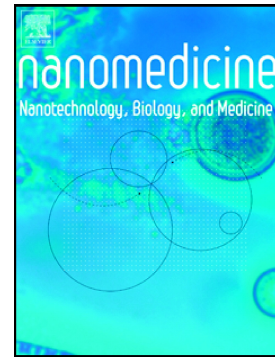# Accepted Manuscript

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Triggering the activation of Activin A type II receptor in human adipose stem cells towards tenogenic commitment using mechanomagnetic stimulation

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Conflict of interest: A.J. El Haj is a co-founder and scientific consultant for MICA Biosystems with a 50% shareholding.

Abstract

Stem cell therapies hold potential to stimulate tendon regeneration and homeostasis, which is maintained in response to the native mechanical environment. Activins are members of the mechano-responsive TGF-β superfamily that participates in the regulation of several downstream biological processes. Mechanosensitive membrane receptors such as activin can be activated in different types of stem cells via magnetic nanoparticles (MNPs) through remote magnetic actuation resulting in cell differentiation.

In this work, we target the Activin receptor type IIA (ActRIIA) in human adipose stem cells (hASCs), using anti-ActRIIA functionalized MNPs, externally activated through a oscillating magnetic bioreactor. Upon activation, the phosphorylation of Smad2/3 is induced allowing translocation of the complex to the nucleus, regulating tenogenic transcriptional responses. Our study demonstrates the potential remote activation of MNPs tagged hASCs to trigger the Activin receptor leading to tenogenic differentiation. These results may provide insights toward tendon regeneration therapies.

Background

During tendon development, mechanical forces are transmitted to tendon cells through mechanosensitive receptors available in cell membranes.[1, 2] These changes initiate intracellular transduction cascades through the activation and the stimulation of transmembrane receptors such as serine/threonine kinase receptors, inducing structural changes in the cytoskeleton and promoting regulated transcriptional responses. However, the mechano-sensing mechanisms that regulate homeostasis and that are involved in mature tendon repair are not well established, hampering the development of successful cell based therapies toward tendon regeneration.

Signaling cascades are the main routes of communication between the membrane and intracellular regulatory targets. Among them, TGF-β/Smad2/3 was reported as one of the most relevant pathway involved in tenogenic differentiation.[3, 4] The transforming growth factor- β (TGF-β) superfamily comprises of the TGFβs, activins, NODAL, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and anti-Müllerian hormone (AMH).[5] Both TGF-β and mechanical stimulation were found to activate the Smad2 and Smad3 molecules, downstream mediators of TGF-β type I (activin receptor-like kinases, ALKs) and type II receptors, suggesting stress-activated TGF-β signaling drives tenogenesis.[6, 7] ActivinβA has been suggested to activate SMADs pathway[8, 9] and to be involved in the regulatory pathway of tenomodulin,[10] a type II transmembrane glycoprotein suggested as tendon specific marker.[11, 12]

Since tendons are notably sensitive to mechanical forces, the use of magnetic mechano-activation might constitute an effective approach to commit stem cells towards the tenogenic lineage through mechanosensing signaling cascades such as TGF-β/Smad2/3 pathway. The technology of remote activation of mechanotransduction via magnetic nanoparticles (MNPs) has been successfully employed using the MICA Bioreactor (MICA Biosystems Ltd) to

4

deliver the magnetic field to MNPs-tagged cells in both 2D and 3D environments.[13, 14] MNPs-tagged cells renders magnetic responsiveness to the engineered systems with potential to be remotely controlled and tuned by the actuation of an external magnetic field stimulating cells *in vitro* and upon implantation.[15] Additionally, the magnetic field was shown to impact biological processes[16, 17] and to render a positive outcome in tissue healing.[18, 19] Thus, in this study we propose to investigate a magnetically actuated TE approach using an externally applied oscillating magnetic field over stem cells tenogenic phenotype commitment (Figure 1). Human adipose-derived stem cells (hASCs) were labelled with MNPs previously functionalized with anti-Activin receptor type IIA antibody. The magnetic actuation is expected to directly activate the mechanosensitive membrane receptor via functionalized MNPs, stimulating hASCs towards controlled cellular responses, more specifically regulating the transcription of tenogenic associated genes and driving differentiation. In this way, we can compare specific MNPs tagging with non specific effects of an oscilating magnetic field at the cell level.

Previous works using biofunctionalized MNPs targeted to the mechano-responsive ion channel TREK1, Wnt Frizzled or PDGFRα and β receptors, have shown the promotion of an osteogenic, or smooth muscle cell phenotype, respectively, in hMSCs *in vitro* and *in vivo* demonstrating the potential for translation as an enabling approach for cell therapies.[13, 14, 20, 21] This raised the hypothesis that this approach can be extended to other tissues of the musculoskeletal system, such as tendons, where stem cell therapies have already been shown to be effective as veterinary treatments and would benefit from further control of differentiation.[22] In this study, we have identified the cell surface receptors which are likely to be mechano-magnetically targeted and investigated if we can activate cell signalling pathways downstream.

Methods

Magnetic nanoparticles (MNPs) conjugation with antibody

Carboxyl functionalized magnetic nanoparticles (nanomag®-D, 09-02-252, Micromod) were covalently coated with Anti-Activin Receptor type IIA (ActRIIA) antibody (ab135634) herein termed as "MNPs-ActRIIA", or with Anti-Rabbit-IgG Fc antibody (ab97196), "MNPs-IgG", by carbodiimide activation as described previously.[14] Briefly, particles were activated using EDAC (03449, Sigma) and NHS (130672, Sigma) dissolved in 0.5 M MES buffer pH6.3 (Sigma) for 1 h at room temperature under continuous mixing. The particle suspension was washed and re-suspended in 0.1 M MES buffer containing 60 μg of anti-rabbit secondary antibody (ab97196). The particle suspension was continuously mixed overnight at 4 °C and then washed and re-suspended in 0.1 mL MES buffer containing 10 μg of Anti-Activin Receptor type IIA antibody. Particle suspensions were mixed for 3h at room temperature and then blocked with 25mM Glycine (Sigma) for 30 minutes before final washing and re-suspension in distilled water. Functionalized nanoparticles were then analyzed for surface charge and size using a Zetasizer 3000 HSa (Malvern Instruments). Particles were diluted in dH_20 and measurements performed at 25 °C. The size and surface charge of ActRIIA-coated nanoparticles was compared to IgG-coated nanoparticles.

Cell Isolation and Expansion

Human adipose-derived stem cells (hASCs) were obtained from lipoaspirate samples from the abdominal region and isolated from surplus tissue samples, under protocols previously established with Hospital da Prelada (Porto, Portugal) and with informed consent of the patients. The content of the written informed consent and related procedures were reviewed and approved by the Hospital Ethics Committee.

