Supporting Information

Deciphering the role of substrate stiffness to enhance internalization efficiency of plasmid DNA in stem cells using lipid-based nanocarriers

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Experimental Section:

Silicone gel substrates: CytoSoft® 6-well plates with a thin layer of anhydride-functionalized silicone with different stiffness (0.5 and 32 kPa) were purchased from Advanced BioMatrix (Carlsbad, CA, USA). The surface of the gels was pre-coated with a sterile solution of Gelatin type A (1% w/v) from porcine skin (Sigma-Aldrich, USA) in PBS at pH 7.4. The coating step was carried out for 4 hours at room temperature adding 4 mL of gelatin solution in each well to ensure the homogeneous coating of the hydrophobic surfaces. The same procedure was applied to coat CytoSoft® Imaging wells 24-well plates (Advanced BioMatrix, San Diego, CA) having different stiffness (0.5 and 32 kPa). This type of well plate was used for imaging cells at a higher magnification (40X).

hASCs culture and fluorescence immunostaining: hASCs were purchased from RoosterBio (RoosterBio, USA). Passages 4–6 were used for all of the studies. Cells were cultured in Minimum Essential Medium Alpha Modification (α-MEM) (Thermo-Fisher Scientific, USA) supplemented

with 15% fetal bovine serum (FBS) (Thermo-Fisher Scientific, USA) and 1% penicillin/streptomycin (Thermo-Fisher Scientific, USA) at 37 °C and 5% CO_2. To study the influence of substrate stiffness on the focal adhesion expression of hASCs, cells were seeded (2 x 10^4 cells/well) on CytoSoft® Imaging wells with different stiffness (0.5 and 32 kPa) following the conditions reported above. After 24 hours, the media was replaced with incomplete α-MEM and cells were cultured in these conditions for 48 hours. Cells were then fixed with 4% paraformaldehyde for 5 minutes at 37 ºC followed by permeabilization with 0.1 % Triton-X100 for 10 minutes at room temperature. Normal goat serum (5%) was applied for 45 minutes at room temperature to block the samples. Mouse anti-human Paxillin antibody (1:400 with 1% goat serum, Invitrogen, USA) was added to cells and incubated overnight at 4 °C. Next, goat anti-mouse AlexaFluor 594 (Invitrogen USA) (1:500 in 1% goat serum) was added to the cells for one hour at room temperature. Nuclei and F-actin were counterstained using Diamidino-2-phenylindole dilactate (DAPI, Invitrogen, USA) and phalloidin-AlexaFluor 488 (Invitrogen, USA), respectively. Immunofluorescence images were taken by EVOS cell imaging system (Thermo-Fisher Scientific, USA) and further analyzed in ImageJ for quantification of area occupied by each cell, number of actin fibers, and the total area of adhesion sites. Specifically, cell area was quantified using 7 to 10 cells per image for a total of 10 images per group (n=10). Number of actin stress fibers was counted based on a total of 7 images each containing one cell captured at 40 X magnification (n=7). Paxillin area was measured using 7 different images per group (n=7) captured at 40X magnification. A single cell in each image was analyzed for paxillin area quantification.

Traction force microscopy: Cell traction was calculated using constrained Fourier transform traction microscopy (FTTM) [16]. 0.2 μm amino-modified fluorescence microbeads were

conjugated to the substrates due to presence of anhydride groups on the substrates' surface. hASCs ($2x10^3$/well) were seeded on substrates of varying stiffness and containing microbeads and allowed to grow for 24 hours. Fluorescence images of the microbeads were taken before and after cell detachment from the substrates by trypsinization. The corresponding displacement field was calculated using a customized Matlab code. The traction force was calculated based on the displacement field computed from 7 images for each group (n=7). A single cell in each image was analyzed for traction force.

