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ARTICLE

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Commitment of human mesenchymal stromal cells towards ACL fibroblast differentiation upon rAAV-mediated FGF-2 and TGF-β overexpression using pNaSS-grafted PCL films

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Abstract

Despite various clinical options, human anterior cruciate ligament (ACL) lesions do not fully heal. Biomaterial-guided gene therapy using recombinant adeno-associated virus (rAAV) vectors may improve the intrinsic mechanisms of ACL repair. Here, we examined whether poly(sodium styrene sulfonate)-grafted poly(ε-caprolactone) (pNaSS-grafted PCL) films can deliver rAAV vectors coding for the reparative basic fibroblast growth factor (FGF-2) and transforming growth factor beta (TGF-β) in human mesenchymal stromal cells (hMSCs) as a source of implantable cells in ACL lesions. Efficient and sustained rAAV-mediated reporter (red fluorescent protein) and therapeutic (FGF-2 and TGF-β) gene overexpression was achieved in the cells for at least 21 days in particular with pNaSS-grafted PCL films relative to all other conditions (up to 5.2-fold difference). Expression of FGF-2 and TGF-β mediated by rAAV using PCL films increased the levels of cell proliferation, the DNA contents, and the deposition of proteoglycans and of type-I and -III collagen (up to 2.9-fold difference) over time in the cells with higher levels of transcription factor expression (Mohawk, Scleraxis) (up to 1.9-fold difference), without activation of inflammatory tumor necrosis alpha especially when using pNaSS-grafted PCL films compared with the controls. Overall, the effects mediated by TGF-β were higher than those promoted by FGF-2, possibly due to higher levels of gene expression achieved upon rAAV gene transfer. This study shows the potential of using functionalized PCL films to apply rAAV vectors for ACL repair.

KEYWORDS

ACL cell differentiation, FGF-2, human mesenchymal stromal cells, PCL, pNaSS grafting rAAV gene transfer, TGF-β

1 | INTRODUCTION

Lesions in the anterior cruciate ligament (ACL) that critically supports the stability of the knee are frequent in patients, with more than 200,000 individuals being affected each year only in the United States and about 1/3000 patients with such injuries in Europe, leading to a socio-economical burden of >$7 billion per year (Bokshan et al., 2019; Musahl & Karlsson, 2019) that may lead to disability and osteoarthritis (Musahl et al., 2022; Werner et al., 2022). The ACL is a cable-like structure composed of a rich, dense rich extracellular matrix (ECM) containing collagen fiber bundles (type-I, -III, -IV, -V, and -VI collagen) and other compounds (proteoglycans, decorin, fibronectin, elastin, tenascin-C, thrombospondin) (Arnoczky, 1983; Kiapour & Murray, 2014). This ECM is embedding fibroblasts present at low densities that produce the ECM components (Arnoczky, 1983; Kiapour & Murray, 2014). The poor cell content of the ACL is one of the major causes for its very low ability to fully heal in response to injury, leading to the occurrence of a repair tissue with weak mechanical properties (Arnoczky, 1983; Kiapour & Murray, 2014). Such repair tissue can not be durably and safely compensated by any of the various options available thus far (conservative treatments: immobilization, bracing, physiotherapy; injection of corticoids; surgery: ACL reconstruction with grafts, synthetic scaffolds, substitutes) (Musahl et al., 2022).

A promising strategy to manage ACL lesions is to apply gene therapy procedures by providing therapeutic sequences in sites of injury via gene vectors as a means to support the production of factors capable of persistently stimulate the intrinsic ACL reparative processes compared with the administration of short-lived recombinant agents (Amini et al., 2022; Watson-Levings et al., 2022). Among many classes of vectors, such as nonviral (Bez et al., 2018; Nakamura et al., 1998; Nichols et al., 2018) and viral (adenoviral, retroviral, and lentiviral) gene vehicles (Alberton et al., 2012; Brunger et al., 2014; Haddad-Weber et al., 2010; Kawakami et al., 2017; Otabe et al., 2015; Pascher et al., 2004; Steinert et al., 2008; Weber et al., 2007; Wei et al., 2011), vectors derived from the adeno-associated virus (AAV) (a human nonpathogenic virus) are particularly well adapted to treat ACL lesions. Recombinant AAV (rAAV) vectors can safely target both ACL fibroblasts and implantable reparative progenitor cells such as mesenchymal stromal cells (MSCs) at very high and sustained efficiencies (about 100%, for several months to years) (Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013; Xiao et al., 1996) in contrast to nonviral and adenoviral vectors that only afford low and short-term levels of gene transfer expression or to retroviral and lentiviral vectors that carry a risk for insertional mutagenesis (Alberton et al., 2012; Amini et al., 2022; Bez et al., 2018; Brunger et al., 2014; Evans & Huard, 2015; Haddad-Weber et al., 2010; Kawakami et al., 2017; Nakamura et al., 1998; Nichols et al., 2018; Otabe et al., 2015; Pascher et al., 2004; Steinert et al., 2008; Weber et al., 2007; Wei et al., 2011). Yet, clinical gene therapy using rAAV remains hindered by various barriers in the joints including the neutralization of the vectors by pre-existing antibodies against the proteins of the viral capsid in the human population (Cottard et al., 2004). A new approach to improve the process of rAAV-mediated gene therapy for ACL repair is to deliver the vectors via biomaterials to promote a more effective and safe expression of the transgenes being carried in a spatiotemporal manner in their targets (Amini et al., 2022).

The goal of the present work was to investigate the feasibility of applying therapeutic genes via rAAV in primary human MSCs (hMSCs) as a source of highly reparative cells for ACL repair (Alberton et al., 2012; Angele et al., 2022; Haddad-Weber et al., 2010; Hevesi et al., 2019; Kawakami et al., 2017; Otabe et al., 2015; Weber et al., 2007; Wei et al., 2011) using a biomaterial-guided strategy based on poly(sodium styrene sulfonate)-grafted poly(ε-caprolactone) (pNaSS-grafted PCL) films (Leroux et al., 2018, 2019, 2020) due to their potential to deliver this class of vectors in ACL fibroblasts (Amini et al., 2023). Among various candidates (insulin-like growth factor I - IGF-I, bone morphogenetic proteins - BMPs, platelet-derived growth factor - PDGF, vascular endothelial growth factor - VEGF, and the transcription factors Mohawk and Scleraxis) (Alberton et al., 2012; Bez et al., 2018; Haddad-Weber et al., 2010; Kawakami et al., 2017; Nakamura et al., 1998; Nichols et al., 2018; Otabe et al., 2015; Steinert et al., 2008; Weber et al., 2007; Wei et al., 2011), the basic fibroblast growth factor (FGF-2) via the Ras-Raf-mitogen activated protein kinase signaling pathway by binding to FGF tyrosine kinase receptors (Cai et al., 2013) and the transforming growth factor beta (TGF-β) were selected in light of their ability to activate the intrinsic reparative processes relevant of ACL repair (cell proliferation, ECM deposition, commitment to ACL cell differentiation) (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013; Pascher et al., 2004; Wei et al., 2011). The current work shows that pNaSS-grafted PCL films safely stimulate the reparative activities in hMSCs via the delivery of rAAV FGF-2 and TGF-β vectors, representing a promising tool for enhanced reparative MSC implantation to treat ACL lesions in patients in the future.

2 | METHODS

2.1 | Reagents

All reagents were from Sigma-Aldrich (Munich, Germany) including the 4¯styrenesulfonic acid sodium salt hydrate (NaSS) (cat. no. 434574), unless otherwise indicated. The anti-FGF-2 (G-2), anti-type-I collagen (COL-1), and anti-type-III (C-15) antibodies were purchased at Santa Cruz Biotechnology and the anti-TGF-β (V) antibody at Invitrogen (Thermo-Fisher Scientific). Biotinylated secondary antibodies and the ABC reagent were from Vector Laboratories (Alexis Deutschland GmbH). The AAVanced Concentration Reagent was from System Bioscience, the FGF-2 Quantikine ELISA (DFB50) and the TGF-β Quantikine Enzyme-Linked Immunosorbent Assay (ELISA; DB100B) from R&D Systems, and the Cell Proliferation reagent WST-1 from Roche Applied Science.