Human ASCs were isolated from tissue samples and cultured as described before,[23, 24] and have been previously characterized by RT-PCR for CD44, STRO-1, CD105 and CD90 markers.[23] Briefly, the tissue was rinsed in phosphate buffered saline (PBS, Sigma-Aldrich) containing 10% of an A/A solution. The fat solution was immersed in a 0.05% collagenase type II (Sigma/C6885) solution for 45 min at 37 °C under mild agitation. The digested tissue was centrifuged at 800 g for 10 min at 4 °C, after which the supernatant was eliminated. Cells were expanded in basal medium composed of αMEM (BE12-169F, Lonza) supplemented with 10% FBS (10270, Gibco) and 1% A/A solution (DE17-603E, Lonza).

Phosphorylation assays

hASCs were seeded at a density of 100,000 cells/well in tissue-culture 6-well plates (Falcon) and incubated with carboxyl functionalized MNPs previously coated with anti-ActRIIA antibody or with anti-Rabbit-IgG antibody, at 25 μg MNPs/2×10^5 cells. After a 30 min labelling period, in basal serum-free medium, with conjugated MNPs, hASCs were incubated/stimulated for 2, 10, or 30 min in basal αMEM medium under magnetic stimulated conditions (magnetic field of ≥25 mT from an array of permanent magnets (NdFeB) situated beneath the culture plates at a frequency of 1 Hz) with a vertical oscillating magnetic bioreactor (MICA Biosystems Ltd) and non-stimulated cells kept in identical conditions in incubator. hASCs cultured without MNPs in αMEM medium supplemented with Recombinant Human/Mouse/Rat Activin A Protein (338-AC-010, R&D systems) or TGF-β3 at 10 and 20 ng/mL[25] were considered positive controls of ActRIIA activation. The dose of ligands was chosen based on literature and according to the phospho-Smad2/Smad3 ELISA kit. The MNPs conjugated only with secondary IgG antibody constitute the negative control of the ActIIRA activation. After each incubation period, cells were analyzed by enzyme-linked immunosorbent assay (ELISA) that recognizes endogenous levels of phospho-Smad2

(Ser465/467) and phospho-Smad3 (Ser423/425) proteins (PathScan® Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) Sandwich ELISA, 12001, Cell Signaling Technology), following the manufacturer's instructions.

hASCs culture

hASCs were seeded at 10,000 cells/well in 24-well plates and cultured in the following conditions for up to 14 days: i) αMEM medium in static conditions ("ST"), ii) αMEM medium in magnetically stimulated conditions ("MICA"), iii) αMEM medium supplemented with Activin A (20 ng/mL) in ST, iv) αMEM medium supplemented with Activin A (20 ng/mL) in MICA, v) αMEM medium in the presence of MNPs-ActRIIA in ST, or vi) αMEM medium in the presence of MNPs-ActRIIA in MICA. Medium was changed every 3 days. Tenogenic commitment of hASCs was evaluated based on the deposition of tendon extracellular matrix (ECM) related proteins and on real time RT-PCR analysis for tendon-related markers as described in detail below.

Magnetically stimulated groups were placed in a commercially available vertical oscillating magnetic bioreactor (MICA Biosystems Ltd), providing a magnetic field of ≥25 mT from an array of permanent magnets (NdFeB) situated beneath the culture plates at a frequency of 1 Hz, provided every other day for 1h sessions. Non-stimulated groups were kept in identical conditions but without magnetic field.

Dextran Immunolabelling

Fixed cells labeled with MNPs-ActRIIA or MNPs-IgG undergoing dextran immunolabelling were permeabilized with 0.025% Triton-X100 (Sigma/X100)/PBS solution, blocked with 2% BSA (A9418, Sigma) in PBS (21-040-CVR, Corning) for 1 h at room temperature and incubated overnight with mouse monoclonal anti-Dextran antibody (60026FI.1, Stem Cell

8

technologies) in 0.1% BSA in PBS at 4 °C. Cells were rinsed in PBS, following incubation

for 1 h at room temperature with anti-mouse IgG FITC antibody (F2012, Sigma). Samples

were stained with 4,6-Diamidino-2-phenyindole dilactate (DAPI, 40009, VWR) for 10 min

and Phalloidin Tetramethylrhodamine B isothiocyanate (P1951, Sigma) for 20 min. The

images were acquired under a Fluorescence Inverted Microscope (Axio Observer, Zeiss).

mRNA Extraction and Quantitative Polymerase Chain Reaction

Total RNA was extracted using TRI reagent (T9424, Sigma) according to the manufacturer's

instructions. Briefly, 800 µL of TRI reagent was added to each sample and stored at -80 $^{\circ}$C.

After defrosting, samples were incubated with 160 µL of chloroform (Sigma) for 15 min and

12,000 g centrifuged for 15 min at 4 $^{\circ}$C. The aqueous fraction was collected and an equal part

of isopropanol (Sigma Aldrich) was added. After 10 min, samples were centrifuged at 12,000

g for 10 min at 4 ºC. RNA pellet was washed with 800 µL of 70% ethanol and subsequently

centrifuged at 7,500 g for 5 min at 4 ºC. Air-dried RNA samples were resuspended in 15 µL

of RNase/DNase free water (Gibco). RNA quantity and purity were determined with a

NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The cDNA synthesis was

performed with the qScript cDNA Synthesis kit (Quanta Biosciences) and using the

Mastercycler Realplex (Eppendorf) using an initial amount of total RNA of 1µg in a total

volume of 20 µL. The quantification of the transcripts was carried out by quantitative

polymerase chain reaction (qPCR) using the PerfeCTA SYBR Green FastMix kit (Quanta

Biosciences) following the manufacturer's protocol, in a Real-Time Mastercycler Realplex

thermocycler (Eppendorf). The primers were pre-designed with PerlPrimer v1.1.21 software

(Table 1) and synthesized by MWG Biotech. GAPDH (glyceraldehyde-3-phosphate

dehydrogenase) was used as the housekeeping gene. The $2^{-\Delta\Delta Ct}$ method was selected to

evaluate the relative expression level for each target gene.[26] All values were first normalized

against GAPDH values, and then to the hASCs cells collected at day 0.

Quantification of Collagen and Non-collagenous Proteins

The amount of collagen and non-collagenous proteins expressed by hASCs cultured in 2D was determined using a semi-quantitative assay, namely Sirius Red/Fast Green Collagen Staining Kit (9046, Chondrex). After cells fixation, the dye solution was added and incubated for 30 min in order to completely immerse the samples. A dye extraction buffer was then mixed and the OD values read in a spectrophotometer (Synergy HT, Biotek Instruments) at 540 nm and 605 nm.