Plasmid construct design: The pscAAV-GFP vector (John T. Gray, Addgene plasmid #32396) harboring the enhanced green fluorescent protein (EGFP) gene was digested with BamHI and NotI restriction enzymes (New England BioLabs, Ipswich, MA) to excise the EGFP gene. The pCMV6-XL5 mammalian expression vector (Origene Technologies, Rockville, MD) was digested with BamHI and NotI enzymes, and after purification, the linearized EGFP gene was ligated with the pCMV6-XL5 expression vector using T4 DNA Ligase (Promega). The resulting construct, pCMV-GFP, was transformed in chemically competent DH5α *Escherichia coli* (Invitrogen, Carlsbad, CA) to amplify the plasmid. Additionally, the expression vector for the human VEGF-165 gene, pCMV6-XL5-VEGF (Origene Technologies, Rockville, MD), was also amplified by transformation of DH5α *E. coli*. Both plasmids were extracted and purified using QIAPrep Spin Midiprep kits (QIAGEN Inc., Germantown, MD).

Lipofectamine-plasmid DNA complex formation and characterization: Prior to transfection, both plasmid DNA and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) were diluted in 150 μL of incomplete α-MEM media. Specifically, the lipoplexes were prepared by mixing the two

components in equal volumes (150 µL) containing a 1:3 ratio of plasmid DNA (4 ug) to Lipofectamine 2000 (12 uL). The complexes were then incubated for 20 minutes at room temperature before addition to hASCs. The hydrodynamic diameter and the zeta potential of the Lipofectamine 2000 and the lipoplexes were obtained using a ZetaPALS zeta potential analyzer (Brookhaven Instruments Corporation). Samples for DLS analysis were prepared by diluting Lipofectamine and the lipoplexes in Opti-MEM (Thermo-Fisher Scientific, USA). On the contrary, zeta potential measurements were carried out by diluting the samples in KCl 1mM.

Transfection of hASCs: hASCs were seeded on the different silicone substrates (10^5 cells/well) and allowed to proliferate for 24 hours prior to transfection. hASCs were cultured in complete α-MEM (15% FBS and 1% penicillin/streptomycin) at 37 °C and 5% CO_2.The growth medium was replaced with fresh α-MEM (2 mL/well) without FBS, and the complexes of Lipofectamine and plasmid DNA (300 µL/well) were added dropwise to the cells (80-90% confluency). After incubation for 1 and 4 hours at 37°C, the media containing the complexes was removed and replaced with complete α-MEM. Cells were cultured for 72 hours prior to changing the media, and further analysis was conducted to evaluate the quantity of VEGF secreted by the cells.

Similarly, GFP transfection of hASCs was carried out using Lipofectamine according to the conditions reported above. Cells were cultured for 24 hours prior to fluorescence-activated cell sorting (FACS) analysis. Briefly, cells were trypsinized 24 hours post-transfection and suspended in PBS at the cell density of $1x10^5$ cells/mL. The percentage of GFP positive hASCs and corresponding mean fluorescence intensity (MFI) values were determined using the Attune NxT flow cytometer (Thermo-Fischer Scientific, USA). Three different samples per group were analyzed and the results were reported as mean ± deviation standard. Additionally, fluorescent

and corresponding phase contrast (10X) images were captured by EVOS cell imaging system (Thermo-Fischer Scientific, USA).

Assessment of plasmid internalization by qPCR analysis: hASCs seeded on soft and stiff substrates were transfected for 1 hour using the GFP plasmid. Cells were thoroughly washed several times with PBS to remove the excess plasmid. Cells were then trypsinized and counted to obtain a final cell suspension of $1x10^3$/mL. Cells were centrifuged at 1000 rpm for 5 minutes and the corresponding pellets were resuspended in 100 µL PBS and freeze-thawed to lyse the cell membrane at -80 °C [18]. One microliter of this lysate suspension containing the plasmid was mixed with 10 µL KiCqStart SYBR Green Master Mix, 2 µL of the forward and reverse primer for GFP gene and 5 µL of RNAse-free water. All of the reactions were performed using a Mastercycler Realplex (Eppendorf, Germany). The quantity of plasmid internalized by the cells in each group was quantified using a calibration curve obtained in the range of 0.9 ng to 30 ng of GFP plasmid (Figure S1, Supporting Information). Each dilution of the GFP-plasmid was generated using a suspension of non-transfected hASCs lysate ($1x10^3$/mL). Additionally, GFP-plasmid dilutions were also obtained in RNAse-free water to detect undesired contamination. Samples were measured in triplicate for each dilution (n=3).

