JCAMECH

Vol. 49, No. 2, December 2018, pp 386-394
DOI: 10.22059/jcamech.2018.265180.324

Magneto-mechanical Stimulation of Bone Marrow Mesenchymal Stromal Cells for Chondrogenic Differentiation Studies

Hanie Kavand [a,b], Mahdi Rahaie [a,], Nooshin Haghighipour [b], Shahin Bonakdar [b], Javad Koohsorkhi [c]*

[a]*Department of Life Science Engineering, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran.*

[b]*National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran.*

[c] *Advanced Micro and Nano Devices Lab, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran*

ARTICLE INFO

Article history:
Received: 8 September 2018
Accepted: 20 September 2018

Keywords:
Mechanotransduction
Magnetic nanoparticles
Magnetic field
Mechanical actuation
Chondrogenesis

ABSTRACT

Mechanical interaction of cells and their surroundings are prominent in mechanically active tissues such as cartilage. Chondrocytes regulate their growth, matrix synthesis, metabolism, and differentiation in response to mechanical loadings. Cells sense and respond to applied physical forces through mechanosensors such as integrin receptors. Herein, we examine the role of mechanical stimulation of integrins in regards to their mechanotransduction ability to promote chondrogenesis. For this purpose, magnetic nanoparticles were chemically bonded to cell membrane mechanoreceptors and stimulated. Histological results showed the endocytosis of nanoparticles over the experimental period, pointing out the inefficient mechanical stimulation of the mechanoreceptors. Moreover, gene expression analysis only showed significant upregulation for SOX9, whereas type II collagen and aggrecan gene expression were not significantly different from the control group. Our results suggest that magneto-mechanical stimulation studies using magnetic nanoparticles should not only focus on the mechanical aspects, but also the interaction of magnetic nanoparticles with intracellular machinery should be investigated as well.

1. Introduction

Articular cartilage is a specialized connective tissue and is subjected to a harsh biomechanical environment.[1] Injuries and degenerative joint diseases cause discomfort followed by disabilities in patients.[2] Articular cartilage has a very poor self-renewing capacity and in case of injuries, inferior fibrocartilage tissues with reduced mechanical properties are formed.[3] Several surgical and biological attempts such as therapeutic interventions with and without active biologics, surgical interventions, and tissue engineering have been considered to treat cartilage lesions.[4] Cartilage tissue engineering has been offering alternative treatment possibilities to restore or improve damaged tissues. Chondrocytes are the only residing cell type in articular cartilage and play a unique role in the development, maintenance, and repair of the extracellular matrix (ECM).[1] However, due to their complex nature and their unstable phenotype in 2D culturing systems, their expansion is often intricate.[5] To overcome the limitation existing in cell source, culture-expanded mesenchymal stromal cells (MSCs) have been extensively investigated as an attractive cell source for cartilage regeneration. MSCs are pluripotent progenitor cells that have self-renewal capacity and their progeny are affected by both intrinsic and extrinsic factors to eventually give rise to different mesodermal cell types including cartilage.[6] Although MSCs are present in different tissues such as bone marrow, synovium, adipose tissue, and umbilical cord blood, comparative studies indorsed higher chondrogenic differentiation potentials to MSCs derived from synovium and bone marrow.[7]

MSCs rely on external factors such as chemical, topographical, and biophysical cues to differentiate into different mesodermal cell types including chondrocytes. Chondrogenesis includes an MSCs condensation stage which is directed by cell-cell and cell-matrix interactions as well as interactions between secreted factors and their receptors.[8] The effect of mechanical loading on chondrogenesis has been extensively reviewed.[9]

From a mechanical point of view, cartilage is a highly anisotropic, viscoelastic and poroelastic tissue, with a site and depth-dependent mechanical properties.[10] The biomechanical properties of cartilage originate from the composition and structural organization of its ECM, notably the type II collagen networks provide the tissue its strength towards tensile forces and its compressive resistance is due to the presence of hydrated proteoglycans.[11] Compression of cartilage triggers complex changes inside the tissue that includes deformations of the cells and the ECM, hydrostatic and osmotic pressure, fluid flow, and also alterations in the water content, ion concentration, and fixed charge density of the ECM.[12] The anisotropic nature of cartilage stemming from the diverse composition and structure of ECM molecules might lead to the assumption that the chondrocytes residing in different zones receive different mechanical loadings and thus they are different from one another.