Immunofluorescence

Cells were rinsed in PBS and fixed in formalin (INOPAT) prior to the detection of Tenomodulin (sc-49324) and Fibromodulin (ab81443) deposition. After cell permeabilization with 0.025% Triton-X100 (Sigma/X100)/PBS solution, the blocking step was performed using RTU Normal Horse Serum (RTU Vectastain Kit, PK-7200, Vector). Then, cells were incubated overnight with anti-tenomodulin and anti-fibromodulin antibodies, diluted in antibody diluent with background reducing components from Dako (S3022, Dako) at 4 °C. Samples were then rinsed in 0.025% Triton-X100 (Sigma/X100)/PBS solution followed by incubation for 1h at room temperature with the respective Alexa fluor 488 (Alfagene) considering the host species of the primary antibodies. Samples were stained with 4,6-Diamidino-2-phenyindole, dilactate (DAPI, 5 μg/μL, D9564, Sigma) for 10 min and with Phalloidin–Tetramethylrhodamine B isothiocyanate (P1951, Sigma) for 20 min. Samples were observed with a Fluorescence Inverted Microscope (Axio Observer, Zeiss).

Statistics

Quantitative results are expressed as the mean ± standard deviation. Two-Way ANOVA followed by Bonferroni's Multiple Comparison test was assessed to determine whether

10

differences between sample groups were significant, unless specified. Differences were considered significant when the P value was < 0.05.

Results

Assessment of Smad2/Smad3 phosphorylation in hASCs triggered by TGF-β-like ligands

TGF-β-like ligands, Activin A and TGF-β3 known for their functions in tendon development,[10, 27] were investigated as inducers of serine/threonine receptor's activation and consequent phosphorylation of Smad2/3 (Figure 2 A). Using two different ligand doses (10 and 20 ng/mL) and at varying temporal exposures, Smad2/3 signaling and subsequent gene expression has been assessed. Moreover, Activin A and TGF-β3 ligand-receptor binding will serve as a positive experimental control of the activation of ActRIIA receptor mediated by MNPs-antibody complexes. We investigated the levels of pSmad2/3 following 2, 10, 30 and 60 min of treatment. The detection of pSmad2/3 proteins was rapid (at 10 min) and significantly more intense in Activin A supplemented medium at 20 ng/mL than in medium supplemented with TGF-β3 ligand (Figure 2 B).

Development and characterization of functionalized MNPs complexes

After antibody functionalization by carbodiimide activation (Figure 3 A), the size and surface charge of MNPs-ActRIIA and MNPs-IgG were characterized. The particle size increased after IgG coating of MNPs in comparison to MNPs coated with anti-ActRIIA (MNPs-ActRIIA). Conversely, the surface charge increased in MNPs-ActRIIA (Figure 3 B).

In order to co-localize functionalized MNPs within hASCs, MNPs-ActRIIA or MNPs-IgG labelled cells were visualized by fluorescence microscopy after immuno-labelling the MNPs

dextran shell with an anti-dextran antibody (Figure 4), suggesting that 30 min after labelling, there is a clear association of MNPs-ActRIIA and MNPs-IgG with cells.

Triggering Smad2/Smad3 phosphorylation in hASCs by MNPs complexes

The activin receptor activation was indirectly assessed through detection of downstream phosphorylation of Smad2/3 using an ELISA kit assay that recognizes endogenous levels of these proteins. Magneto-mechanical stimulated groups showed significantly higher phosphorylation levels in relation to non-stimulated and to Activin A control groups after 2 and 10 min, decreasing after 30 min (Figure 5 A) in MNPs-ActRIIA ST and MNPs-ActRIIA MICA conditions. The detection of phospho-Smad2/3 proteins seems to be more intense after 10min in hASCs labeled with MNPs-ActRIIA under magnetic stimulation, which highlights a more efficient ActRIIA activation. Results also show a significant elevation of absorbance values in comparison to the negative control group, MNPs-IgG, or to the positive control group, Activin (Figure 5 B).

Triggering tenogenesis in hASCs using MNPs-ActRIIA complex

In this study, real time RT-PCR analysis, the production assessment of collagen and non-collagenous proteins and the deposition of Tenomodulin (TNMD) and Fibromodulin (FMOD) tendon-related proteins were conducted up to 14 days upon ActRIIA activation under magnetic field stimulation provided by MICA bioreactor.

Gene expression analysis

The expression of tendon associated genes was found to be higher in hASCs bound with MNPs-ActRIIA with and without the magnetic stimulation after 7 days (Figure 6). PCR results evidence higher gene expression values in MNPs-ActRIIA after 7 days under magnetic

stimulation. Targeted mechano-activation of the ActRIIA using magnetic actuation of MNPs attached to the membrane receptor resulted in enhanced upregulation of tenogenic genes, *TNC, TNMD*, *SCXA, DCN,* and *COL3A1* but not *COL1A1*. Interestingly, MNPs-ActRIIA binding alone enhanced *TNC* expression but not other tenogenic genes, as for example *TNC* gene expression is significantly higher in MNPs-ActRIIA ST (P<0.05) in comparison to Activin A ST and to αMEM ST (P<0.01).

Extracellular matrix formation assessment

Of the tendon dry weight, 60% is collagen type I arranged in tensile-resistant fibers[28] and other types such as collagen types III (0-10%), IV (~2%), V, and VI. The non-collagenous matrix is primarily made up of glycoproteins which include the proteoglycans such as decorin (DCN), tenascin C (TNC), fibromudulin (FMOD) and tenomodulin (TNMD).[29] ECM formation was assessed by production of collagen and non-collagenous proteins as depicted in Figure 7. Collagen and non-collagenous proteins production by hASCs significantly increased with time in culture from day 0 to 7 days and from 0 to 14 days, respectively (statistical significant groups: *a* and *b*). Interestingly, the collagen values obtained in MNPs-ActRIIA MICA condition were significantly higher than in static conditions after 14 days. These values were also higher than αMEM or Activin A controls. The same trend was observed in non-collagenous protein production.

Tendon specific markers assessement

FMOD is highly expressed in the tendon and identified as a critical component of the tendon stem cell niche.[30] The immunodetection of TNMD and FMOD is presented in Figures 8A and 8B, respectively. Tendon-specific transcription factors such as *SCX*[3] are essential and

13

involved in mechanoresponsive tenogenesis through regulation of its downstream ECM proteins such as the tendon marker TNMD[31, 32] and proteoglycans such as FMOD. Tenomodulin and Fibromodulin proteins deposition was increased by hASCs previously labeled with MNPs-ActRIIA in comparison with hASCs cultured in basal αMEM or in αMEM supplemented with Activin A (negative and positive controls, respectively). Also, the detection of these proteins after 7 and 14 days of culture was higher when the oscillating magnetic field, provided by MICA bioreactor, was applied.

