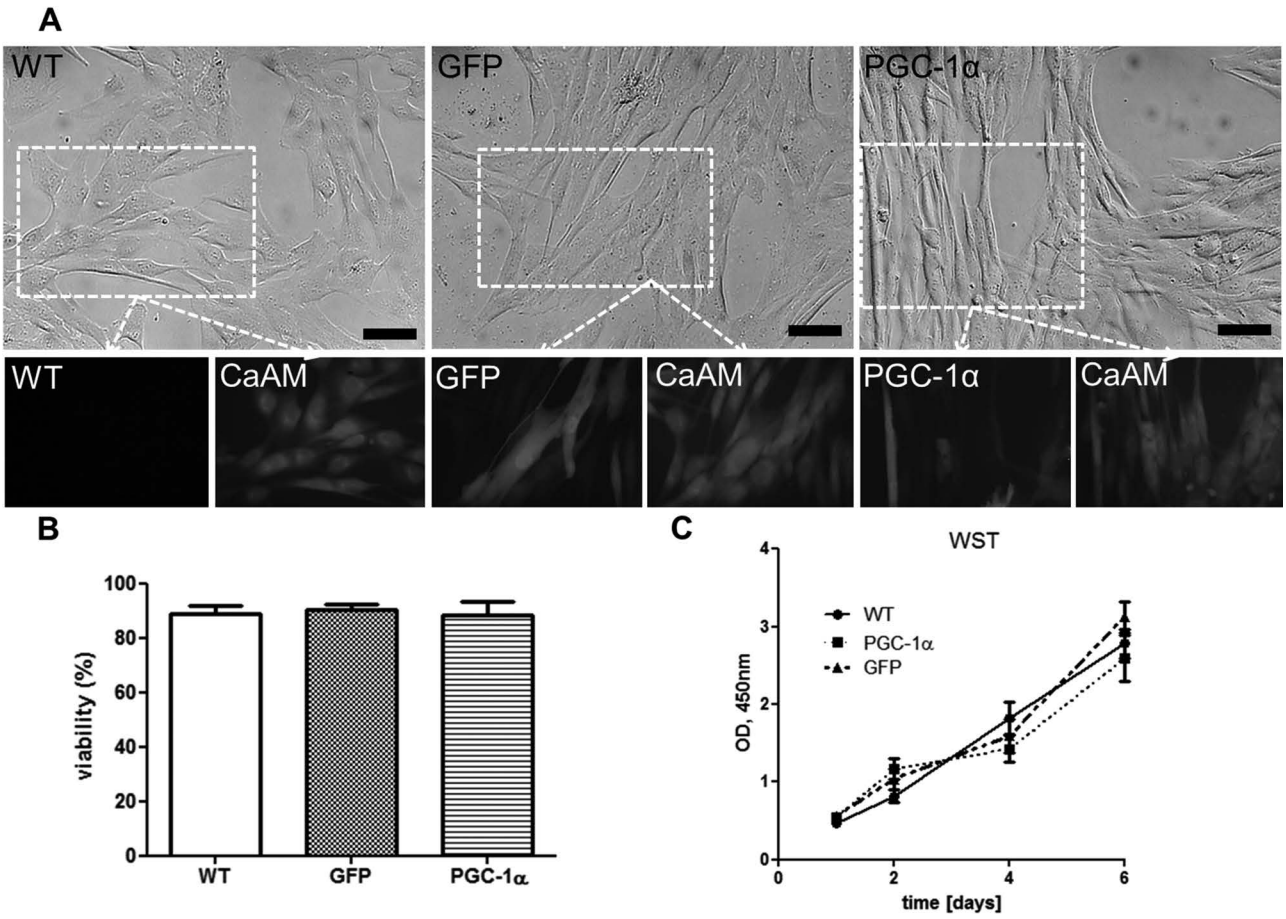

Figure 1. PGC-1α adenoviral transfection without adverse effects on hMPCs. The successful adenoviral transduction of human muscle precursor cells (hMPCs) with green fluorescent protein (GFP) and peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (A) did not affect the cell viability, as shown by the CaAM assay (A, lower right from the marked area). This was further confirmed by trypan blue staining of live/dead cells (B). Proliferation rate also did not differ between the wild type (WT) nontransfected cells and the transfected hMPCs (C). Scale bars: 50 μm. Abbreviations: CaAM, calcein AM; OD, optical density; WST, proliferation assay.