2.2 | Isolation and culture of primary hMSCs

Human bone marrow aspirates (about 15 mL) were obtained from the distal femurs of donors undergoing total knee arthroplasty ($n = 4$, age range 65–79 years) (Cucchiarini et al., 2011; Frisch et al., 2014)

(Figure 1a). The study was approved by the Ethics Committee of the Saarland Physicians Council (*Ärztekammer des Saarlandes*, reference number Ha06/08) and all donors gave their informed consent before being included in the analyses performed according to the Helsinki Declaration. The bone marrow aspirates were washed and centrifuged in Dulbecco's modified Eagle's medium (DMEM) and the pellet was resuspended in Red Blood Cell Lysing Buffer/DMEM (1:1), washed, again pelleted and resuspended in DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin (growth medium) (Cucchiarini et al., 2011; Frisch et al., 2014) (Figure 1a). HMSCs were extracted and plated as 2 x 10^5 cells/cm^2 in T-75 flasks in a humidified atmosphere at 37°C under 5% CO_2, with medium changes after 48 h and every 2–3 days in the presence of recombinant FGF-2 (1 ng/mL) (cat. no. 100-18B-50UG; Peprotech, ThermoFisher Scientific) (Cucchiarini et al., 2011; Frisch et al., 2014) (Figure 1a). The cells were tested by flow cytometry for the expression of stem cell surface markers ($CD71^+$, $CD105^+$, and $CD34^-$), plated at 10^4 cells/well in 24-well plates for the evaluations using cells at passage <2, and kept for up to 21 days in growth medium at 37°C under 5% CO_2 (Cucchiarini et al., 2011; Frisch et al., 2014).

2.3 | rAAV vectors

rAAV vectors were generated from the parental AAV-2 genomic clone (pSSV9) (Samulski et al., 1987, 1989). rAAV-RFP contains the reporter Discosoma sp. red fluorescent protein (RFP) gene, rAAV-hFGF-2 a human basic FGF-2 (hFGF-2) cDNA, and rAAV-hTGF-β a 1.2-kb human TGF-β 1 (hTGF-β) cDNA, with all coding sequences controlled by the cytomegalovirus immediate-early promoter (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013). Helper-free (two-plasmid) transfection was performed in HEK 293 cells to package conventional (not self-complementary) vectors using pXX2 (packaging plasmid) and pXX6 (adenovirus helper plasmid) (Amini et al., 2023; Frisch et al., 2014). The vectors were purified with the AAVanced Concentration Reagent

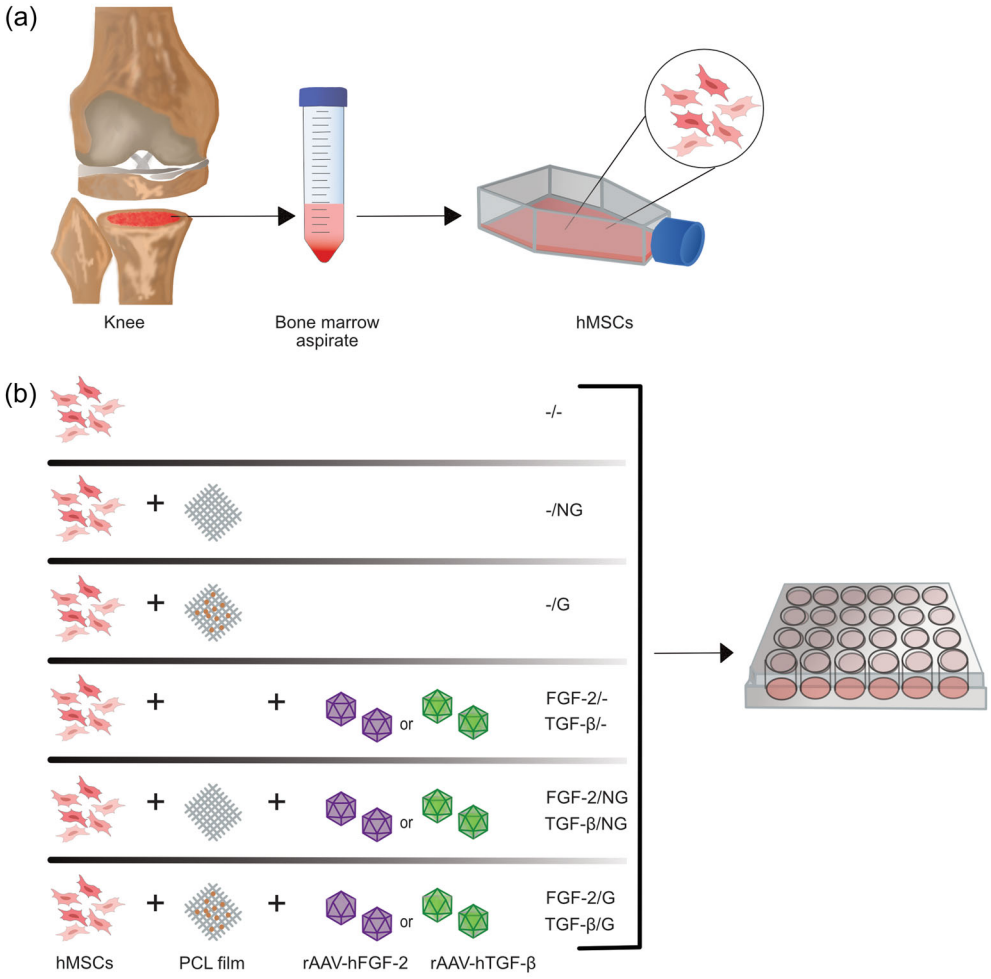

FIGURE 1 Study design. (a) Isolation of hMSCs. (b) Experimental gene transfer conditions in hMSCs (10^4 cells; 40 µL rAAV vectors, that is, 8 × 105 transgene copies, per PCL film or as film-free solutions, MOI = 80). -/-, absence of vector incubation and of PCL film; -/NG, absence of vector incubation on ungrafted PCL films; -/G, absence of vector incubation on pNaSS-grafted PCL films; FGF-2/-, film-free rAAV-hFGF-2 solution; FGF-2/NG, rAAV-hFGF-2 incubated on ungrafted PCL films; FGF-2/G, rAAV-hFGF-2 incubated on pNaSS-grafted PCL films; MOI, multiplicity of infection; TGF-β/-, film-free rAAV-hTGF-β solution; TGF-β/NG, rAAV-hTGF-β incubated on ungrafted PCL films; TGF-β/G, rAAV-hTGF-β incubated on pNaSS-grafted PCL films.

(Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014) and titered via real-time PCR (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013), with ~10^{10} transgene copies/mL (i.e., ~1/500 functional recombinant viral particles).

2.4 | Poly(ε-caprolactone) (PCL) films

PCL films (Figure 1b) were produced by spin coating with a SPIN150-v3 SPS (Leroux et al., 2018, 2019, 2020). PCL at 60% [w/v] in dichloromethane was dropped for spinning on a glass slide for 30 s at 1500 rpm and the films were air dried for 2 h and then vacuum dried for 24 h. The PCL films were cut as 4-mm disks, some of which being next grafted with poly(sodium styrene sulfonate) (pNaSS) at 1.3 10^{-5} mol/g via ozonation for 10 min at 30°C and then by graft polymerization for 3 h at 45°C in degassed NaSS at 15% w/v in distilled water (Figure 1b). The PCL films were then rinsed and vacuum dried following washing in distilled water, 0.15 M NaCl, and phosphate-buffered saline.

2.5 | rAAV immobilization onto PCL films

PCL films were sterilized for 10 min in 70% ethanol, washed with PBS, and then incubated overnight with 0.002% poly-L-lysine at 37°C (Amini et al., 2023). The films were washed twice in PBS and the rAAV vectors (40 µL, 8×10^5 transgene copies, multiplicity of infection [MOI] = 80) were then immobilized by dropping on the films and incubated for 2 h at 37°C (Amini et al., 2023) (Figure 1b). Some PCL films were prepared without adding rAAV vectors as control conditions (Figure 1b). Controlled rAAV release studies were not performed here since we already demonstrated that all PCL films (grafted and ungrafted) tested here can effectively release rAAV vectors over prolonged time periods (at least 21 days) (Venkatesan et al., 2020).

2.6 | rAAV gene transfer

pNaSS-grafted and ungrafted PCL films incubated (or not) with rAAV vectors were added to the bottom of a 24-well plate seeded with hMSCs (10^4 cells/well) and incubated for 2 h at 37°C under 5% CO_2 and then DMEM, 10% FBS, 1% pen-strep was provided overnight at 37°C under 5% CO_2 (Amini et al., 2023) (Figure 1b). Some wells were employed as control conditions by directly providing film-free rAAV vector solutions to the cells or left without rAAV vector solutions (Figure 1b). The medium was changed after 24 h and every 2 days for up to 21 days. When added, the PCL films were left over the whole period of culture.