Discussion

Signal transduction molecules of the Smad family are components of a critical intracellular pathway that transmit signals mediated by transmembrane serine/threonine kinase type II receptors into the nucleus. Smad2/3 signaling pathways are associated with tendon development and transcriptional responses and, to date and to authors knowledge, no published studies have reported to locally target and activate Activin receptors upon mechano-magnetic stimulation, in order to modulate Smad2/3 signaling. Binding of TGF-β family ligands, such as Activins or TGF-β3, to receptors, leads to the recruitment, phosphorylation and activation of type I Activin receptor (ALK4), which provides a binding site for the downstream substrates, initiating the intracellular signaling through activation of Smad proteins.[33] In the present work, the assessment of Smad2/Smad3 phosphorylation in hASCs triggered by TGF-β-like ligands, showed increased absorbance values in Activin A supplemented medium (20 ng/mL) after 10 min, what suggests a more effective phosphorylation of Smad proteins. Therefore, Activin A at this concentration was selected as the positive control for ActRIIA activation in the subsequent experiments of this study. Indeed, the activation of the ligand-receptor complex is described as a relatively fast step in

the TGF-β signal transduction, occurring within 2 min after ligand stimulation, and the signal is relayed to the activation of Smad proteins, which reach at maximal levels in up to 30-60 minutes.[34]

In order to remotely stimulate ActRIIA receptor in a controlled way, MNPs complexes were developed and characterized. The results of size and surface charge of the developed complexes (MNPs-ActRIIA and MNPs-IgG) suggested improved electrostatic interactions of the MNPs coated with anti-ActRIIA with the negatively charged cell membrane, which were further co-localized in hASCs after the 30 min period of labelling by immunocytochemistry.

To understand the capability of functionalized MNPs (MNPs-ActRIIA) to directly target and remotely activate the Activin receptor via magneto-mechanical stimulation, we investigated the phosphorylation of Smad2/3 using MICA bioreactor and comparing to static environment. The outcomes suggested that ActRIIA is a mechanosensitive receptor that can be remotely activated using Ab coated MNPs, whose action is favored by the actuation of an external magnetic field provided by MICA bioreactor. Previous works using biofunctionalized MNPs targeted to mechano-responsive receptors demonstrated an enhanced mechanoactivation of the targeted receptors caused by movement of the MNPs-Ab complex in the magnetic field.[14, 21, 35] Furthermore, the commitment of hASCs towards tenogenesis using the MNPs-ActRIIA complex was studied. Tenoblasts maturation to tenocytes is characterized by the formation of enriched ECM and controlled by key tenogenic transcription factors, such as *SCX*.[36] The TGF-β signaling pathway regulates a diverse group of cellular behaviors such as proliferation, differentiation, and growth arrest[37] and has also been suggested as the most active pathway in tendon cells during mouse limb development.[38] Overall, magnetic actuation of MNPs coated with ActRIIA bound to the receptor enhanced the expression of all tendon related genes studied. Havis *et al.* reported that blocking the TGF-β pathway with SB43 inhibitor, significantly downregulated *SCX* and *COL1a1*, among other gene expression[38] concluding

15

that TGF-β is required via the Smad2/3 intracellular pathway for expression of tendon-related markers in mouse forelimbs.[38] We can infer that MNPs-ActRIIA and magnetic actuation synergistically influenced Smad2/3 mediators. This agrees with other studies using alternate mechanical activation methods where phosphorylation assays, and *SCX* and *TNMD* transcriptional activity was upregulated in response to strain.[12, 31, 39] Furthermore, the fluctuations in gene expression from 7 to 14 days (Figure S1) may be related with ligand depletion from the environment causing cells to lose the stimulation and shut down the phosphorylation of Smad2, which can occur within a few hours post stimulation.[40, 41] Dosage and stimulation time of TGF-β-like ligands have effects on Smad signaling and short-term ligand pulse stimulation results in transient pSmad2, whereas serial pulses or continuous stimulation results in sustained pSmad2.[40] Moreover, there is a time-delay for reaching the maximum pSmad2/3 phosphorylation, which we determined to be 10 min after treatment with MNPs-ActRIIA or with Activin A supplementation at 20 ng/mL, and nuclear accumulation and subsequent regulation of transcriptional responses. Considering these two assertions and the results of gene expression we can infer that as the complex MNPs-ActRIIA was provided to hASCs once at the beggining of culture, the transcriptional responses observed were time-dependent and transient mostly affecting gene expression in early culture periods.

Additionally, the synthesis of a complex matrix of collagen and non-collagenous proteins also supports the activation of activin receptor by MNPs-ActRIIA complex under magnetic actuation in a more efficient way than using the Activin A ligand to trigger hASCs response. Considering the role of TNMD as specific tendon marker and FMOD as a crucial proteoglycan component of tendons that regulate physiological ECM assembly, it was assessed the deposition of these markers by hASCs tagged MNPs-ActRIIA. Tenomodulin has been sugested to be expressed upon Activin II receptor stimulation by myostatin, triggering the Smad2/3 signaling cascade and increasing the expression of *SCX* that ultimately results in

16

the *TNMD* gene expression.[10] Thus, upon MICA stimulation, MNPs-ActRIIA complexes are likely to act over Activin II receptor, activating the signaling cascade and ultimately inducing the transcription of *TNMD* and its translation into protein. Moreover, FMOD was suggested to play a role in the formation of ECM and to regulate TGF-β levels.[42] Also, mutations in the small leucine-rich proteoglycan gene *FMOD* result in irregularities in the diameter of collagen fibers in the tendons.[43] Herein, the higher deposition of TNMD and FMOD together with Sirius Red/Fast Green Collagen staining assay and RT-PCR, confirms the hypothesis of magnetically actuated MNPs-tagged hASCs towards a tenogenic commitment. Moreover, it was clear in the present work that the induction of Smad2/3 signaling elicited hASCs tenogenic response. However, it is known that other variables outside the target cell determine the extent of stimulation by TGF-β cytokines.[44] Thus, future studies will take into account the impact of extracellular regulators in Smad signaling dynamics, such as ligand exposure, or the effect of antagonistic ligands.

Collectively, the present work has shown that the remote activation of MNPs tagging mechanosensitive receptors of hASCs may have potential for controlling stem cell differentiation envisioning successful cell therapies for tendon regeneration. Previously, we have shown the potential benefit of this apporach in orthopaedic bone tissue engineering and similar strategies could be employed for tendon regeneration.[13] It has been demonstrated that magnetic MNPs tagging can be utilised in tendon lineage comitment via ActRIIA/Smad2/3 signaling cascade, as shown by phosphorylation of Smad2/3 proteins in MNPs-ActRIIA tagged hASCs. Further investigation is required to fully explore the mechanism behind signal activation and downstream signaling events in response to MNPs-ActRIIA stimulation and the effects on hASCs fate and differentiation. The development of this technology raises the possibility of remotely controlling TGF-β/Smad2/3 signaling and consequently the control of stem cell behavior. Overall, the remote activation of MNPs tagged hASCs may have potential

17

for controlling stem cell differentiation following cell therapy and modulation of signaling

pathways involved in tendon regeneration.

Figure Legends

Figure 1. Schematic representation of cellular Activin A receptor targeting with MNPs functionalized with Anti-Activin type IIA antibody (MNPs-ActRIIA) for tenogenic differentiation.