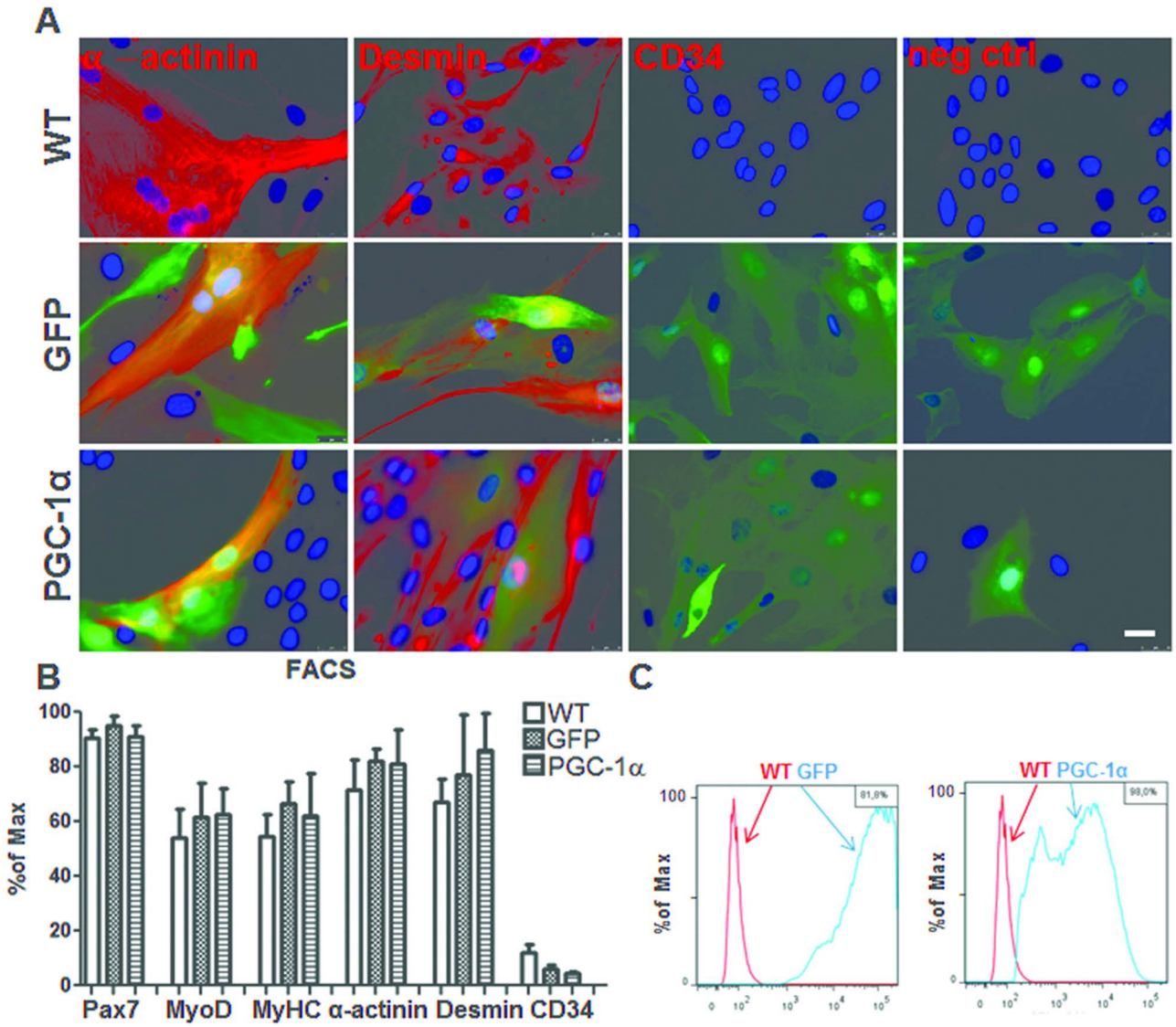

Figure 2. Adenoviral transfection did not change the hMPC phenotype. The expression of typical muscle markers [cyanine 3 (Cy3), red] in the transfected cells (green) was confirmed by immunocytochemistry. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue) (A). The human muscle precursor cells (hMPCs) were analyzed by fluorescence-activated cell sorting (FACS) for expression of various muscle marker proteins (B), and the successful transduction was confirmed by a shift in fluorescein isothiocyanate (FITC) signal (C). Scale bar: 25 μm.

detected already at day 2 after transfection after 9 h of differentiation.