2.7 | Detection of transgene expression

Transgene (RFP) expression was examined by fluorescence microscopy with a 568-nm filter (Olympus CK41; Olympus) (Amini et al., 2023; Cucchiarini et al., 2011). FGF-2 and TGF-β expression was examined by immunocytochemistry using specific primary antibodies, biotinylated secondary antibodies, and the ABC method with diaminobenzidine (DAB) as the chromogen for analysis under light microscopy (Olympus BX45) (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013). To control for secondary immunoglobulins, samples were processed with omission of the primary antibody. FGF-2 and TGF-β expression was also assessed by specific ELISAs according to the manufacturer's instructions and using a GENios spectrophotometer/fluorometer (Tecan) (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013).

2.8 | Biological analyses

Cultures were harvested and the indices of proliferation were assessed with the Cell Proliferation reagent WST-1, with optical density ($OD^{450\ nm}$) proportional to the cell numbers (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014). Other cultures were digested with papain (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013) and the DNA and proteoglycan contents were measured with the Hoechst 33258 assay and by binding to the dimethylmethylene blue (DMMB) dye, respectively (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013). A GENios spectrophotometer/fluorometer (Tecan) was employed for the measurements (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013).

2.9 | Immunocytochemical analyses

Type-I and -III collagen deposition was examined on fixed cultures by immunocytochemistry using specific primary antibodies, biotinylated secondary antibodies, and the ABC method with DAB as the chromogen for analysis under light microscopy (Olympus BX45) (Cucchiarini et al., 2011). To control for secondary immunoglobulins, samples were processed with omission of the primary antibody.

2.10 | Cytomorphometric analysis

The cell densities (cells/mm^2 on immunocytochemically-stained cultures) were measured at four randomized locations for each replicate condition (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013) using the ImageJ software (National Institutes of Health) (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013). The intensities of transgene (FGF-2 and TGF-β) expression and of type-I and -III collagen deposition assessed by immunocytochemistry were measured by monitoring the integrated densities per pixel per cells based on the respective cell densities at four randomized locations for each replicate condition (Amini et al., 2023) using the ImageJ software (National Institutes of Health) (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013).

2.11 | Real-time RT-PCR analysis

Total culture cellular RNA was extracted with the RNeasy Protect Mini Kit, using an on-column RNase-free DNase treatment (Qiagen) (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014). RNA was eluted with 40 µL RNase-free water and reverse transcription was performed with 8 µL eluate using the 1st Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche Applied Science) (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014). Real-time PCR amplification was achieved on the Mx3000P QPCR system (Stratagene, Agilent Technologies) with 2 µL cDNA product using the Brilliant SYBR Green QPCR Master Mix (Stratagene) (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014). The following protocol was employed (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014): 10 min (95°C), 65 amplification cycles (30 s denaturation at 95°C; 1 min annealing at 55°C; 30 s extension at 72°C), denaturation 1 min (95°C), and final incubation 30 s (60°C). The primers were (Table 1): type-I collagen (COL1A1; ligament marker), type-III collagen (COL3A1, ligament marker), Mohawk (ligament-related transcription factor), Scleraxis (specific transcription factor), tumor necrosis alpha (TNF-α, proinflammatory marker), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene and internal control (150 nM final concentration) (Invitrogen, ThermoFisher Scientific). Control reactions included the use of water and of nonreverse-transcribed mRNA, with specificity of the products confirmed by melting curve analysis and agarose gel electrophoresis. The threshold cycle (C_t) value for each gene of interest was analyzed for each amplified sample with the MxPro QPCR software (Stratagene Agilent Technologies) (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014). C_t values were normalized to GAPDH expression via the $2^{-\Delta\Delta C_t}$ method (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014).

2.12 | Statistical analysis

All experiments were repeated a minimum of three times using isolated hMSCs from all donors. The nonparametric one-way analysis of variance test was used for statistical analysis (except for biochemistry data performed by Student *t* test), with $^{*}p \le 0.05$, $^{**}p \le 0.01$, and $^{***}p \le 0.001$ considered statistically significant.

3 | RESULTS

3.1 | Effective, durable rAAV-mediated gene expression in hMSCs via PCL film-guided vector application

Monolayer cultures of primary hMSCs were first treated with the reporter rAAV-RFP vector incubated on pNaSS-grafted versus ungrafted PCL films to analyze the feasibility of genetically modifying these cells in vitro over time via biomaterial-guided gene vector delivery compared with control treatments in the absence of vector incubation or with film-free vector solutions. The data show the effective, durable expression of RFP via rAAV starting on Day 2 and until up to Day 21 (the longest time-point analyzed) regardless of the class of PCL film employed, with signals in the range of those noted with the film-free rAAV-RFP solution (Figure 2b) relative to treatments in the absence of rAAV-RFP (Figure 2a).

Monolayer cultures of primary hMSCs were then treated with the therapeutic rAAV-hFGF-2 and rAAV-hTGF-β vectors incubated on pNaSS-grafted versus ungrafted PCL films to analyze the feasibility of overexpressing FGF-2 and TGF-β in these cells in vitro over time compared with control treatments in the absence of vector incubation or with film-free vector solutions. An evaluation of FGF-2 and TGF-β transgene expression in hMSCs on Day 9 by immunocytochemical analysis with corresponding cytomorphometry (Figure 3) showed the optimal, significant expression of FGF-2 (Figure 3a) and of TGF-β (Figure 3b) upon administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films (FGF-2/G and TGF-β/G, respectively) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (1.3- and 1.2-fold difference, respectively, $p \le 0.001$). This was also noted upon administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on ungrafted PCL films (FGF-2/NG and TGF-β/NG, respectively) or using film-free rAAV-hFGF-2 and rAAV-hTGF-β vector solutions (FGF-2/- and TGF-β/-, respectively) compared with the control -/- condition (up to 1. Twofold difference, $p \le 0.001$) (Figures 2a,b). Importantly, administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films was more potent than when using

TABLE 1 Primers employed in the study.

Gene	Forward primer	Reverse primer
COL1A1	5′-ACGTCCTGGTGAAG TTGGTC-3′	5′-ACCAGGGAAGCCTCTCTCTC-3′
COL3A1	5′-CACAAGGAGTCTGCATGTCT-3′	5′-GTTCACCAGGCTCACCAGCA-3′
Mohawk	5′-AAGATACTCTTGGCGCTCGG-3′	5′-ACACTAAGCCGCTCAGCATT-3′
Scleraxis	5′- TACCTGGGTTTTCTTCTGGTCACT-3′	5′-TATCAAAGACACAAGATGCCAGC-3′
TNF-α	5′-AGAACCCCCTGGAGATAACC-3′	5′-AAGTGCAGCAGGCAGAAGAG-3′
GAPDH	5′-GAAGGTGAAGGTCGGAGTC-3′	5′-GAAGATGGTGATGGGATTTC-3′

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tumor necrosis alpha.