Figure 2. Phosphorylation assessment of Smad2/Smad3 in hASCs triggered by TGF-β-like ligands. A) Schematic of the experimental design for the phosphorylation assay. Treatment of hASCs with Activin A and TGF-β3 ligands for up to 60min and subsequent phospho-Smad2/Smad3 (pSmad2/3) detection; B) Assessment of pSmad2/3 by ELISA kit assay, in hASCs cultured in αMEM medium (cells untreated) or cultured in αMEM medium suplemented with Activin A or TGF-β3 at 10 and 20 ng/mL for 10, 30 and 60min. Values represent the mean ± SD. Symbol *, **, *** and **** denotes study groups with statistical significant difference $P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$ respectively.

Figure 3. Development and characterization of functionalized MNPs complexes. A) Schematic representation of MNPs functionalization with anti-activin receptor type IIA antibody by carbodiimide activation; B) Size and zeta potential analysis of MNPs functionalized with anti-activin receptor type IIA antibody (MNPs-ActRIIA) or with IgG secondary antibody (MNPs-IgG); C) Dextran (green) immunofluorescence images of hASCs labelled with MNPs-ActRIIA or MNPs-IgG. Cell nuclei are shown by DAPI (blue) and the cytoskeleton by phalloidin (red). Insets represent lower magnification images.

Figure 4. Dextran (green) immunofluorescence images of a) hASCs labelled with MNPs-ActRIIA or MNPs-IgG. Cell nuclei are shown by DAPI (blue) and the cytoskeleton by phalloidin (red); b) lower magnification images.

19

Figure 5. Phosphorylation assessment of Smad2/Smad3 in hASCs. A) Phospho-Smad2/Smad3 ELISA assay of hASCs cultured in Activin A (positive control) and with MNPs-ActRIIA under static (ST) or under actuation of a vertically oscillating magnetic field (MICA) for 2, 10, and 30min; B) Phospho-Smad2/Smad3 ELISA assay of hASCs cultured with MNPs-ActRIIA or MNPs-IgG under static (ST) or under actuation of a vertically oscillating magnetic field (MICA) for up to 10min; Values represent the mean ± SD. Symbol *, **, *** and **** denotes study groups with statistical significant difference P<0.05, P<0.01, P<0.001 and P<0.0001 respectively.

Figure 6. Real time RT-PCR analysis of *tenascin C* (*TNC*), *tenomodulin (TNMD), scleraxis* (*SCXA*), *decorin* (*DCN*), *collagen type I (COL1A1)* and *collagen type III* (*COL3A1*) gene expression of hASCs cultured for 7 days in αMEM medium, αMEM supplemented with Activin A (20 ng/mL) or labeled with MNPs-ActRIIA in αMEM medium, under static (ST) or under actuation of a vertically oscillating magnetic field (MICA). Values represent the mean ± SD. Symbol * and ** denotes study groups with statistical significant difference P<0.05 and P<0.01, P<0.001, by one-way ANOVA.

Figure 7. Quantification of collagen and non-collagenous proteins by Sirius Red/Fast Green Collagen staining kit in hASCs labeled with MNPs-ActRIIA in αMEM medium, under static (ST) or under actuation of a vertically oscillating magnetic field (MICA). hASCs cultured for up to 14 days in αMEM medium and in αMEM supplemented with Activin A (20 ng/mL) were considered the negative and positive experimental controls, respectively. Values represent the mean ± SD. Symbol *, **, *** and **** denotes study groups with statistical significant difference P<0.05, P<0.01, P<0.001 and P<0.0001 respectively; a and b letters represent statistically different groups.

Figure 8. Immunocytochemistry of A) Tenomodulin and of B) Fibromodulin proteins (green) deposited by hASCs labeled with MNPs-ActRIIA in αMEM medium, under static (ST) or under actuation of a vertically oscillating magnetic field (MICA). hASCs cultured for up to 14 days in αMEM medium and in αMEM supplemented with Activin A (20 ng/mL) were considered the negative and positive experimental controls, respectively. DAPI (blue) stains cell nuclei. Insets represent hASCs labeled with MNPs-ActRIIA in αMEM medium at day 0.

References

1. Wall ME, Dyment NA, Bodle J, Volmer J, Loboa E, Cederlund A, et al. Cell Signaling in Tenocytes: Response to Load and Ligands in Health and Disease. *Advances in experimental medicine and biology*. 2016;**920**:79-95.

2. Wall M, Butler D, Haj AE, Bodle JC, Loboa EG, Banes AJ. Key developments that impacted the field of mechanobiology and mechanotransduction. *Journal of Orthopaedic Research*. 2017.

3. Gaut L, Duprez D. Tendon development and diseases. *Wiley interdisciplinary reviews Developmental biology*. 2016;**5**(1):5-23.

4. Havis E, Bonnin MA, Esteves de Lima J, Charvet B, Milet C, Duprez D. TGFbeta and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. *Development*. 2016;**143**(20):3839-51.

5. Wakefield LM, Hill CS. Beyond TGFβ: roles of other TGFβ superfamily members in cancer. *Nature Reviews Cancer*. 2013;**13**(5):328-41.

6. Rothrauff BB, Yang G, Tuan RS. Tendon resident cells-functions and features in section I-developmental biology and physiology of tendons. Tendon regeneration: Elsevier Inc.; 2015. p. 41-76.

7. Jones ER, Jones GC, Legerlotz K, Riley GP. Cyclical strain modulates metalloprotease and matrix gene expression in human tenocytes via activation of TGFβ. *Biochimica et Biophysica Acta*. 2013;**1833**:2596-607.

8. Pauklin S, Vallier L. Activin/Nodal signalling in stem cells. *Development*. 2015;**142**(4):607-19.

9. Yu L, Hebert MC, Zhang YE. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. *The EMBO journal*. 2002;**21**(14):3749-59.

10. Qi J, Dmochowski JM, Banes AN, Tsuzaki M, Bynum D, Patterson M, et al. Differential expression and cellular localization of novel isoforms of the tendon biomarker tenomodulin. *J Appl Physiol (1985)*. 2012;**113**(6):861-71.

11. Brandau O, Meindl A, Fassler R, Aszodi A. A novel gene, tendin, is strongly expressed in tendons and ligaments and shows high homology with chondromodulin-I. *Developmental dynamics : an official publication of the American Association of Anatomists*. 2001;**221**(1):72-80.

12. Docheva D, Hunziker EB, Fässler R, Brandau O. Tenomodulin is necessary for tenocyte proliferation and tendon maturation. *Molecular and cellular biology*. 2005;**25**(2):699-705.

13. Henstock JR, Rotherham M, Rashidi H, Shakesheff KM, El Haj AJ. Remotely Activated Mechanotransduction via Magnetic Nanoparticles Promotes Mineralization Synergistically With Bone Morphogenetic Protein 2: Applications for Injectable Cell Therapy. *Stem Cells Transl Med*. 2014;**3**(11):1363-74.

14. Rotherham M, El Haj AJ. Remote activation of the Wnt/beta-catenin signalling pathway using functionalised magnetic particles. *PloS one*. 2015;**10**(3):e0121761.

15. Gomes ME, Rodrigues MT, Domingues RMA, Reis RL. Tissue Engineering and Regenerative Medicine: New Trends and Directions-A Year in Review. *Tissue engineering Part B, Reviews*. 2017;**23**(3):211-24.