The induction of differentiation goes hand in hand with an increase in metabolic activity (ATP consumption). To analyze this process, the intra- and extracellular creatine kinase (iCK and eCK) levels were measured 2 and 6 days after adenoviral transfection and initiation of differentiation. The iCK levels were significantly increased in PGC-1α-transfected cells at day 2 compared to GFP-transfected cells ($p<0.001$) (Fig. 3D). At day 6, an overall increase in iCK levels in all three groups, concomitant with the induced differentiation, could be seen (Fig. 3D). Importantly, an increase in eCK levels is often associated with muscle damage, cell death, and membrane disruption[23]. Our results show that the viral transfection did not affect the viability and integrity of hMPCs and that the eCK levels remained low 2 and 6 days after transfection (Fig. 3E).

Efficient Elevation of PGC-1α and Downstream Regulated Genes Using Viral Vectors in hMPCs

Functionality of the expressed PGC-1α protein was validated by determination of well-described target genes of the coactivator in muscle cells. As expected,

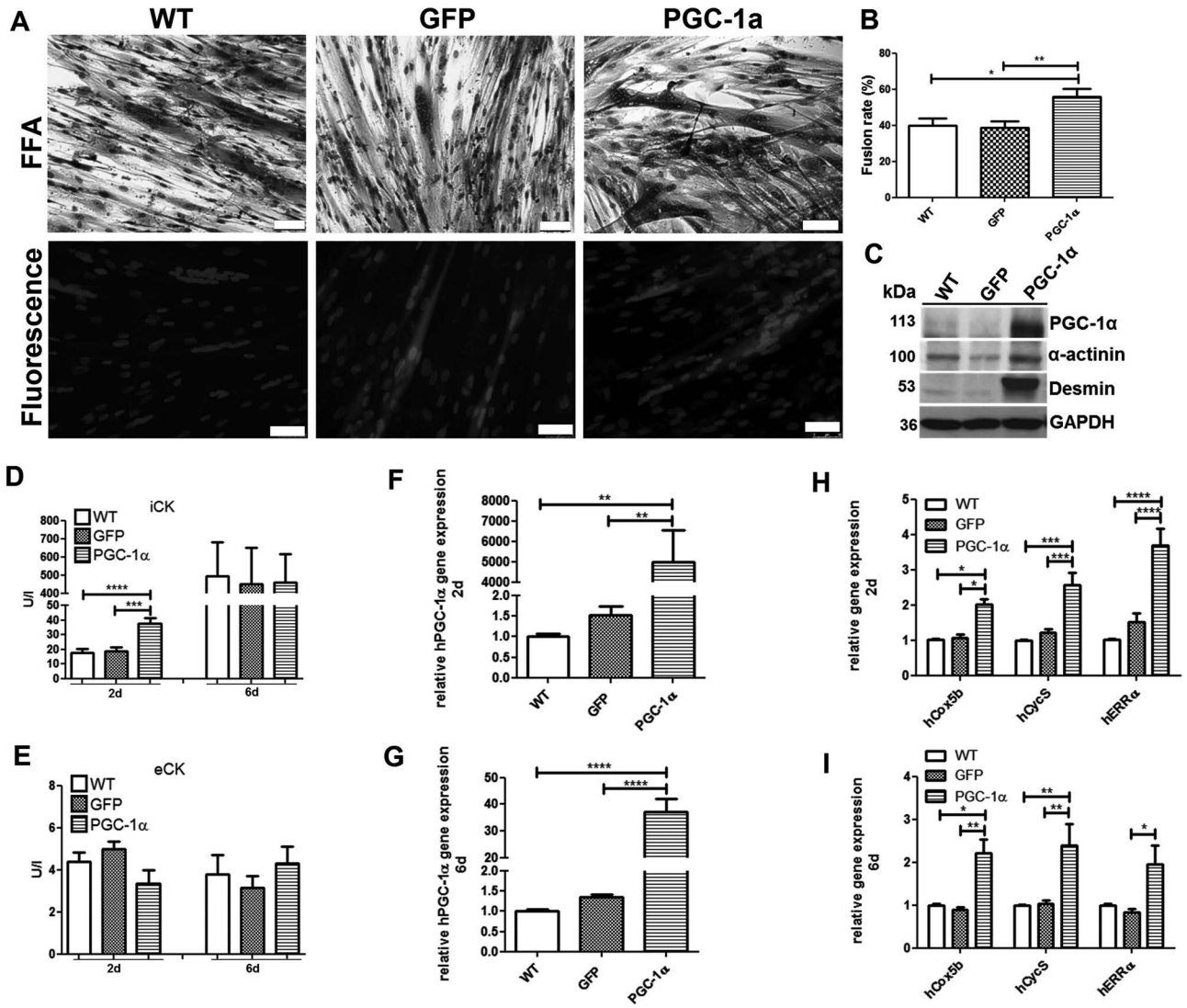

Figure 3. PGC-1α-overexpressing hMPCs differentiate faster into myotubes in vitro. Fiber formation assay[22] and fluorescence microscopy revealed successful myotube formation in wild-type (WT) human muscle precursor cells (hMPCs) and transfected cells, respectively (A), with an increased fusion rate in peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α)-overexpressing cells (B). Two days after transfection, PGC-1α-overexpressing hMPCs displayed enhanced desmin protein levels (C). Intracellular CK (iCK) levels were significantly increased 2 days after PGC-1α transfection (D), whereas extracellular (eCK) levels remained unaffected (E). An overall increase in iCK levels was observed 6 days after inducing differentiation of the hMPCs (D) and eCK remained unchanged (E). PGC-1α gene expression levels were upregulated in hMPCs, transfected with PGC-1α adenovirus after 2 (F) and 6 days (G) after transfection, compared to green fluorescent protein (GFP) and WT controls. PGC-1α downstream genes [human cytochrome c oxidase subunit 5 (hCox5b); human cytochrome c (hCycS); human estrogen-related receptor α (hERRα)] were upregulated in hMPCs transduced with PGC-1α, respectively (H, I). Data are presented as gene expression relative to human TATA-binding protein (hTBP) using the ddCt method. Scale bars: 50 μm.

the introduction of the PGC-1α viral vector specifically elevated the corresponding gene expression level 2 days ($p<0.01$) and 6 days ($p<0.0001$) after inducing cell differentiation (Fig. 3F and G). The bioactivity of the construct was furthermore confirmed after 2 and 6 days, respectively, by the induction of its target genes Cox5b ($p<0.05$, $p<0.01$), CycS ($p<0.001$, $p<0.001$), and ERRα ($p<0.0001$, $p<0.05$), compared to the control cells transfected with GFP and untransduced hMPCs (WT) (Fig. 3H and I). These data confirm the efficiency of the presented PGC-1α adenoviral construct for genetic modification of hMPCs.