Evaluation of plasmid internalization using fluorescent labeled plasmid-Lipofectamine complexes: To visualize the plasmid internalization, the plasmid was labeled with red fluorescent Rhodamine using the Label IT® Tracker™ intracellular nucleic acid localization Kit CX-Rhodamine (Mirus, WI, USA). The process of labeling was achieved following the established protocol provided by the kit. The labeled plasmid was complexed with Lipofectamine following

the procedure reported above. hASCs were transfected for one hour followed by thorough washing with PBS to remove the non-internalized plasmid. Fluorescent and corresponding phase contrast (40X) images were captured by EVOS cell imaging system (Thermo-Fischer Scientific, USA). Cell boundaries were defined based on phase contrast images. The fluorescence intensity was quantified based on the integrated density values of each cell using ImageJ. Results were reported as mean ± standard deviation (n = 5) and normalized to the number of cells.

qPCR investigation of caveolin and clathrin genes: Expression of caveolin and clathrin-mediated genes by hASCs was evaluated using qPCR after 1 and 4 hours post-transfection on the soft (0.5 kPa) and stiff substrates (32 kPa). mRNA was extracted from the cells using an RNeasy Mini Kit (Qiagen, Germany) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, USA). Next, mRNA samples were converted to cDNA using the High-Capacity cDNA Conversion Kit (Applied Biosystems, USA). Lastly, gene expression was measured using a cocktail of predesigned primers and the KiCqStart SYBR Green Master Mix (Sigma Aldrich, USA). All of the reactions were performed using a Mastercycler RealPlex (Eppendorf, Germany). The fold expression levels were calculated using the relative ΔΔCt method. GAPDH was considered as the housekeeping gene, and results were normalized based on the gene expression of hASCs cultured on the softer substrates.

VEGF quantification by ELISA: hASCs (10^5 cells/well) were seeded on 6-well plate silicone hydrogels coated with 1% w/v of gelatin type A and allowed to grow in α-MEM (2 mL) supplemented with 15% FBS and 1% penicillin/streptomycin (Thermo-Fisher Scientific, USA) at 37 °C and 5% CO_2. Cells at 70-80% confluency were transfected with VEGF-165 plasmid using

Lipofectamine 2000 reagent in incomplete α-MEM. Supernatants were collected 72 hours after transfection. Quantification of VEGF expression was carried out using an enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN) according to manufacturer's protocol. The quantity of VEGF released was quantified by ELISA using a standard calibration curve ranging from 32 pg/mL to 1 ng/mL. Results were normalized based on the concentration of VEGF obtained from the media recovered after 72 hours in the soft substrates (n=3).

HUVEC migration assay: Human umbilical vein endothelial cells (HUVECs) (Lonza, Walkersville, MD) between passages 6-9 were cultured in 24 well plates (2 x 10^4 cells/well) in endothelial growth medium (400 μL) (EGM-2 BulletKit, Lonza, Walkersville, MD) without the addition of VEGF and rhFGF-B. After reaching confluency, scratches were made in cell monolayer using a cell scraper. Cells were washed thoroughly with PBS to remove the floating cells. Media containing VEGF was collected from stiff and soft substrate groups after 72 hours of transfection (100 μL). Then, HUVECs were allowed to migrate through the scratch for 12 and 24 hours. Cells were stained with calcein and corresponding fluorescent images were taken in the same positions along the scratch at 12 and 24 hours. HUVECs that did not receive any growth factors were considered as control groups. The percentage of area covered by HUVECs during the migration was calculated using ImageJ software. The original scratch area was calculated based on preliminary images taken immediately after the scratch was formed (n=3).

Statistical analysis: Statistical analysis for area calculation covered by HUVECs in the scratch assay was performed using one-way repeated measured analysis of variance (ANOVA) followed by Tukey's multiple comparison test to determine any significant differences between groups at

each time point. For all other analyses, a t-test (non-parametric) was used to compare two groups. All analyses were carried out with GraphPad Prism. A p-value less than 0.05 indicates statistical significance.