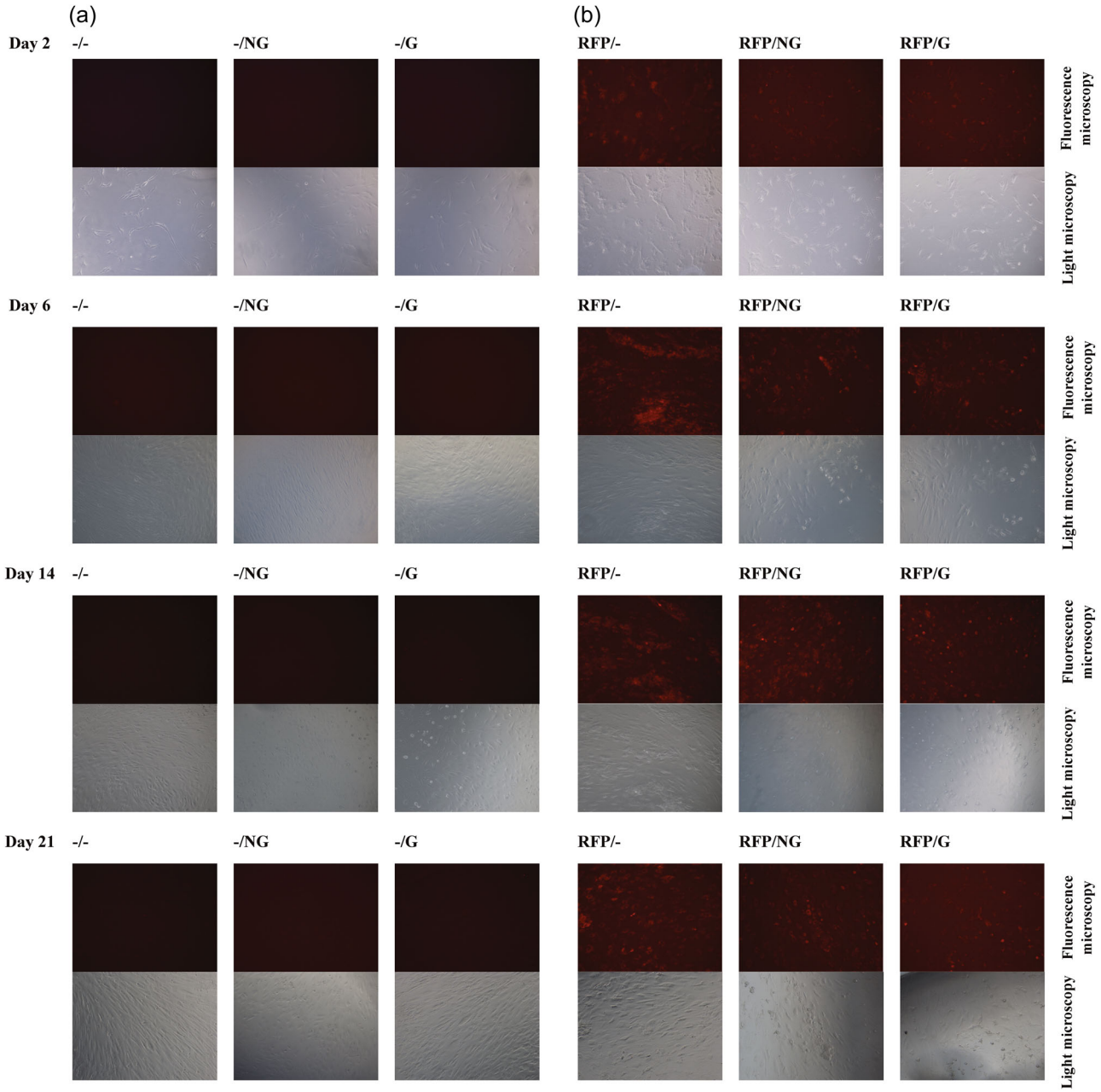

FIGURE 2 Evaluation of transgene (RFP) expression in hMSC cultures treated with rAAV-RFP incubated on PCL films. The PCL films were incubated with rAAV-RFP (40 µl, 8×10^5 transgene copies) or left without vector treatment before administration to the cultures and RFP expression was analyzed by fluorescence microscopy at the denoted time points as described in the Materials and Methods ([a] absence of vector incubation; [b] rAAV-RFP gene delivery; fluorescence microscopy photographs on the top panels with corresponding light microscopy photographs on the lower panels; magnification x10; all representative data). -/-, absence of vector incubation and of PCL film; -/NG, absence of vector incubation on ungrafted PCL films; -/G, absence of vector incubation on pNaSS-grafted PCL films; RFP/-, film-free rAAV-RFP solution; RFP/NG, rAAV-RFP incubated on ungrafted PCL films; RFP/G, rAAV-RFP incubated on pNaSS-grafted PCL films.

ungrafted PCL films or film-free vector solutions (up to 1.2-fold difference, $p \leq 0.014$) (Figures 2a,b). An evaluation of the levels of FGF-2 production over time in hMSCs by ELISA (Figure 4a) showed the optimal, significant and steady expression of FGF-2 upon administration of rAAV-hFGF-2 incubated on pNaSS-grafted films (FGF-2/G) as early as 7 days and over the whole period of analysis (21 days) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (up to 2.5-fold difference, $p \leq 0.001$) (Figure 4a). This was also noted upon administration of rAAV-hFGF-2 incubated on ungrafted PCL films (FGF-2/NG) or using a film-free rAAV-hFGF-2 vector solution (FGF-2/-) compared with the control -/- condition (up to 2.3-fold difference, $p \leq 0.001$) (Figure 4a). Importantly, administration of rAAV-hFGF-2 incubated on pNaSS-grafted PCL films was more potent than when

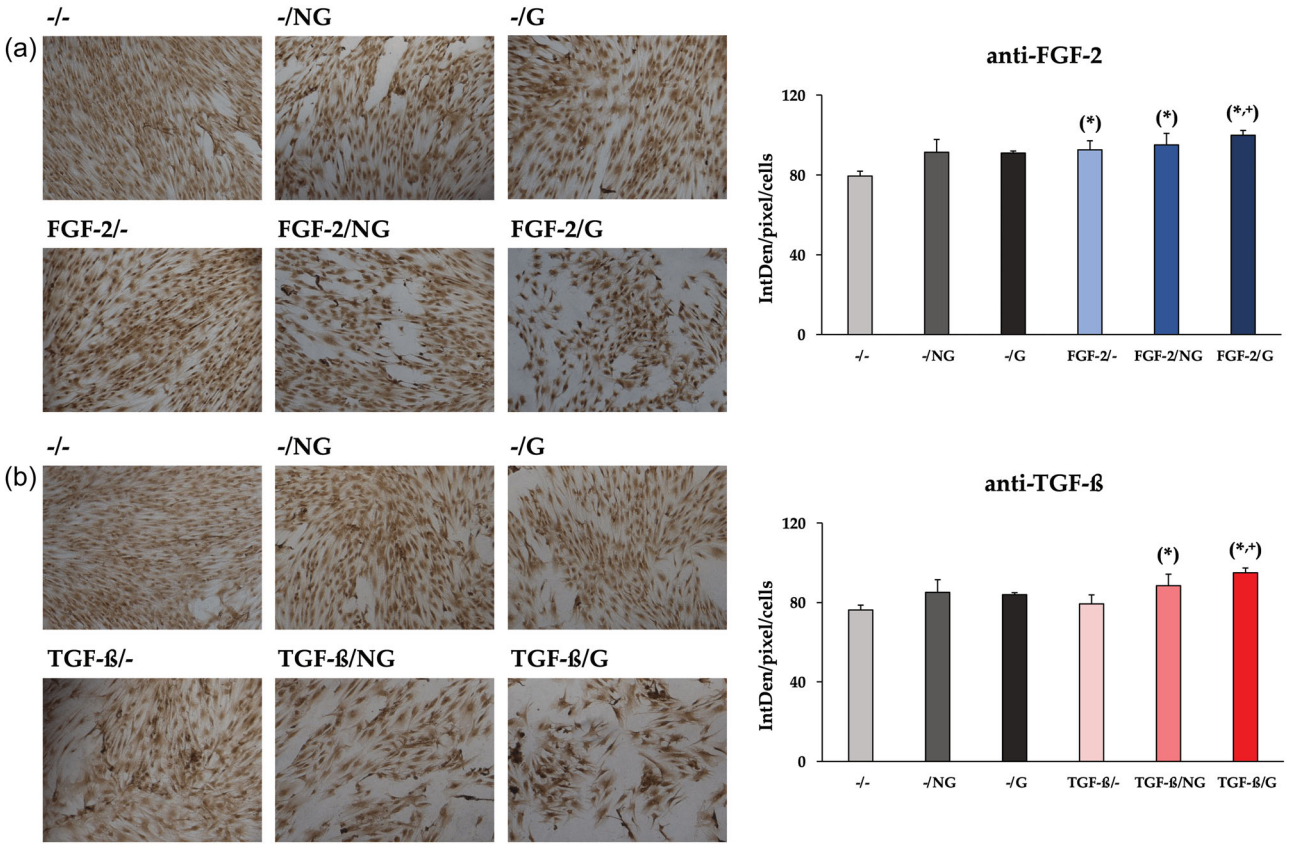

FIGURE 3 Evaluation of transgene (FGF-2, TGF-β) expression in hMSC cultures treated with therapeutic rAAV vectors incubated on PCL films by immunocytochemistry. The PCL films were incubated with rAAV-hFGF-2 or rAAV-hTGF-β (40 µL each vector, 8 × 105 transgene copies) or left without vector treatment before administration to the cultures and the expression of (a) FGF-2 and (b) TGF-β was analyzed after 9 days by immunocytochemistry (magnification x10; all representative data) with an analysis of the corresponding integrated densities with normalization to the cell densities using the ImageJ software as described in the Materials and Methods. -/-, absence of vector incubation and of PCL film; -/NG, absence of vector incubation on ungrafted PCL films; -/G, absence of vector incubation on pNaSS-grafted PCL films; FGF-2/-, film-free rAAV-hFGF-2 solution; FGF-2/NG, rAAV-hFGF-2 incubated on ungrafted PCL films; FGF-2/G, rAAV-hFGF-2 incubated on pNaSS-grafted PCL films; TGF-β/-, film-free rAAV-hTGF-β solution; TGF-β/NG, rAAV-hTGF-β incubated on ungrafted PCL films; TGF-β/G, rAAV-hTGF-β incubated on pNaSS-grafted PCL films; IntDen/pixel/cells: integrated densities per pixel per cells. Statistically significant relative to *the -/- condition and to +the corresponding ungrafted condition or film-free vector solution.

using ungrafted PCL films or a film-free vector solution (up to 1.8-fold difference, $p \leq 0.001$) (Figure 4a). Similarly, an evaluation of the levels of TGF-β production over time in hMSCs by ELISA (Figure 4b) showed the optimal, significant and steady expression of TGF-β upon administration of rAAV-hTGF-β incubated on pNaSS-grafted films (TGF-β/G) as early as 7 days and over the whole period of analysis (21 days) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (up to 5.2-fold difference, $p \leq 0.001$) (Figure 4b). This was also noted upon administration of rAAV-hTGF-β incubated on ungrafted PCL films (TGF-β/NG) or using a film-free rAAV-hTGF-β vector solution (TGF-β/-) compared with the control -/- condition (up to 2.8-fold difference, $p \leq 0.001$) (Figure 4b). Importantly, administration of rAAV-hTGF-β incubated on pNaSS-grafted PCL films was more potent than when using ungrafted PCL films or a film-free vector solution (up to threefold difference, $p \leq 0.001$) (Figure 4b).