PGC-1α Triggers Early Myotube Formation In Vivo

Encouraged by our in vitro observations, we further evaluated the capability of transduced hMPCs to form

ectopic muscle tissue in vivo. The tissues formed by subcutaneously injected hMPCs (transduced with GFP or PGC-1α) were harvested after 1, 2, and 4 weeks for analysis. The weight of the collected tissues did not differ significantly between the two groups [GFP and PGC-1α: 114.9±18.73 mg and 88.47±15.96 mg ($p=0.3021$), 39±5.84 mg and 42.33±4.76 mg ($p=0.6814$), and 19.44±2.33 mg and 22.94±2.28 mg ($p=0.3219$) at 1, 2, and 4 weeks, respectively; data not shown], although a tendency for visually smaller PGC-1α tissues at 1 week was observed, concomitant with the enhanced differentiation (Fig. 4A). The typical red color of slow-twitch oxidative type I myofibers[17] could be detected macroscopically after tissue pulverization with liquid nitrogen in the PGC-1α samples, but not in the control GFP samples after 1 week (Fig. 4B). The constant tissue volume decrease over time in the ex situ model due to myofiber formation and simultaneous collagen remodeling has previously been reported[21]. Histological assessment via H&E staining of the engineered tissue revealed earlier formation of myotubes in the PGC-1α samples (already at week 1) (Fig. 4C, black arrows). This was further confirmed by immunohistological staining of the samples for MyHC, a marker for muscle differentiation and maturation, displaying an increased signal (Cy3, red) in the PGC-1α-transduced tissues and more organized structures (myotubes) (Fig. 4D, white arrowheads). This was concomitant with an initial significant increase in VEGF-A gene levels ($p<0.0001$) in the harvested PGC-1α-overexpressing tissues (Fig. 5A). The PGC-1α overexpression was confirmed by RT-PCR ($p=0.009$) (Fig. 5B). The earlier differentiation was further confirmed by an increase in desmin ($p=0.047$) and MyHC1 ($p=0.024$) gene expression in the PGC-1α-transfected samples, while GFP-transfected samples did not show expression at week 1 ($n=3$–5) (Fig. 5C and D). In line with these results, at protein level, PGC-1α-overexpressing engineered muscle tissue also indicated an increase in relative expression levels of desmin (2.36±1.32, $n=6$) (Fig. 5E) and MyHC1 (2.24±0.67, $n=4$) (Fig. 5F) at week 1, compared to GFP samples. An increase in the expression of MyHC protein over time was observed (2.5±0.96 at 4 weeks, $n=4$) (Fig. 5G), leading to a suggestion for a PGC-1α-induced shift toward MyHC1-type myofibers at 1 week (Fig. 5H). Based on the findings above, PGC-1α-overexpressing hMPCs hold promise for the enhanced repair of skeletal muscle tissue because of their capacity to speed up myofiber formation, with an initial shift to oxidative type I myofibers.