Table S1. Main genes regulating the process of caveolin-mediated endocytosis

Genes	***Forward primer***	***Reverse primer***	***Role(s)***
CAV1 (Caveolin 1)	GACGTCGTCAAGGTAAGCCA	GTAACGTTTCTGCCGACTGC	Flask shaped protein essential for caveolae formation
CAVIN1	GCAATACGCTGAGCAAGCTG	CTGTGGGCTCACCTGGTAGAT	Regulator of caveolae formation
ABL1 (Abl tyrosin kinase)	CTCAGACGAAGTGGAAAAG	GAGTGAGGCATCTCAGG	Responsible for actin polymerization to form stress fibers and organization of CAV1 linked to these fibers
FLNA (Filamin A)	TAAGGTTACTGTGCTCTTTG	AAGATCTCAAAGTAGGTGGTC	Key factor for the interplay between actin and CAV1
ITGB1 (β1 integrin)	ATTCCCTTTCCTCAGAAGTC	TTTTCTTCCATTTTCCCCTG	Mediator of microtubule stabilization and translocation of caveolae within the cytoskeletal system
DIAPH1 (Diaphanous Related Formin 1)	ATTTCAGTCCAGGTGGTTGC	GTGAGGCAGGTGCTTTCTTC	Cooperate with ABL1 along with local stabilization of microtubules
RHOA (Ras Homolog Family Member A)	AGGAAGATTATGATCGCCTG	TTCTAAACTATCAGGGCTGTC	Regulator of the link between caveolae and actin stress fibers

Table S2. Main genes regulating the process of Clathrin-mediated endocytosis.

Genes	***Forward primer***	***Reverse primer***	***Role(s)***
CLTC (Clathrin)	TAAAGTGTTTCCCCCTGCTGCAC	GCTGGTCTCTCAACCAAAGC	Main component of clathrin triskelion formation
SNX9	GGAATGGAAAACTGGAAAGAG	TCTACTAAGTCCAAGTCAGG	Involved in clathrin-coated vesicle budding and formation of the clathrin neck
EPS15	AAGGTCAACAATGAAGATCC	GACCGATGTTTCCTCAAATAC	A scaffolding protein, Involved in clathrin recruitment
EPN1 (Epsin)	GTTTACAAGGCCATGACG	CTTAGCTTTCTCACGCAC	An accessory protein which mediates clathrin binding to cell membrane and cargo receptors