16. Maziarz A, Kocan B, Bester M, Budzik S, Cholewa M, Ochiya T, et al. How electromagnetic fields can influence adult stem cells: positive and negative impacts. *Stem cell research & therapy*. 2016;**7**(1):54.

17. de Girolamo L, Vigano M, Galliera E, Stanco D, Setti S, Marazzi MG, et al. In vitro functional response of human tendon cells to different dosages of low-frequency pulsed electromagnetic field. *Knee surgery, sports traumatology, arthroscopy : official journal of the ESSKA*. 2015;**23**(11):3443-53.

18. Xu D, Zhang T, Qu J, Hu J, Lu H. Enhanced Patella–Patellar Tendon Healing Using Combined Magnetic Fields in a Rabbit Model. *The American Journal of Sports Medicine*. 2014;**42**(10):2495-501.

19. Strauch B, Patel MK, Rosen DJ, Mahadevia S, Brindzei N, Pilla AA. Pulsed magnetic field therapy increases tensile strength in a rat Achilles' tendon repair model. *J Hand Surg Am*. 2006;**31**(7):1131-5.

20. Kanczler JM, Sura HS, Magnay J, Green D, Oreffo RO, Dobson JP, et al. Controlled differentiation of human bone marrow stromal cells using magnetic nanoparticle technology. *Tissue engineering Part A*. 2010;**16**(10):3241-50.

21. Hu B, Dobson J, Haj AJE. Control of smooth muscle α-actin (SMA) up-regulation in HBMSCs using remote magnetic particle mechano-activation. *Nanomedicine: Nanotechnology, Biology, and Medicine*. 2014;**10**:45-55.

22. Russell JW, Russell TM, Vasey JR, Hall MS. Autologous bone marrow aspirate for treatment of superficial digital flexor tendonitis in 105 racehorses. *The Veterinary record*. 2016;**179**(3):69.

23. Rada T, Reis RL, Gomes ME. Novel method for the isolation of adipose stem cells (ASCs). *Journal of tissue engineering and regenerative medicine*. 2009;**3**(2):158-9.

24. Carvalho PP, Wu X, Yu G, Dias IR, Gomes ME, Reis RL, et al. The effect of storage time on adipose-derived stem cell recovery from human lipoaspirates. *Cells, tissues, organs*. 2011;**194**(6):494-500.

25. Attisano L, Wrana JL, Montalvo E, Massague J. Activation of signalling by the activin receptor complex. *Molecular and cellular biology*. 1996;**16**(3):1066-73.

26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-$\Delta\Delta$CT method. *Methods*. 2001;**25**(4):402-8.

27. Pryce BA, Watson SS, Murchison ND, Staverosky JA, Dunker N, Schweitzer R. Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. *Development*. 2009;**136**(8):1351-61.

28. Kjaer M. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol Rev*. 2004;**84**(2):649-98.

29. Thorpe CT, Birch HL, Clegg PD, Screen HR. The role of the non-collagenous matrix in tendon function. *Int J Exp Pathol*. 2013;**94**(4):248-59.

30. Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med*. 2007;**13**(10):1219-27.

31. Shukunami C, Takimoto A, Oro M, Hiraki Y. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. *Dev Biol*. 2006;**298**(1):234-47.

32. Murchison ND, Price BA, Conner DA, Keene DR, Olson EN, Tabin CJ, et al. Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. *Development*. 2007;**134**(14):2697-708.

33. Abe Y, Minegishi T, Leung PC. Activin receptor signaling. *Growth factors*. 2004;**22**(2):105-10.

34. Zi Z, Chapnick DA, Liu X. Dynamics of TGF-beta/Smad signaling. *FEBS letters*. 2012;**586**(14):1921-8.

35. Rotherham M, Henstock JR, Qutachi O, El Haj AJ. Remote regulation of magnetic particle targeted Wnt signaling for bone tissue engineering. *Nanomedicine: Nanotechnology, Biology and Medicine*. 2017.

36. Spanoudes K, Gaspar D, Pandit A, Zeugolis DI. The biophysical, biochemical, and biological toolbox for tenogenic phenotype maintenance in vitro. *Trends in biotechnology*. 2014;**32**(9):474-82.

37. Massagué J. TGF-β signal transduction. *Annual review of biochemistry*. 1998;**67**(1):753-91.

38. Havis E, Bonnin M-A, Olivera-Martinez I, Nazaret N, Ruggiu M, Weibel J, et al. Transcriptomic analysis of mouse limb tendon cells during development. *Development*. 2014;**141**(19):3683-96.

39. Maeda T, Sakabe T, Sunaga A, Sakai K, Rivera AL, Keene DR, et al. Conversion of mechanical force into TGF-β mediated biochemical signals. *Current Biology*. 2011;**21**(11):933-41.

40. Zi Z, Feng Z, Chapnick DA, Dahl M, Deng D, Klipp E, et al. Quantitative analysis of transient and sustained transforming growth factor-beta signaling dynamics. *Molecular systems biology*. 2011;**7**:492.

41. Feng Z, Zi Z, Liu X. Measuring TGF-beta Ligand Dynamics in Culture Medium. *Methods in molecular biology*. 2016;**1344**:379-89.

42. Walden G, Liao X, Donell S, Raxworthy MJ, Riley GP, Saeed A. A Clinical, Biological, and Biomaterials Perspective into Tendon Injuries and Regeneration. *Tissue engineering Part B, Reviews*. 2017;**23**(1):44-58.

43. Svensson L, Aszodi A, Reinholt FP, Fassler R, Heinegard D, Oldberg A. Fibromodulin-null mice have abnormal collagen fibrils, tissue organization, and altered lumican deposition in tendon. *J Biol Chem*. 1999;**274**(14):9636-47.

44. Massagué J. TGFβ signalling in context. *Nature reviews Molecular cell biology*. 2012;**13**(10):616-30.

Table 1. Primers used for quantitative RT-PCR analysis.