* Corresponding Author: E-mail. mrahaie@ut.ac.ir

However, the unique structure and biochemical properties of chondrocyte's pericellular matrix has a strain-regulating role as it either amplifies or attenuates local mechanical strains and transduces mechanical deformations to physicochemical or biochemical changes in the chondrocyte microenvironment.[13] In fact, under compressive forces, chondrocytes show significantly fewer deformations when surrounded by pericellular matrix than isolated chondrocytes.[14]

Mechanical inputs are transduced into biochemical signal via cell surface integrin receptors and a number of non-integrin receptors such as G-protein coupled receptors, enzyme-linked receptors, ion channels, lipid rafts, and the glycocalyx.[15] Integrins are heterodimeric transmembrane proteins that are involved in binding to matrix ligands from their extracellular domains (mechanical linker between the cell and their environment), initiating kinase-mediated signaling, helping cytoskeletal protein organization from their cytoplasmic domains, and activating intracellular signaling to regulate cellular functions such as proliferation, morphology, and motility.[16] The integrin specificity for different ECM molecules is determined by the combination of α and β subunits of integrins.[17] The role of integrins in mechanotransduction is becoming increasingly evident as new technologies and approaches emerge.

Over the last two decades, there had been a rapid rise in the application of nanotechnology in the biomedical fields. Iron-containing nanoparticles or magnetic nanoparticle (MNPs) show an acceptable level of biocompatibility, facile synthesis, versatile surface modification possibilities, and unique magnetic properties that consist of aligning along the external magnetic field and a high magnitude magnetic saturation when exposed to magnetic fields.[18], [19] MNPs have been of precise interest in biomedical applications such as drug delivery, magnetic resonance imaging (MRI), magnetic hyperthermia, cell tracking and manipulation, and tissue engineering.[20]–[24] Magnetic hyperthermia and mechanical manipulation of cells rely on the magnetic to thermal and mechanical transduction potential of MNPs, respectively. In cell manipulation studies, magnetic fields stimulate the MNPs attached to the cell membrane and induce direct mechanical stimulations to the cells. The nanometric dimension of ligand-conjugated particles makes receptor targeting and activation feasible.[21] A list of magneto-mechanical stimulation strategies towards cell differentiation can be seen in Table 1.

As mechanical forces are regarded as critical inducers for chondrogenic differentiation and these forces are vital for the normal function and regulation of chondrocytes, it was hypothesized that magnetic fields can enhance chondrogenic differentiation through mechanical stimulation of integrin mechanoreceptors present on the cell membrane. In the present study, RGD-conjugated MNPs were used to label cells and permanent magnets were used for mechanical stimulation. In this case, the magnetic field is transduced to mechanical forces that act on cells. Due to the complexity of the differentiation process, a standard pellet culture system (providing a 3D culturing system) was applied that minimized the potential effects of scaffolds while providing a sufficient microenvironment for chondrogenic studies.

Table 1. Magneto-mechano stimulation of cells for cell differentiation purposes.