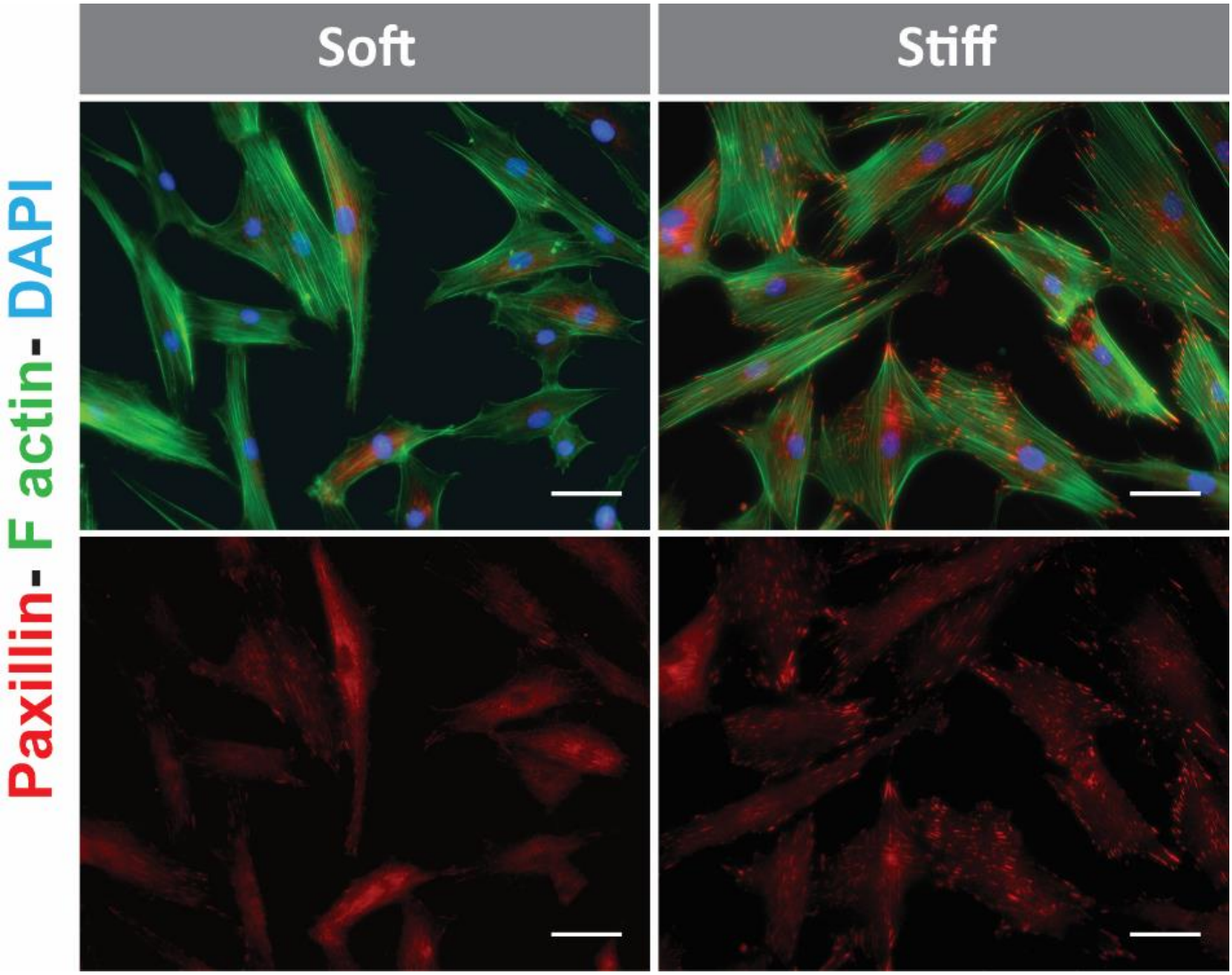

Figure S1. Immunofluorescence staining of hASCs seeded on soft (0.5 kPa) and stiff (32 kPa) substrates. Cells were stained with Alexa Fluor 488 Phalloidin to visualize actin stress fibers (green) and with Diamidino-2-phenylindole dilactate (DAPI) to stain the nuclei (blue). In addition, paxillin staining (red) was carried out to identify the area of focal adhesion. Scale bar = 60 µm.

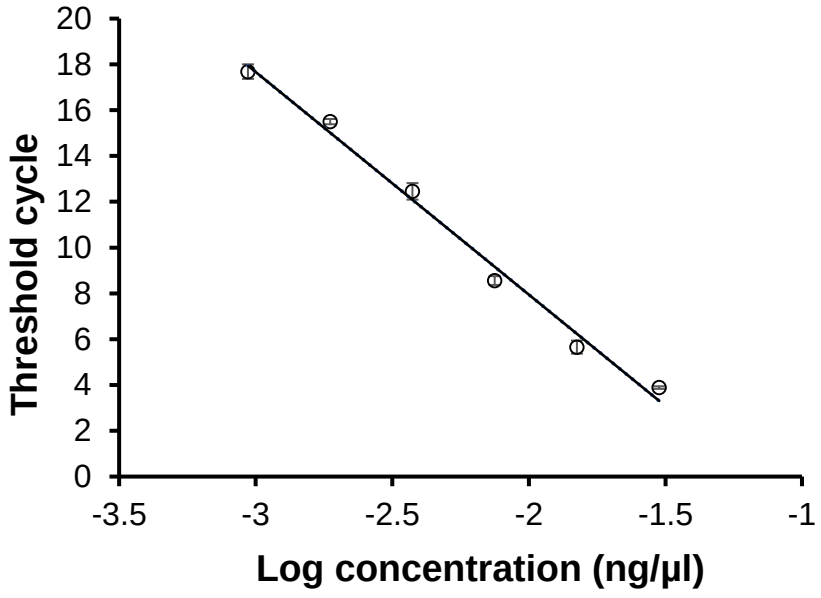

Figure S2. Calibration curve for plasmid internalization analyzed by qPCR. This curve was obtained by acquiring the corresponding threshold cycle for different concentration of GFP plasmid (0.9 ng to 30 ng) in the cell lysates.