DISCUSSION

Muscle tissue bioengineering has made substantial progress over the last decade, allowing us to grow functional muscle tissue[21,24]. Subcutaneous implantation of myoblasts may have a range of useful applications, from the study of myogenesis to the delivery of gene products[24]. Nevertheless, there are still substantial limitations in size and quality of the engineered constructs. Additionally, drugs including testosterone, growth hormone, leptin, myostatin inhibitors, creatine, and vitamin D have been successfully used to support muscle strength and growth[25]. Yet, there are safety concerns for their long-term application, and therefore, more investigations are required in order to minimize their adverse effects. Another possible solution for rebuilding muscle tissues is cell therapy using satellite cells. The induced expression of several factors in MPCs has extensively been studied, mostly to obtain enhanced cell viability and proliferation, or to prevent apoptosis and induce angiogenesis, or to induce gap junction formation against cardiac arrhythmias[26].

Many muscle disorders are associated with skeletal muscle inactivity, and exercise is an exceptional therapeutic mediation for many disease conditions[27]. Although the exact mechanisms mediating these healing effects still remain elusive, several pathways have been proposed[28]. While resistance training combined with adequate nutrition remains the most effective intervention for diminishing the functional decline in muscles, there is a certain age-linked barrier to obtaining full benefits from this therapy[25].

A promising molecule controlling skeletal muscle metabolism with potential therapeutic effect has been identified[14]. PGC-1α is a known and potent transcription coactivator for nuclear receptors and other transcription factors. It is expressed in skeletal muscles and is a powerful master regulator of mitochondrial biogenesis[29]. Interestingly, PGC-1α has been shown to increase the expression of myofiber type I fibrillar proteins by coactivating the myocyte enhancer factor 2 (Mef2) transcription factors, thereby coordinating the expression of both metabolic and contractile properties of type I myofibers[17]. These findings make PGC-1α a good candidate for targeted regulation of skeletal muscle cell metabolism and plasticity in the myofiber formation process; therefore, we decided to research its applicability for the bioengineering of improved muscle tissues.

The ultimate goal in skeletal muscle tissue engineering is the construction of a functional tissue of a desired myofiber type. Interested in sphincter muscle bioengineering, we aimed at improving the regenerative potential of hMPCs by inducing PGC-1α overexpression through adenoviral gene delivery. By overexpressing PGC-1α in hMPCs, we targeted a major crossroad of intracellular pathways, affecting cell metabolism (mitochondrial activity upregulation) and, simultaneously, enhancing the early expression of contractile proteins (MyHC1, desmin) and secretory factors (VEGF-A), thereby facilitating the process of skeletal muscle bioengineering. Our data showed feasibility of viral overexpression of PGC-1α with desired