Cell type	Application	Treatment	Results	Ref.
MSCs	Osteogenesis	Functionalized magnetic nanoparticles were attached to mechanically gated TREK1 K^+ or (integrin) RGD binding domains, oscillating 25 mT magnetic field	Extensive mineralization compared to unlabeled cells. Targeting TREK1 resulted in 2.4 fold increase in mineralization and significant increase in matrix density.	[25]
MSCs, 2D culture and alginate chitosan capsules	Osteogenesis Chondrogenesis	250 nm silica magnetic particles were bound to anti-TREK-1 antibody or RGD. 1 hourly, alternate day cyclic loading intervals at 1 Hz and 1-100 pN/particle for 7 days	Remote nanomagnetic actuation of labeled cells with TREK-1 and RGD magnetic particles in 3D microcapsules can stimulate cell migration, proliferation, and osteogenic and chondrogenic differentiation.	[23]
MSCs sheets	Chondrogenesis	4.4 μm magnetic particles, magnetic field induced mechanical stimulation: static (4.39 pN) and cyclic (1.06-63.6 pN, 16.7 mHz)	No effect on cartilage formation.	[26]
Osteoblast, monolayer culture	Osteogenesis	RGD coated magnetic particles (4-4.5 μm), 1 Hz/60 mT permanent magnet, 30 min/day, 21 days	Mineralized bone matrix production, upregulation of osteopontin	[27]
Osteoblast, monolayer culture	Osteogenesis	RGD coated magnetic particles (4.5 μm), static magnetic field of ~56 mT	Mechanical manipulation of integrin attached and integrin-internalized particles induce calcium signaling.	[28]
MSCs, pellet culture	Chondrogenesis	Endocytosed magnetic nanoparticles (40-50 nm), static magnetic field and magnetic-derived shear stress, B = 0.25 mT, 1 h/day for 5 days and further maintenance for 3 weeks	Magnetic stimuli enhanced sGAG and collagen synthesis and facilitated chondrogenic differentiation.	[29]

2. Materials and methods

2.1. Materials

Phosphate-buffered saline (PBS), MEM Alpha, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco, Life Technologies (USA). Collagenase D was purchased from Roche (Germany). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimid (NHS), RGD peptide, and Prussian blue were purchased from Sigma (USA). Citric acid, paraformaldehyde, Alcian blue 8 GX, hydrochloric acid, and potassium ferrocyanide (potassium hexacynoferrate(II) trihydrate were obtained from Merck (Germany). All reagents were of chemical grade and were used as received.

2.2 BM-MSCs isolation and culture

The marrow samples were harvested from the femur of adult rabbits obtained from a slaughterhouse. As the animal was obtained from a slaughterhouse, no ethical approval was required to conduct the experiment. The tips of the bones were removed and the marrow was collected. Tissue was washed with PBS and to help cell dissociation; medium containing collagenase D (0.5 mg/ml) was added and the tube was placed in an incubator under continuous agitation at 37°C for 30 min. To neutralize the enzyme's activity, complete media (MEM Alpha + 10% FBS + 1% penicillin/streptomycin) was added to the tube. Cells were then washed, centrifuged for 5 min at 1,400 rpm, filtered through a 70 μm mesh cell strainer (Falcon), and finally seeded in T75-flasks with complete media. Cells were incubated at 37°C in humid air with 5% CO_2 and complete medium was added to the flask every 3 days. After approximately a week in culture, cells were washed with PBS to remove the non-adhering cells and thereafter, complete media was added to the flasks and changed every three days until confluent. To create study groups, cells at passage II were used. To evaluate the chondrogenic potential of the isolated cells, cell pellets were treated with a commercially available chondrogenesis differentiation kit (StemPro, Invitrogen) for two weeks.

2.3. Magnetic nanoparticle labeling

RGD tripeptide was conjugated onto PEG carboxyl functionalized MNPs (30 nm in diameter, kindly provided by Ms. Maryam Farahnak Zarabi, Pilot Biotechnology Department, Pasteur Institute of Iran). To do so, nanoparticles were initially sterilized using ethanol treatment.[30] Briefly, 1 ml MNPs (5 mg/ml) was mixed with 75% ethanol and vortexed for 15 s. After 30 min, the precipitate was centrifuged at 4,000 rpm for 10 min and air-dried. MNPs dispersion was achieved by sonication in NaCl-HCl buffer (pH = 5.6) for 10 min. EDC and NHS were then added and the mixture was kept under vigorous shaking for 1 h. After surface activation using EDC/NHS coupling chemistry, nanoparticles were centrifuged (4,000 rpm, 10 min), the washing buffer was replaced with PBS, and particles were sonicated for 5 min. One milligram of MNPs was then conjugated to 10 μg RGD tripeptide for 4 h. For labeling cells with RGD-coated MNPs, cells were detached and kept in a serum-free medium for 4 h followed by incubation with 125 μg particles per 10^6 cells under intermittent agitation. Cells were then washed, centrifuged, and pellets (4×10^5 cells) were prepared in 15 ml conical centrifuge tubes as previously outlined by Johnstone and colleagues [31].