3.2 | Effects of rAAV-mediated FGF-2 and TGF-β overexpression via PCL film-guided vector delivery on the reparative activities of hMSCs

Monolayer cultures of primary hMSCs were finally treated with the therapeutic rAAV-hFGF-2 and rAAV-hTGF-β vectors incubated on pNaSS-grafted versus ungrafted PCL films to analyze the effects of FGF-2 and TGF-β overexpression on the reparative activities of these cells in vitro over time compared with control treatments in the absence of vector incubation or with film-free vector solutions.

An evaluation of the indices of cell proliferation in hMSCs on Day 21 using the Cell Proliferation reagent WST-1 (Figure 5a) showed significant effects when administering rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films (FGF-2/G and TGF-β/G, respectively) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (1.4- and 1.7-fold difference, respectively, $p \leq 0.001$), with optimal effects reached with the TGF-β/G

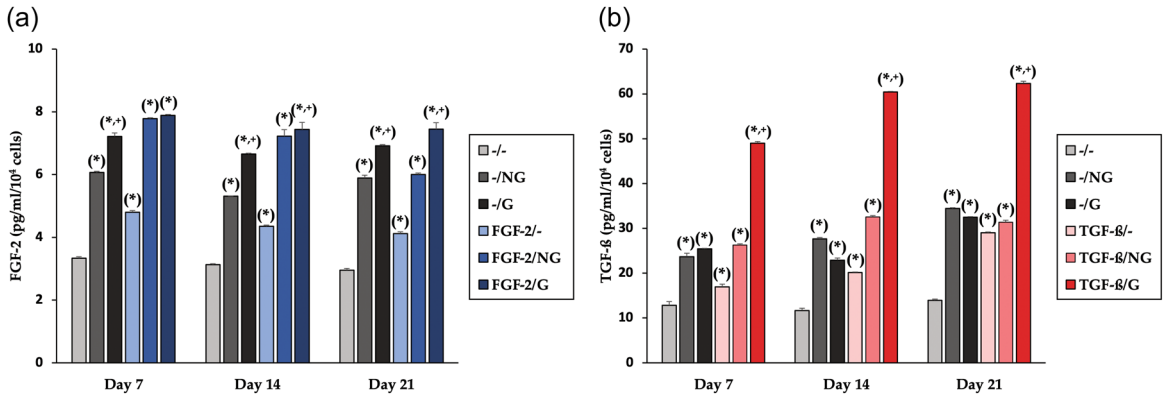

FIGURE 4 Evaluation of transgene (FGF-2, TGF-β) expression in hMSC cultures treated with therapeutic rAAV vectors incubated on PCL films by ELISA. The PCL films were incubated with rAAV-hFGF-2 or rAAV-hTGF-β or left without vector treatment before administration to the cultures as described in Figure 3 and the expression of (a) FGF-2 and (b) TGF-β was analyzed at the denoted time points by specific ELISAs with normalization to the cell densities as described in the Materials and Methods. -/-, absence of vector incubation and of PCL film; -/NG, absence of vector incubation on ungrafted PCL films; -/G, absence of vector incubation on pNaSS-grafted PCL films; FGF-2/-, film-free rAAV-hFGF-2 solution; FGF-2/NG, rAAV-hFGF-2 incubated on ungrafted PCL films; FGF-2/G, rAAV-hFGF-2 incubated on pNaSS-grafted PCL films; TGF-β/-, film-free rAAV-hTGF-β solution; TGF-β/NG, rAAV-hTGF-β incubated on ungrafted PCL films; TGF-β/G, rAAV-hTGF-β incubated on pNaSS-grafted PCL films. Statistically significant relative to *the -/- condition and to +the corresponding ungrafted condition or film-free vector solution.

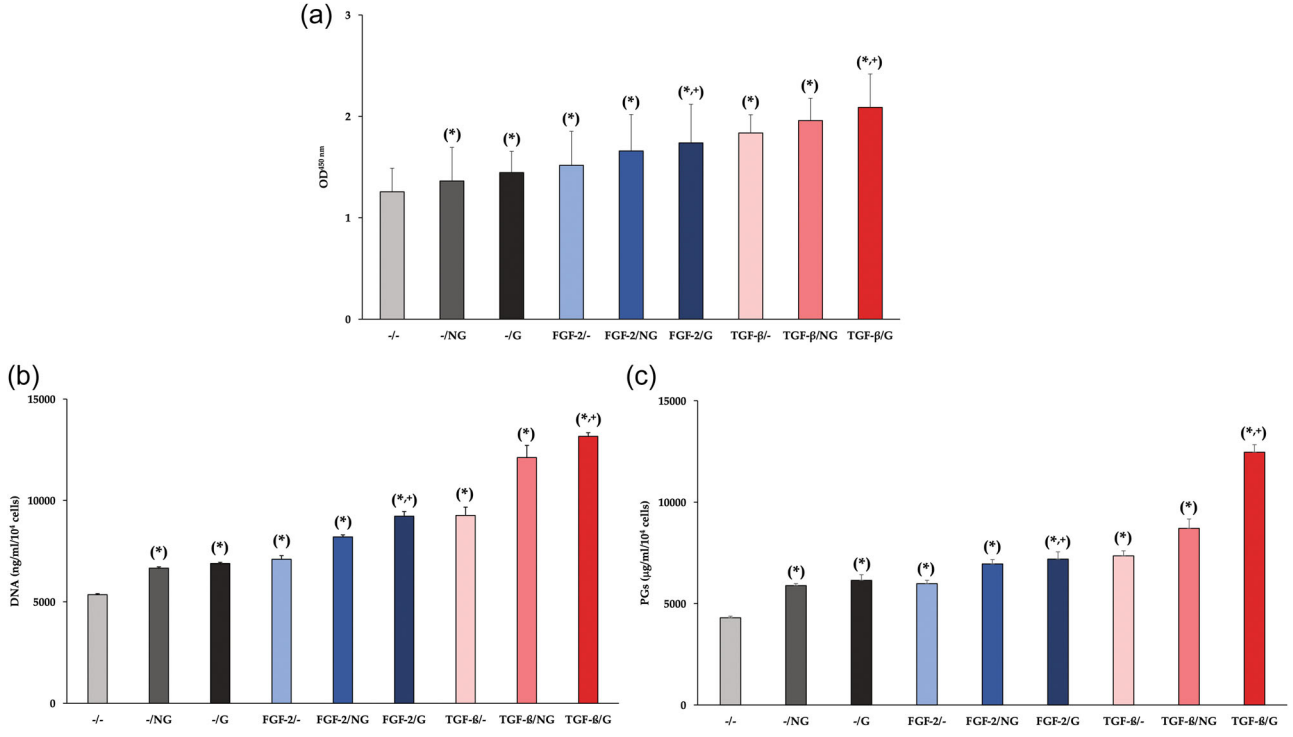

FIGURE 5 Evaluation of the reparative activities (cell proliferation, DNA and proteoglycan contents) in hMSC cultures treated with therapeutic rAAV vectors incubated on PCL films. The PCL films were incubated with rAAV-hFGF-2 or rAAV-hTGF-β or left without vector treatment before administration to the cultures as described in Figures 2 and 3 and the indices of cell proliferation as well as the DNA and proteoglycan contents (PGs) were analyzed after 21 days (a) with the Cell Proliferation reagent WST-1; (b) with the Hoechst 33258 assay, and (c) via binding to the DMMB dye, respectively, with (b, c) normalization to the cell densities as described in the Materials and Methods. -/-, absence of vector incubation and of PCL film; -/NG, absence of vector incubation on ungrafted PCL films; -/G, absence of vector incubation on pNaSS-grafted PCL films; FGF-2/-, film-free rAAV-hFGF-2 solution; FGF-2/NG, rAAV-hFGF-2 incubated on ungrafted PCL films; FGF-2/G, rAAV-hFGF-2 incubated on pNaSS-grafted PCL films; TGF-β/-, film-free rAAV-hTGF-β solution; TGF-β/NG, rAAV-hTGF-β incubated on ungrafted PCL films; TGF-β/G, rAAV-hTGF-β incubated on pNaSS-grafted PCL films. Statistically significant relative to *the -/- condition and to +the corresponding ungrafted condition or film-free vector solution.