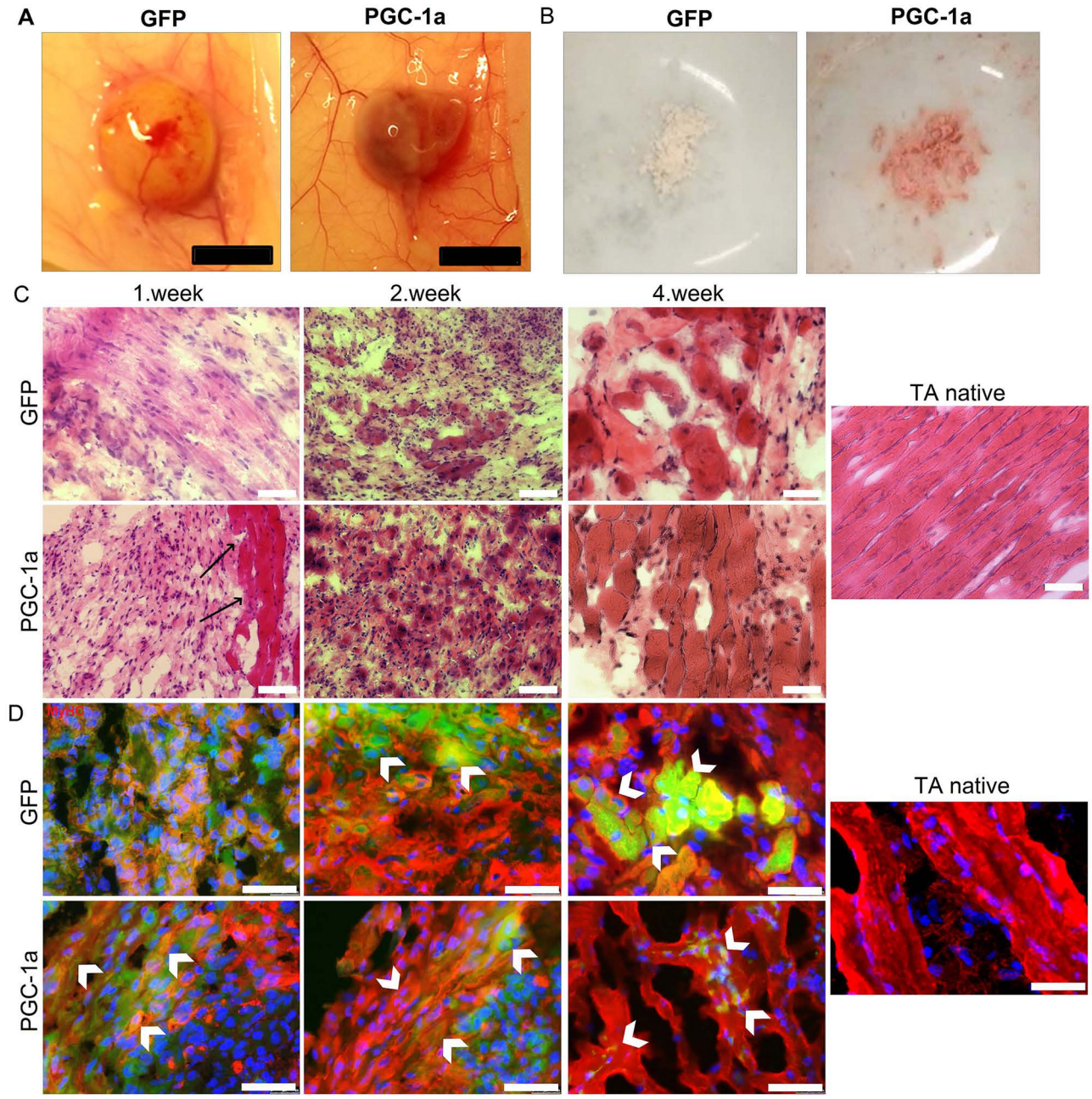

Figure 4. Engineered muscle formation is enhanced by overexpression of PGC-1α in hMPCs. Human muscle precursor cells (hMPCs) transduced with peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) or control green fluorescent protein (GFP) adenovirus were injected subcutaneously on both sides of the back of nude mice. The harvested tissue was visualized macroscopically at 1 week at the subcutaneous tissue (A). The typical red color of oxidative type I myofibers was enriched in PGC-1α samples, compared to GFP controls (samples powderized with liquid nitrogen) (B). The process of successful myofiber formation was visualized over 4 weeks with hematoxylin and eosin (H&E) staining (C), revealing an increased early myotube formation in PGC-1α-overexpressing samples (black arrows). Tibialis anterior (TA) was used as native control for muscle fiber formation. The increased differentiation capacity of PGC-1α transgenic hMPCs at early time points (1 week) was also envisioned by immunostaining for myosin heavy chain (MyHC) [cyanine 3 (Cy3), red] of the ex situ-engineered muscle fibers (green) [4′,6-diamidino-2-phenylindole (DAPI), blue] (D). Scale bars: 50 μm (white), 0.5 cm (black).