2.4. Nanoparticle characterization

Nanoparticles' surface characterization was carried out by Fourier Transform infrared spectroscopy (FTIR-Shimadzu-8400S). Freeze-dried samples were milled with KBr and pressed into pellets for analysis. Scanning electron microscope (SEM; HITACHI S-4160) was used to measure MNP's size.

2.5. Force generation

Hollow cylindrical permanent magnets were used to apply physical forces to the MNPs and subsequently the cells. The magnets were kept under the cell pellet containing tubes for the whole experimental period (two weeks). To ensure a constant spacing between the cell pellets and the magnets in all experimental groups, cell containing Falcon tubes were placed in a custom-made tube holder. The magnetic flux density at this distance was measured as 19 mT using a gaussmeter (FW Bell 6010). Cells were kept inside the incubator (37°C and 5% CO_2) all through the experimental period.

2.6. Histological analysis

For histological analysis, samples were fixed in 4% paraformaldehyde for 20 min, dehydrated, embedded in paraffin, and sectioned (4 μm). Alcian Blue staining was used to observe proteoglycan synthesis in the samples. Samples were dewaxed, rehydrated, and sections were stained with Alcian Blue (pH = 2.5) for 25 min. Following washing, sections were counterstained with 0.1% nuclear fast red. For iron visualization, Prussian Blue staining was used. Samples were dewaxed, rehydrated, incubated with a 1:1 vol/vol of 20% hydrochloric acid and 10% potassium ferrocyanide solution for 20 min, washed, and counterstained with 0.1% nuclear fast red. Samples were observed using an optical microscope (Olympus Slide Scanner, 20x/0.75).

2.7. Quantitative PCR analysis

To study chondrogenic specific markers' gene expression, total RNA was extracted (RNeasy Plus mini kit, Qiagen), quantified (Qubit, Invitrogen), and cDNA was synthesized using a cDNA synthesis kit (SuperScript VILO cDNA Synthesis kit) according to manufacturer's instructions. The primer sequences for *GAPDH*, *COL1A1*, *COL2A1*, and *SOX9* genes were obtained from a previous study[32]. Table 2 shows the primer sequences. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the QuantStudio 6 Flex machine (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The relative expression level of each gene was calculated according to the ΔC_T method ($2^{-\Delta\Delta CT}$).[33] *GAPDH* was used as an internal control to normalize the data. A Student's t-test was performed (GraphPad Prism 7 software) to ascertain statistical significance between the expression levels. $P<0.05$ was considered statistically significant.

Table 2. List of primer sequences applied for gene expression analysis by qPCR.

Gene	Forward (5'→3')	Reverse (5'→3')
Collagen type II	CAGGCAGAGGCAGGAAACTAAC	CAGAGGTGTTTGACACGGAGTAG
Aggrecan	ATGGCTTCCACCAGTGCG	CGGATGCCGTAGGTTCTCA
SOX9	GTACCCGCACCTGCACAAC	TCCGCCTCCTCCACGAAG
GAPDH	CGTCTGCCCTATCAACTTTCG	CGTTTCTCAGGCTCCCTCT

3. Results

3.1. FTIR analysis

RGD conjugated MNPs were prepared by PEG carboxyl functionalized MNPs. PEG carboxyl functionalized groups were used to provide free carboxylic acid groups on the MNP's surface. EDC/NHS activates the available carboxylic groups and facilitates RGD conjugation. FTIR spectroscopy range (4000-400cm^{-1}) shows FTIR spectra of MNPs (Fe_3O_4), PEG carboxylic MNPs (PEG-COOH-Fe_3O_4), and RGD functionalized MNPs (PEG-COOH-Fe_3O_4 +RGD) (Fig 1a). The characteristic peaks of Fe_3O_4 appear between 600 cm^{-1} and 400 cm^{-1}, which are correlated to the stretching and torsional vibration modes of magnetite. The large intensity at 3421 cm^{-1} can be assigned to the structural OH groups available on the Fe_3O_4 surface. The 1730 cm^{-1} peak of PEG-COOH-Fe_3O_4 is assigned to the stretching vibration of C=O from the COOH group that shifts to the intense band at about 1635 cm^{-1} in PEG carboxylic acid functionalized MNPs. The peaks at 2921 and 2852 cm^{-1} are attributed to the C-H stretch in PEG.[34] The FTIR spectra obtained after RGD conjugation shows an increased peak at 3423 cm^{-1} due the additional OH groups involved in RGD and new intense absorption peaks between 1660 cm^{-1} to 1089 cm^{-1}. The 1660 cm^{-1} peak corresponds to the amide C=O functional groups with the characteristic absorption peaks of 1610 to1690 cm^{-1}. The 1557 cm^{-1} peak arises from the N-H bending vibration strongly coupled to the C-N stretching vibration of amide II groups and the 1400 cm^{-1} to 1200 cm^{-1} peak could be attributed to glycine and arginine amino acid residues.[35] FTIR results confirm the conjugation of RGD peptides to the nanoparticles. Fig 1b shows the SEM micrograph of the RGD functionalized PEG carboxylate MNPs.