condition. This was also noted upon administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on ungrafted PCL films (FGF-2/NG and TGF-β/NG, respectively) or using film-free rAAV-hFGF-2 and rAAV-hTGF-β vector solutions (FGF-2/- and TGF-β/-, respectively) compared with the control -/- condition (up to 1.6-fold difference, $p \leq 0.001$) (Figure 5a). Importantly, administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films was more potent than when using ungrafted PCL films or film-free vector solutions (up to 1.1-fold difference, $p \leq 0.001$) (Figure 5a). An evaluation of the DNA contents in hMSCs on Day 21 using the Hoechst 33258 assay (Figure 5b) showed significant effects when administering rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films (FGF-2/G and TGF-β/G, respectively) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (1.7- and 2.5-fold difference, respectively, $p \leq 0.001$), with optimal effects reached with the TGF-β/G condition. This was also noted upon administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on ungrafted PCL films (FGF-2/NG and TGF-β/NG, respectively) or using film-free rAAV-hFGF-2 and rAAV-hTGF-β vector solutions (FGF-2/- and TGF-β/-, respectively) compared with the control -/- condition (up to 2.3-fold difference, $p \leq 0.001$) (Figure 5b). Importantly, administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films was more potent than when using ungrafted PCL films or film-free vector solutions (up to 1.9-fold difference, $p \leq 0.001$) (Figure 5b).

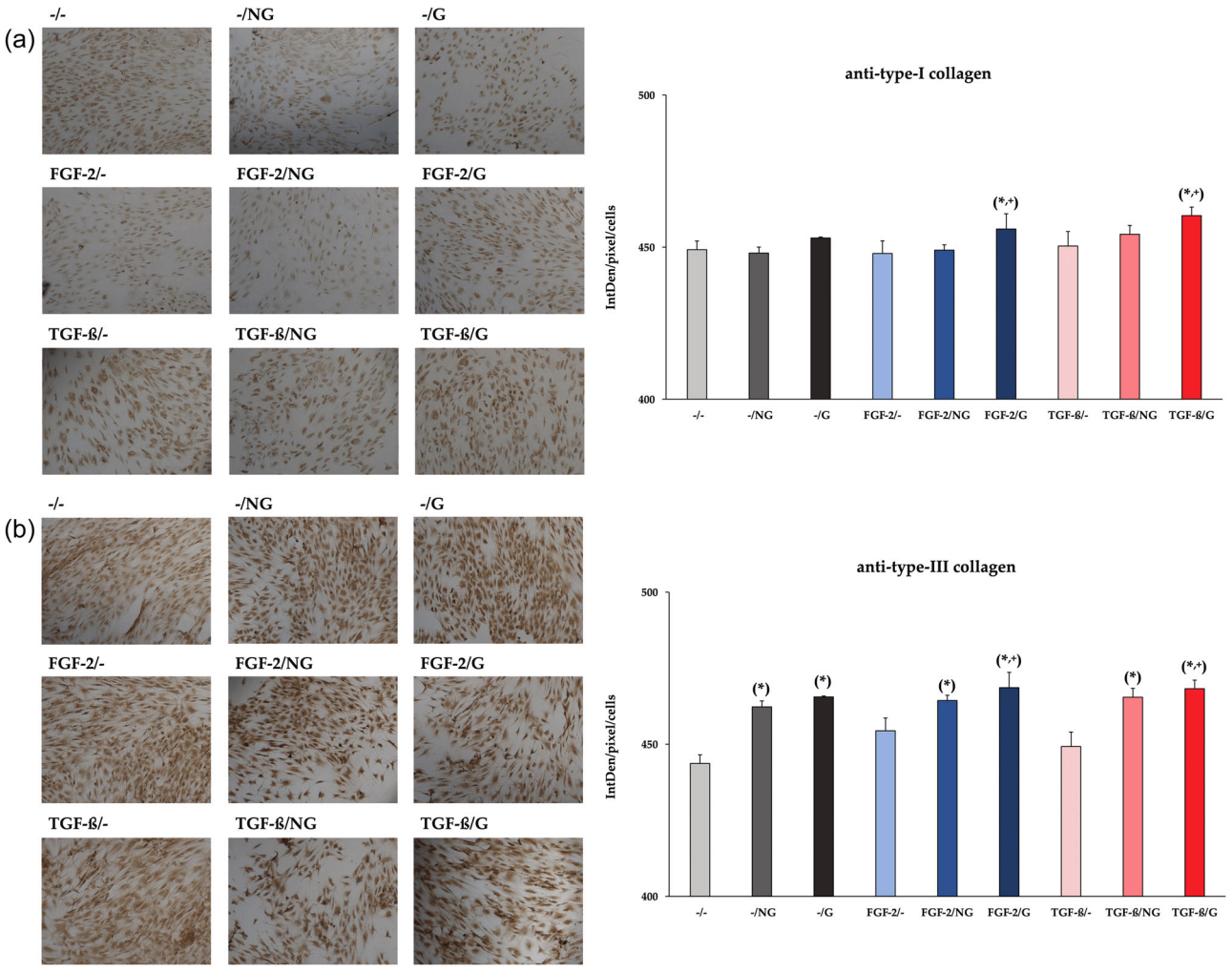

FIGURE 6 Evaluation of the reparative activities (type-I and -III collagen deposition) in hMSC cultures treated with therapeutic rAAV vectors incubated on PCL films by immunocytochemistry. The PCL films were incubated with rAAV-hFGF-2 or rAAV-hTGF-β or left without vector treatment before administration to the cultures as described in Figures 2–4 and the deposition of (a) type-I collagen and (b) type-III collagen was analyzed after 9 days by immunocytochemistry (magnification x10; all representative data) with an analysis of the corresponding integrated densities with normalization to the cell densities using the ImageJ software as described in the Materials and Methods. -/-, absence of vector incubation and of PCL film; -/NG, absence of vector incubation on ungrafted PCL films; -/G, absence of vector incubation on pNaSS-grafted PCL films; FGF-2/-, film-free rAAV-hFGF-2 solution; FGF-2/NG, rAAV-hFGF-2 incubated on ungrafted PCL films; FGF-2/G, rAAV-hFGF-2 incubated on pNaSS-grafted PCL films; TGF-β/-, film-free rAAV-hTGF-β solution; TGF-β/NG, rAAV-hTGF-β incubated on ungrafted PCL films; TGF-β/G, rAAV-hTGF-β incubated on pNaSS-grafted PCL films; IntDen/pixel/cells: integrated densities per pixel per cells. Statistically significant relative to *the -/- condition and to +the corresponding ungrafted condition or film-free vector solution.

An evaluation of the proteoglycan contents in hMSCs on Day 21 via binding to DMMB (Figure 5c) showed significant effects when administering rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films (FGF-2/G and TGF-β/G, respectively) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (1.7- and 2.9-fold difference, respectively, $p \leq 0.001$), with optimal effects reached again with the TGF-β/G condition. This was also noted upon administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on ungrafted PCL films (FGF-2/NG and TGF-β/NG, respectively) or using film-free rAAV-hFGF-2 and rAAV-hTGF-β vector solutions (FGF-2/- and TGF-β/-, respectively) compared with the control -/- condition (up to twofold difference, $p \leq 0.001$) (Figure 5c). Importantly, administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films was more potent than when using ungrafted PCL films or film-free vector solutions (up to 2.1-fold difference, $p \leq 0.001$) (Figure 5c).

An evaluation of the deposition of type-I collagen in hMSCs on Day 9 by immunocytochemical analysis with corresponding cytomorphometry (Figure 6a) showed significant effects when administering rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films (FGF-2/G and TGF-β/G, respectively) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (always 1.1-fold difference, $p \leq 0.001$), with optimal effects reached with the TGF-β/G condition. Importantly, administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films was more potent than when using ungrafted PCL films or film-free vector solutions (up to 1.1-fold difference, $p \leq 0.001$) (Figure 6a).

Similarly, an evaluation of the deposition of type-III collagen in hMSCs on Day 9 by immunocytochemical analysis with corresponding cytomorphometry (Figure 6b) showed significant effects when administering rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films (FGF-2/G and TGF-β/G, respectively) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (always 1.1-fold difference, $p \leq 0.001$). This was also noted upon administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on ungrafted PCL films (FGF-2/NG and TGF-β/NG, respectively) compared with the control -/- condition (up to 1.1-fold difference, $p \leq 0.001$) (Figure 6b). Importantly, administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films was more potent than when using ungrafted PCL films or film-free vector solutions (up to 1.1-fold difference, $p \leq 0.001$) (Figure 6b).