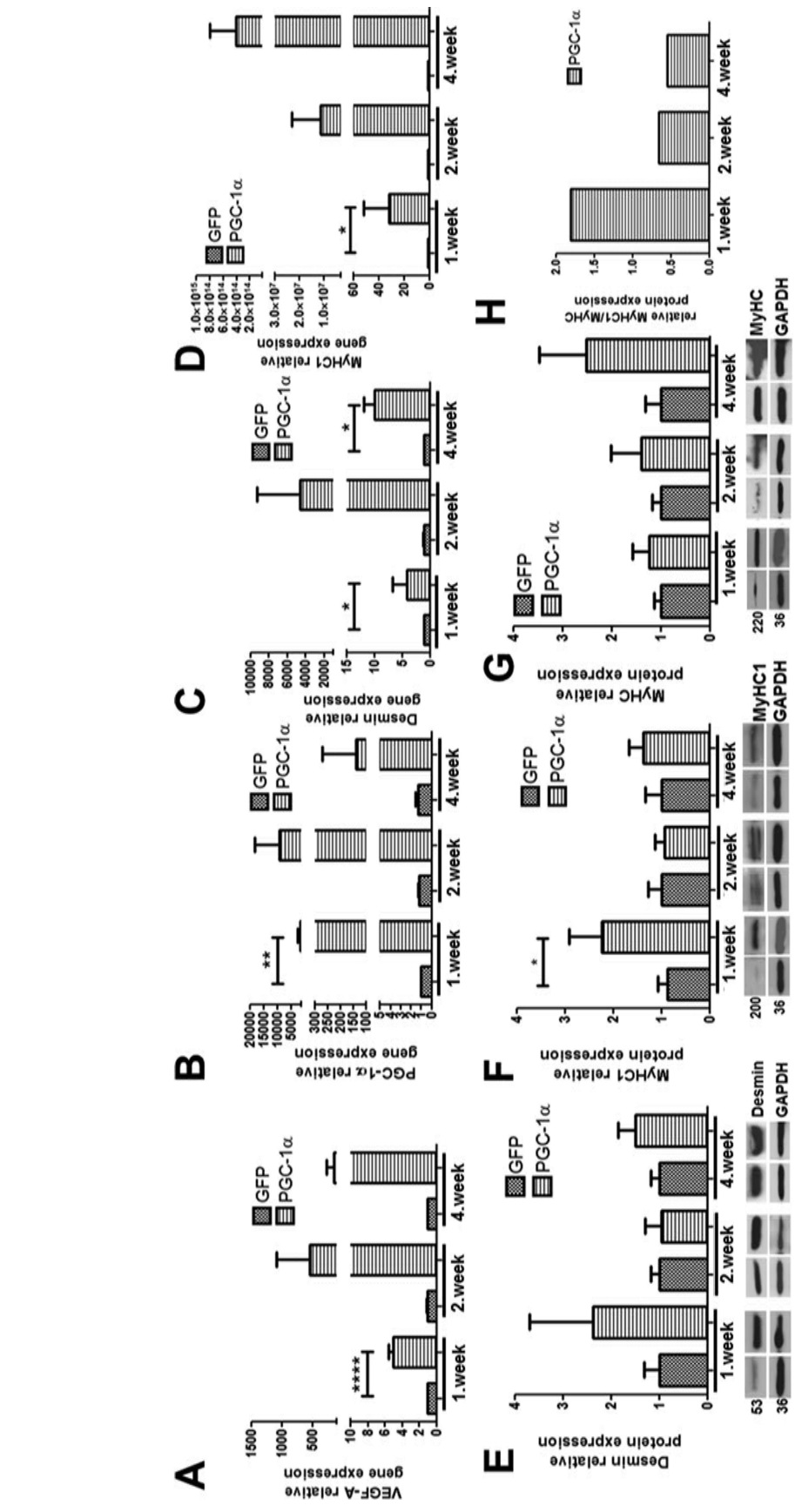

Figure 5. Gene and protein expression in ex situ-bioengineered muscle tissues overexpressing PGC-1α. Gradual increase in vascular endothelial growth factor A (VEGF-A) gene expression levels was shown by real-time polymerase chain reaction (RT-PCR) (A). peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) gene overexpression was confirmed (B). Desmin and myosin heavy chain 1 (MyHC1) gene expression levels (relative to 18S) were increased in PGC-1α-overexpressing tissues (C, D). The enhanced differentiation in PGC-1α-overexpressing tissues was also observed by increased contractile protein expression levels in the corresponding bioengineered tissues (E–G). The ratio of MyHC1 to general MyHC showed an initial switch to oxidative type I myofibers (H). $^{*}p<0.05$, $^{**}p<0.01$, $^{****}p<0.0001$.