Gene	Sequence	NCBI reference
Human Glyceraldehyde -3-phosphate dehydrogenase (*GAPDH*)	F- GGGAGCCAAAAGGGTCATCA R- GCATGGACTGTGGTCATGAGT	NM_001256799.1
Collagen, Type I, alpha 1 (*COL1A1*)	F- CCCCAGCCACAAAGAGTCTAC R- TTGGTGGGATGTCTTCGTCT	NM_000088.3
Collagen, Type III, alpha 1 (*COL3A1*)	F- CCTGAAGCTGATGGGGTCAA R- CAGTGTGTTTCGTGCAACCAT	NM_000090.3
Tenascin C (*TNC*)	F- ACTGCCAAGTTCACAACAGACC R- CCCACAATGACTTCCTTGACTG	NM_002160.3
Scleraxis (*SCXA*)	F- AGAACACCCAGCCCAAACAGAT R- TCGCGGTCCTTGCTCAACTTT	NM_001080514.2
Decorin (*DCN*)	F- CTAGTCACAGAGCAGCACCTAC R- CCAGGGAACCTTTTAATCCGGGAA	NM_001920.4
Tenomodulin (*TNMD*)	F- CCGCGTCTGTGAACCTTTAC R- CACCCACCAGTTACAAGGCA	NM_022144.2

Graphical Abstract: Text

Schematic illustration of the process of hASCs-MNPs-ActRIIA labeling and stimulation with magnetic field to target the Activin A receptor, initiating the intracellular signaling through activation of TGF-β/Smad2/3 towards tenogenesis. The activation of the ligand-receptor complex via magneto-mechanical stimulation was confirmed by the assessment of Smad2/3 phosphorylation in hASCs. Moreover, the remote activation of MNPs tagged hASCs resulted in improved collagenous enriched matrix and tenogenic commitment of hASCs, demonstrating potential for the modulation of signaling pathways involved in tendon regeneration.
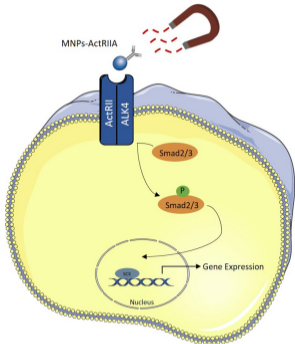
Graphics Abstract

MNPs-ActRIIA

Magnetic stimulation

ActRII ALK4

Smad2/3

P

Smad2/3

SCX

Nucleus

Gene Expression

MNPs

Anti-Activin Receptor Type IIA antibody

Tenogenic Differentiation
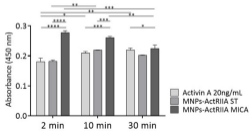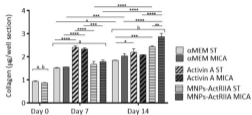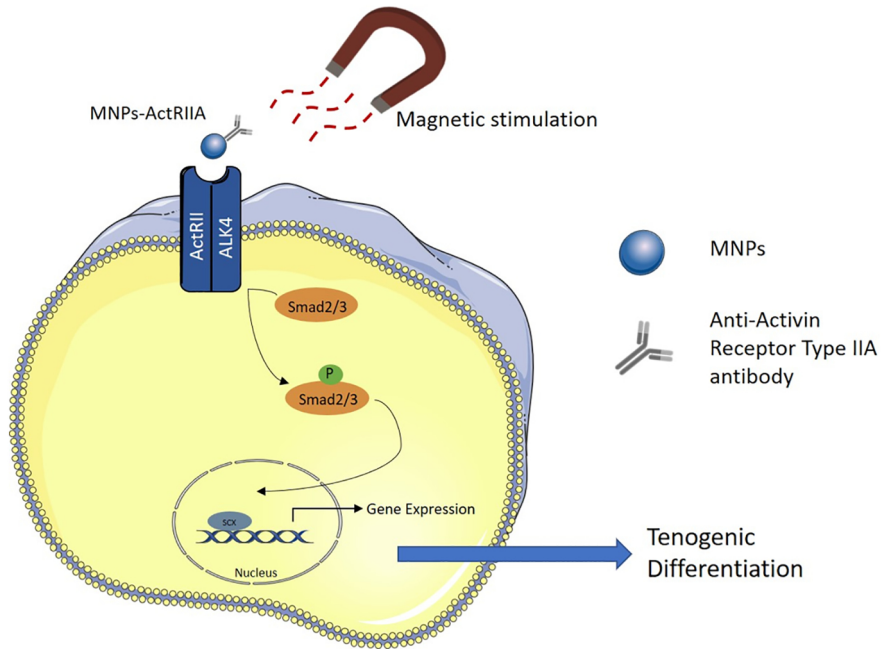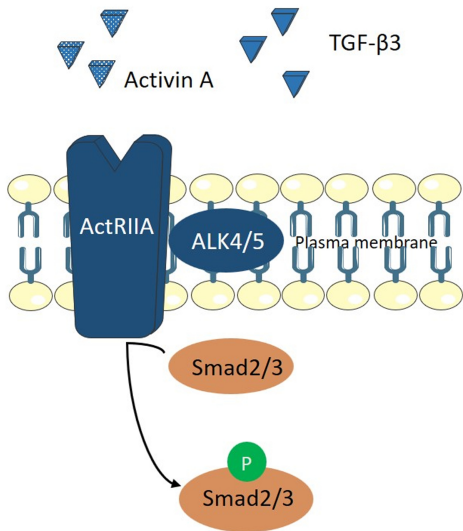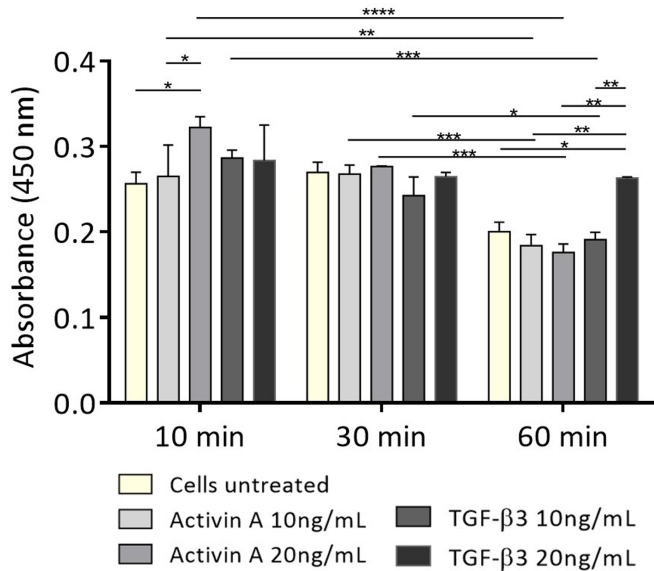
Figure 1