An evaluation of the gene expression profiles for matrix components in hMSCs on Day 21 by real-time RT-PCR (Figure 7a) showed significant increases in COL1A1 expression when administering rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films (FGF-2/G and TGF-β/G, respectively) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (always 1.2-fold difference, $p \leq 0.001$). Importantly, administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films was more potent than when using ungrafted PCL films or film-free vector solutions (up to 1.2-fold difference, $p \leq 0.001$) (Figure 7a). Significant increases in COL3A1 expression in hMSCs on Day 21 by real-time RT-PCR (Figure 7b) were also noted when administering rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films (FGF-2/G and TGF-β/G, respectively) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (always 1.2-fold difference, $p \leq 0.001$). Again, administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films was more potent than when using ungrafted PCL films or film-free vector solutions (up to 1.2-fold difference, $p \leq 0.001$) (Figure 7b).

Remarkably, an evaluation of the gene expression profiles for transcription factors in hMSCs on Day 21 by real-time RT-PCR (Figure 8) showed significant increases in Mohawk expression when

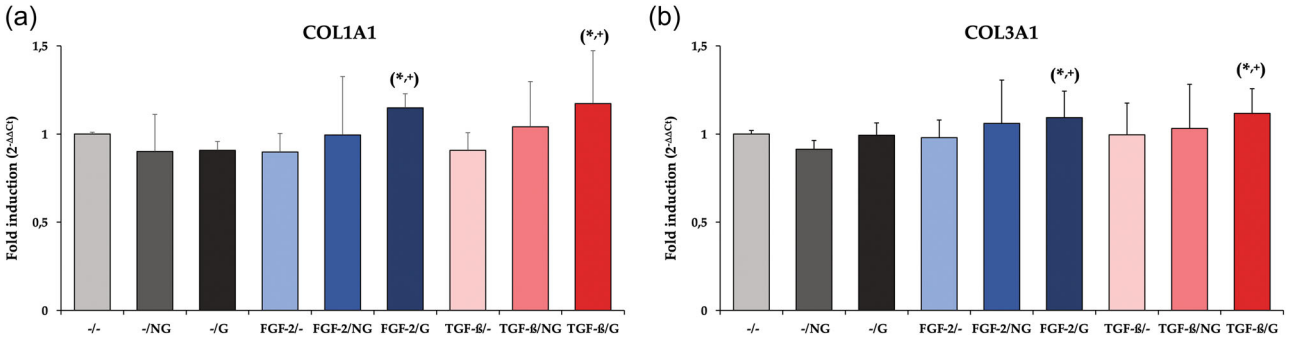

FIGURE 7 Evaluation of the gene expression profiles of matrix components in hMSC cultures treated with rAAV vectors incubated on PCL films by real-time RT-PCR. The PCL films were incubated with rAAV-hFGF-2 or rAAV-hTGF-β or left without vector treatment before administration to the cultures as described in Figures 2–5 and the gene expression profiles for (a) type-I collagen (COL1A1) and (b) type-III collagen (COL3A1) were monitored on Day 21 by real-time RT-PCR with GAPDH as a housekeeping gene as described in the Materials and Methods. C_t values were generated for each target gene and for GAPDH as a control of normalization and fold inductions (relative to the -/- condition) were calculated using the $2^{-\Delta\Delta C_t}$ method. -/-, absence of vector incubation and of PCL film; -/NG, absence of vector incubation on ungrafted PCL films; -/G, absence of vector incubation on pNaSS-grafted PCL films; FGF-2/-, film-free rAAV-hFGF-2 solution; FGF-2/NG, rAAV-hFGF-2 incubated on ungrafted PCL films; FGF-2/G, rAAV-hFGF-2 incubated on pNaSS-grafted PCL films; TGF-β/-, film-free rAAV-hTGF-β solution; TGF-β/NG, rAAV-hTGF-β incubated on ungrafted PCL films; TGF-β/G, rAAV-hTGF-β incubated on pNaSS-grafted PCL films. Statistically significant relative to *the -/- condition and to +the corresponding ungrafted condition or film-free vector solution.

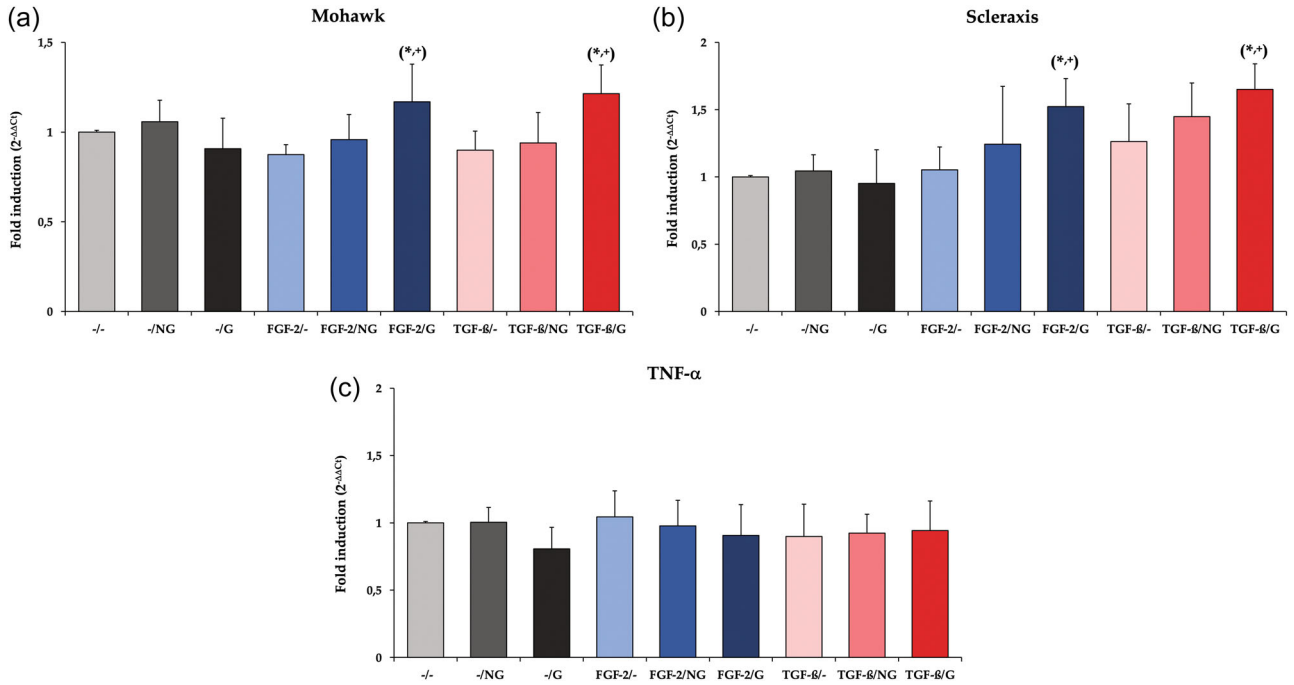

FIGURE 8 Evaluation of the gene expression profiles of transcription factors and of inflammation in hMSC cultures treated with rAAV vectors incubated on PCL films by real-time RT-PCR. The PCL films were incubated with rAAV-hFGF-2 or rAAV-hTGF-β or left without vector treatment before administration to the cultures as described in Figures 3–7 and the gene expression profiles for (a, b) the transcription factors (a) Mohawk and (b) Scleraxis and for (c) the inflammatory mediator TNF-α were monitored on Day 21 by real-time RT-PCR with GAPDH as a housekeeping gene as described in the Materials and Methods. Ct values were generated for each target gene and for GAPDH as a control of normalization and fold inductions (relative to the -/- condition) were calculated using the $2^{-\Delta\Delta C_t}$ method. -/-, absence of vector incubation and of PCL film; -/NG, absence of vector incubation on ungrafted PCL films; -/G, absence of vector incubation on pNaSS-grafted PCL films; FGF-2/-, film-free rAAV-hFGF-2 solution; FGF-2/NG, rAAV-hFGF-2 incubated on ungrafted PCL films; FGF-2/G, rAAV-hFGF-2 incubated on pNaSS-grafted PCL films; TGF-β/-, film-free rAAV-hTGF-β solution; TGF-β/NG, rAAV-hTGF-β incubated on ungrafted PCL films; TGF-β/G, rAAV-hTGF-β incubated on pNaSS-grafted PCL films. Statistically significant relative to *the -/- condition and to +the corresponding ungrafted condition or film-free vector solution. TNF-α, tumor necrosis alpha.