phenotypic changes in the ectopic muscle but importantly without detrimental side effects on hMPC viability and differentiation in vivo and in vitro. We acknowledge that the proliferation assay (WST-1) measurement is dependent on mitochondrial function, and PGC-1α might mask the possible lack of proliferation in the transfected cells. However, we visualized the cell viability by CaAM assay and quantified it by trypan blue staining without observing any detrimental effects upon PGC-1α viral transfection.

The bioactivity of PGC-1α was confirmed by significantly enhanced expression of PGC-1α downstream targets, proving the functional efficiency of the presented construct. Supporting evidence suggests an increased expression of mitochondrial and other metabolic genes as a plausible mechanism for rescuing a damaged muscle[27]. Therefore, the demonstrated increase in PGC-1α downstream mitochondrial targets was a key milestone for further in vitro and in vivo studies.

A crucial role in the process of forming new muscle tissue is played by the capacity of myoblasts to differentiate into myotubes/myofibers and a sustained energy metabolism. Our genetically modified hMPCs showed a significantly increased fusion rate, in parallel with an enhanced energy metabolism, depicted by iCK levels and mitochondrial gene level analysis. Given the low iCK levels in myoblasts and their increase with myotube formation, our results indicate that viral overexpression of PGC-1α drives higher iCK expression, possibly due to a faster differentiation process. Moreover, elevated desmin protein expression was detected 2 days after the adenoviral transfection, showing the early appearance of sarcomeric architecture. Consistent with the observed facilitated in vitro differentiation, the ectopically formed skeletal muscle tissue with PGC-1α overexpression revealed myotube formation and increased expression of contractile proteins (desmin and MyHC1) already after 1 week, indicating facilitated differentiation. Interestingly, this strong effect was reduced at later time points. However, PGC-1α-modified samples expressed higher levels of general MyHC with an increase over time, when compared to GFP samples, while there was higher MyHC1 (slow twitch) protein expression at 1 week. These data indicate a possible initial shift toward slow-type myofibers. Related gene delivery studies suggest that overexpression of certain factors (e.g., VEGF) may be beneficial at the time of cell transplantation, and their transient effect may prevent the risk of malignancy formations[30,31].

Vascularization of the bioengineered tissue is the bottleneck of many approaches. Importantly, PGC-1α-overexpressing tissues showed enhanced VEGF-A gene levels. Secretion of various effectors by the injected hMPCs may also contribute to optimization of the regenerative process (e.g., increased neovascularization)[21,32]. Moreover, PGC-1α has been demonstrated to stimulate angiogenesis in skeletal muscle by inducing the release of key factors, including VEGF, in cultured muscle cells and skeletal muscle in vivo[33]. Furthermore, PGC-1α seems to have led to overexpression of VEGF-A, thereby probably inducing early neovascularization and counteracting necrosis after implantation, possibly leading to improved cell/tissue survival and better long-term survival. In line with our in vivo observations, others have shown that an increased VEGF release in a hypoxic environment leads to enhanced differentiation[34]. This could explain the observed increase in contractile MyHC protein and myofiber formation in PGC-1α-engineered tissues, when compared to the corresponding GFP samples.

Whether the accelerated differentiation seen in this research using an ex situ model really leads to functional improvement during regeneration has to be validated using intramuscular (IM) injection of the modified cells. However, the use of ectopic model for muscle tissue engineering also brings along valuable information about the formation of muscle fibers in a nonmyogenic environment, as it might be in the patients, for example, scar tissue. Considering these facts, the optimal model to study skeletal muscle regeneration would be to inject the cells in injured muscles. Further, the use of viral gene delivery for clinical applications is still associated with several drawbacks[35]. We acknowledge that the choice of the adenoviral delivery method might not be the most suitable one. For a clinical setup, we would suggest a nonviral gene delivery method. We further acknowledge a possible immune reaction from the mice we chose, although no detrimental effects were detected in the engineered tissues. Still this model sets a significant milestone toward the possible engineering of improved muscle tissue of a desired myofiber type.

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