Figure 2

A
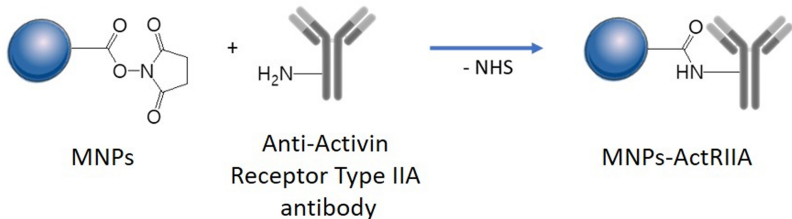
B

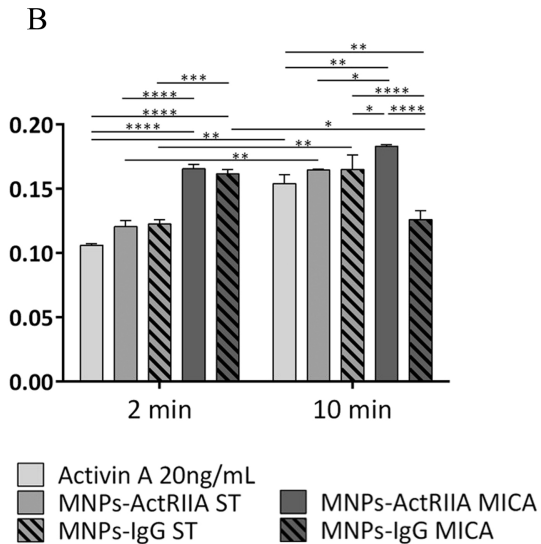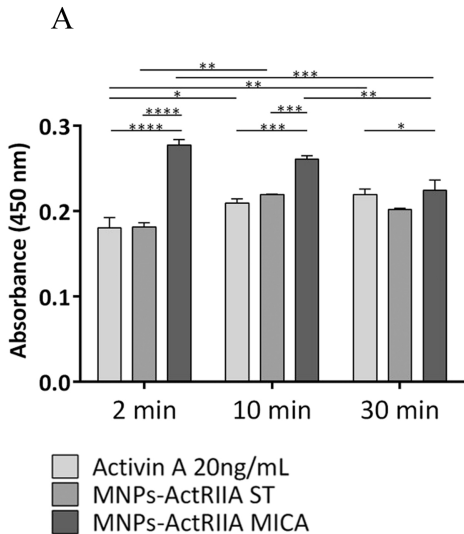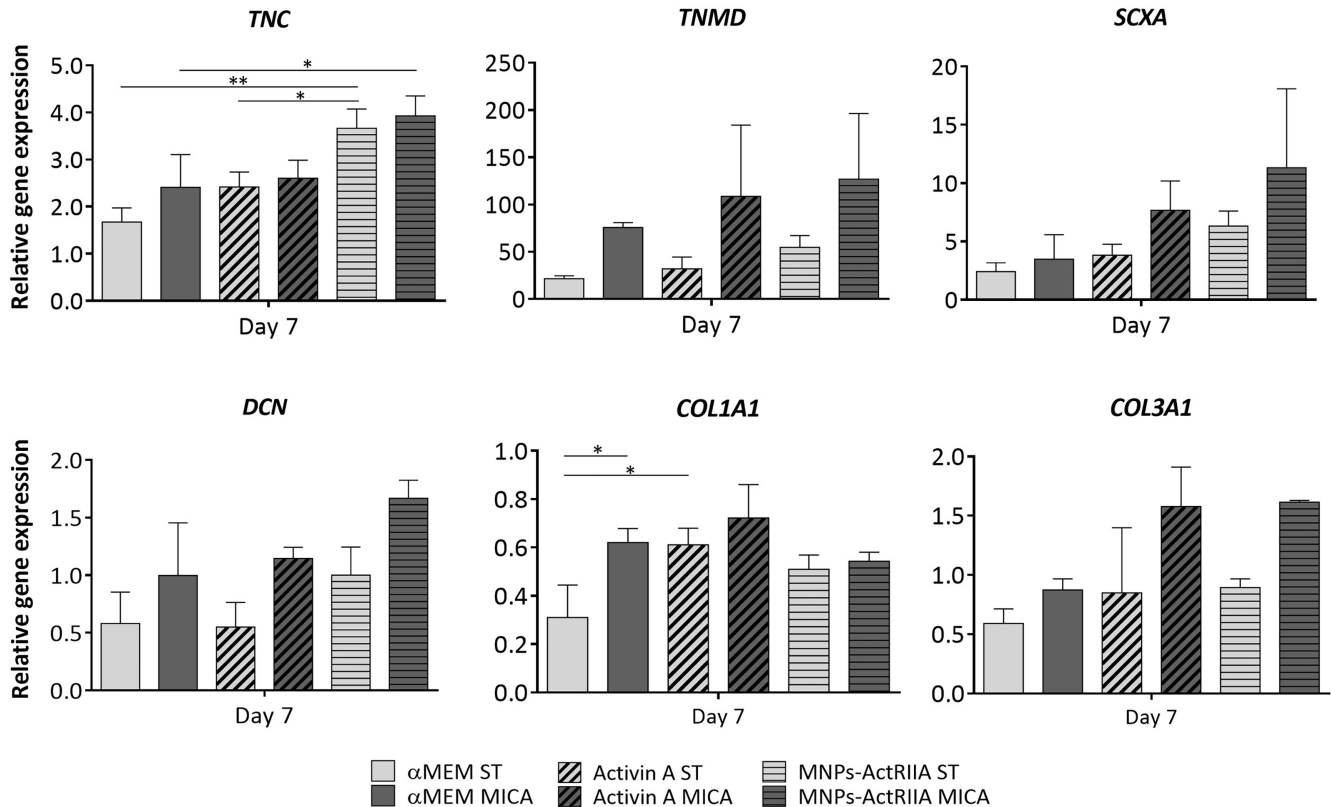| Particles	Size (nm)	Zeta potential (mV)
MNPs-ActRIIA	349.43 ± 2.08	-7.1 ± 0.6
MNPs-IgG	369.70 ± 2.15	-16.1 ± 0.4

Figure 3
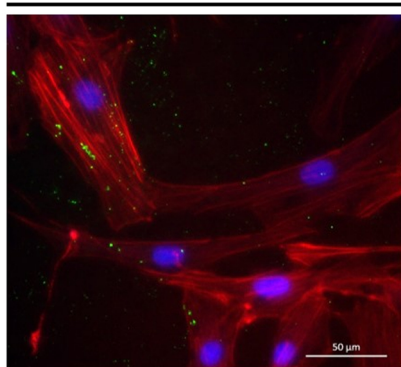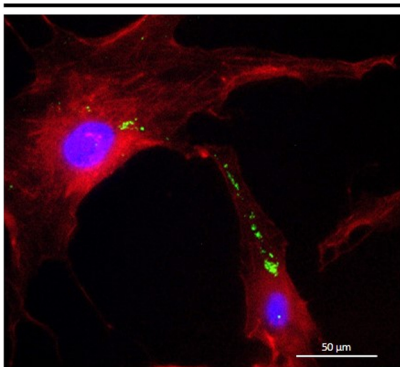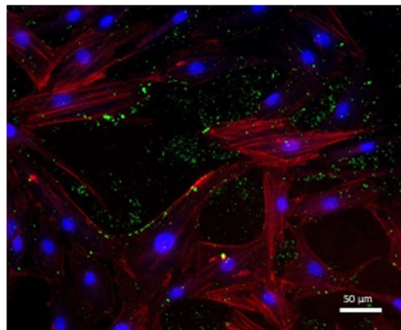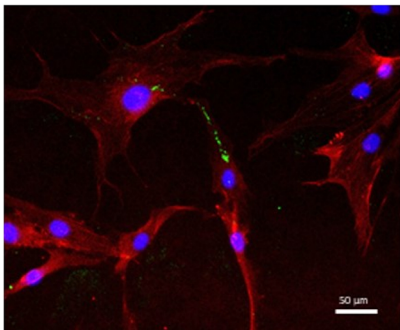
Figure 4

Figure 5

Figure 6

Figure 7

Figure 8