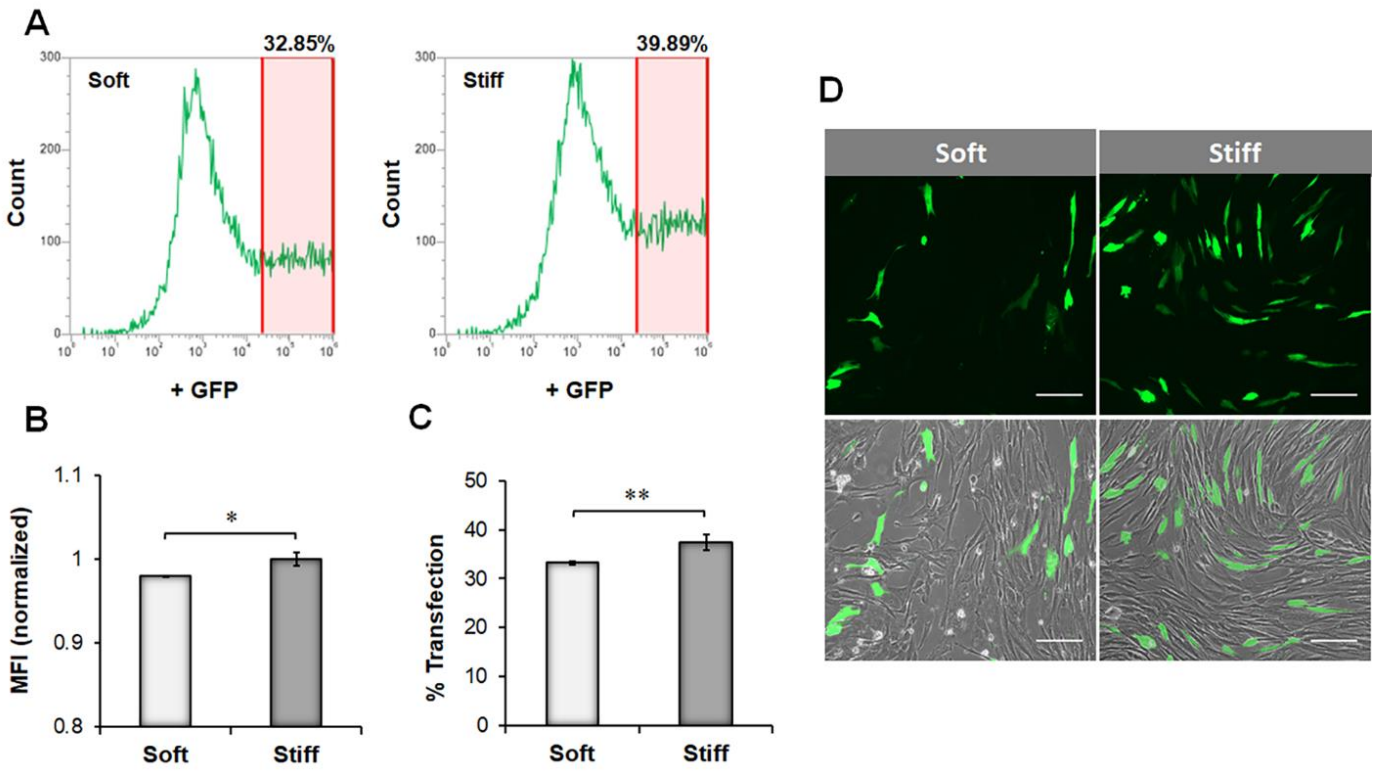

Figure S3. Effect of substrate stiffness on green fluorescent protein (GFP) expression on hASCs upon transfection with Lipofectamine. A) FACs analysis of hASCs seeded on the two different substrates after being transfected with GFP plasmid. The population of GFP positive cells are indicated on the graph (pink area). B) Quantification of mean fluorescence intensity (MFI) of GFP positive hASCs obtained using FACs analysis. C) Percentage of transfected cells obtained by analyzing FACs data. Results are reported as mean ± standard deviation (n = 3) * = $p < 0.05$, ** = $p < 0.01$. D) Fluorescent images and corresponding phase contrast images of hASCs expressing GFP after 24 hours of transfection. (Scale bar = 200 µm).