administering rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films (FGF-2/G and TGF-β/G, respectively) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (1.7- and 1.2-fold difference, respectively, $p \leq 0.001$) (Figure 8a). Importantly, administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films was more potent than when using ungrafted PCL films or film-free vector solutions (up to 1.9-fold difference, $p \leq 0.001$) (Figure 8a). Significant increases in Scleraxis expression in hMSCs on Day 21 by real-time RT-PCR (Figure 8b) were also noted when administering rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films (FGF-2/G and TGF-β/G, respectively) compared with the control condition in the absence of vector incubation and of PCL film (-/-) (1.5- and 1.7-fold difference, respectively, $p \leq 0.001$). Again, administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films was more potent than when using ungrafted PCL films or film-free vector solutions (up to 1.8-fold difference, $p \leq 0.001$) (Figure 8b). Of further interest, an evaluation of inflammatory gene expression profiles in hMSCs on Day 21 by real-time RT-PCR (Figure 8c) showed no detrimental effects on TNF-α expression when administering rAAV-hFGF-2 and rAAV-hTGF-β incubated on pNaSS-grafted PCL films (FGF-2/G and TGF-β/G, respectively) compared with the control condition in the absence of vector incubation and of PCL film (-/-) or with any other condition ($p \geq 0.050$) (Figure 8c).

4 | CONCLUSIONS

The combined use of gene therapy vectors with biomaterials represents a powerful strategy to stimulate the mechanisms of ACL repair via the delivery of candidate genetic sequences to generate noninvasive therapeutic options in patients in the future (Amini et al., 2022). Among various potent genes to achieve this goal, those coding for the FGF-2 and TGF-β were applied via the rAAV vector already employed in patients (Watson-Levings et al., 2022) incubated on pNaSS-grafted PCL films (Amini et al., 2023) to highly regenerative hMSCs due to the reparative activities of each of these two growth factors (Amini et al., 2023; Cucchiarini et al., 2011; Frisch et al., 2014; Madry et al., 2013; Pascher et al., 2004; Wei et al., 2011).

The data first demonstrate that PCL films can effectively and durably deliver and overexpress reporter (RFP) rAAV vectors in hMSCs (up to 21 days, the longest time-point examined) to levels

similar to those achieved using film-free vector solutions and in contrast to control treatments in the absence of the vectors, concordant with findings when providing the rAAV-RFP vector to human ACL fibroblasts (Amini et al., 2023) as a result of the effective vector incubation on and controlled release from this material (Venkatesan et al., 2020). The data also reveal that PCL films can effectively and durably deliver and overexpress therapeutic (FGF-2 and TGF-β) rAAV vectors in hMSCs (up to 21 days), most particularly when using pNaSS-grafted PCL films and compared with all other treatments, again concordant with findings when providing the rAAV-hFGF-2 and rAAV-hTGF-β vectors to human ACL fibroblasts at similar MOIs (Amini et al., 2023) and probably resulting from the effective vector incubation on and controlled release from this material (Venkatesan et al., 2020). Interestingly, the levels of TGF-β achieved using rAAV-hTGF-β incubated on pNaSS-grafted PCL films were higher (~ninefold) than the levels of FGF-2 reached using rAAV-hFGF-2 incubated on similar films, as previously observed (Shi et al., 2010) and possibly due to differences in the activities of regulatory untranslated regions in the growth factor sequences (van der Velden & Thomas, 1999).

The data next show that the effective and durable overexpression of the candidate (FGF-2 and TGF-β) rAAV vectors increased the levels of cell proliferation, the DNA contents, and the deposition of specific ECM components (proteoglycans, type-I, and -III collagen) in hMSCs (up to 21 days), most particularly when using pNaSS-grafted PCL films and compared with all other treatments. This is again concordant with findings when providing the rAAV-hFGF-2 and rAAV-hTGF-β vectors to human ACL fibroblasts at similar MOIs (Amini et al., 2023) and with the properties of the growth factors (DesRosiers et al., 1996; Marui et al., 1997; Murray et al., 2003; Molloy et al., 2003; Pascher et al., 2004), probably resulting from the effective vector incubation on and controlled release from this material (Venkatesan et al., 2020). Again, the effects noted here were more marked when using rAAV-hTGF-β incubated on pNaSS-grafted PCL films compared with those achieved with rAAV-hFGF-2 incubated on similar films, probably due to the higher levels of TGF-β achieved via such systems. Of note, these effects were associated with and probably resulting from enhanced levels of the specific Mohawk and Scleraxis ECM-inducing transcription factors (Nakahara et al., 2013; Nichols et al., 2018) in hMSCs (up to 21 days), most particularly when using pNaSS-grafted PCL films and compared with all other treatments. This is concordant with findings when providing the rAAV-hFGF-2 and rAAV-hTGF-β vectors to human ACL fibroblasts at similar MOIs (Amini et al., 2023) and with the properties of these growth factors (Farhat et al., 2012; Hyun et al., 2017). Again, the effects noted here were more marked when using rAAV-hTGF-β incubated on pNaSS-grafted PCL films compared with those achieved with rAAV-hFGF-2 incubated on similar films, probably due to the higher levels of TGF-β achieved via such systems. Finally, administration of rAAV-hFGF-2 and rAAV-hTGF-β incubated on PCL films had no deleterious effects on cell inflammation in hMSCs (up to 21 days) as noted when analyzing the expression profiles for TNF-α, in agreement with findings when providing the rAAV-hFGF-2 and rAAV-hTGF-β vectors to human ACL fibroblasts at similar MOIs (Amini et al., 2023), with the reported protection against inflammation afforded by PCL films in particular upon pNaSS grafting (Leroux et al., 2019), and with the properties of the growth factors (Fujihara et al., 2019).

The current study indicates that pNaSS-grafted PCL films have a strong value to deliver clinically adapted rAAV vectors as a means to overexpress FGF-2 and TGF-β, providing an innovative, off-the-shelf gene therapy system capable of stimulating the mechanisms relevant of ACL repair. Work is ongoing to examine the possible benefits of this biomaterial-guided approach in models of ACL lesions in animals in vivo (Maurice et al., 2022) based on the effective release of rAAV vectors from the films over prolonged time periods (at least 21 days in vitro) (Venkatesan et al., 2020). Such work will also test whether a combined delivery of the both FGF-2 and TGF-β rAAV vectors may be more potent than using single vector administration (Morscheid et al., 2019) in light of findings reporting synergistic effects of the two growth factors on the tenogenesis of MSCs in vitro (Li et al., 2023). This study overall shows the advantages of delivering rAAV using functionalized PCL films for future improvement of ACL repair.

AUTHOR CONTRIBUTIONS

Conceptualization: Meret Stehle and Magali Cucchiarini. *Methodology*: Meret Stehle, Mahnaz Amini, Jagadeesh K. Venkatesan, Wei Liu, Dan Wang, Tuan N. Nguyen, Amélie Leroux, Henning Madry, Véronique Migonney, and Magali Cucchiarini. *Validation*: Meret Stehle, Mahnaz Amini, Jagadeesh K. Venkatesan, Wei Liu, Dan Wang, Tuan N. Nguyen, Amélie Leroux, Henning Madry, Véronique Migonney, and Magali Cucchiarini. *Formal analysis*: Meret Stehle, Mahnaz Amini, Jagadeesh K. Venkatesan, Wei Liu, Dan Wang, Tuan N. Nguyen, Amélie Leroux, Henning Madry, Véronique Migonney, and Magali Cucchiarini. *Investigation*: Meret Stehle, Mahnaz Amini, Jagadeesh K. Venkatesan, Wei Liu, Dan Wang, Tuan N. Nguyen, Amélie Leroux, Henning Madry, Véronique Migonney, and Magali Cucchiarini. *Resources*: Meret Stehle, Mahnaz Amini, Jagadeesh K. Venkatesan, Wei Liu, Dan Wang, Tuan N. Nguyen, Amélie Leroux, Henning Madry, Véronique Migonney, and Magali Cucchiarini. *Writing—original draft preparation*: Meret Stehle and Magali Cucchiarini; *Writing, review and editing*: Meret Stehle, Mahnaz Amini, Jagadeesh K. Venkatesan, Wei Liu, Dan Wang, Tuan N. Nguyen, Amélie Leroux, Henning Madry, Véronique Migonney, and Magali Cucchiarini. *Supervision*: Magali Cucchiarini; Funding acquisition: Henning Madry, Véronique Migonney, and Magali Cucchiarini. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available on reasonable request.

ETHICS STATEMENT

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the Saarland Physicians Council (*Ärztekammer des Saarlandes*, reference number Ha06/08). Informed consent was obtained from all subjects involved in the study.

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