3.2. Histology

After 14 days of treatments, cell pellets were histologically examined (Fig 2). The result of Alcian Blue staining from the positive control sample (chondrogenic media treated) indicates chondrogenic differentiation capacity of the isolated cells as the section was positively dyed (Fig 2a). Due to the iron accumulation in the pellets, Alcian Blue staining did not reveal much information from the labeled and magneto-mechanically stimulated group. Prussian Blue staining showed the presence and accumulation of MNPs in the cytoplasm (Fig 2b). Magnetically labeled integrin receptors were internalized, which indicates the inefficient mechanical stimulation of the mechanosensors over the experimental period. Internalization of particles is in agreement with previous studies [28][36].

3.3. Gene expression analysis

To determine the effect of mechanical stimulation of labeled cells by an external magnetic field, the expression levels of *COL2A1* (type II collagen), *SOX*, and *ACAN* (Aggrecan) were analyzed by quantitative PCR (Fig 3). Unlabeled cell pellets cultured in basic media were considered as the negative control. SOX9 is one of the transcription factors that regulate the onset of chondrogenesis in undifferentiated MSCs and mediates the expression of cartilage-specific genes such as type II collagen and aggrecan.[9] The results showed a significant (2.2 fold) increase in the expression of the *SOX9* transcript. The expression level of type II collagen, which represents 90-95% of the collagen in the ECM, and aggrecan were also assessed.[1] The expression levels *COL2A1* and ACAN were not significantly different in the mechanically stimulated group compared to the control group.

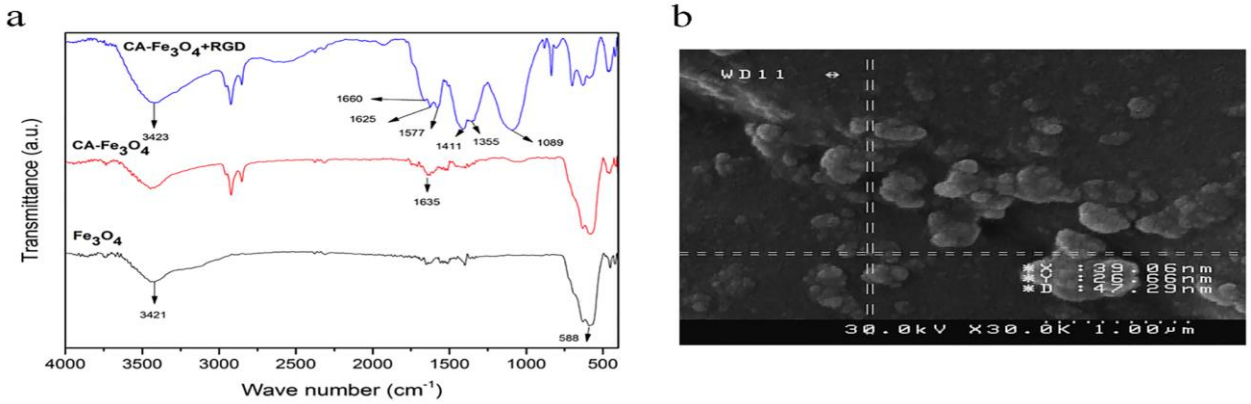

Fig. 1. Characterization of MNPs. (a)FTIR spectra of (i) MNPS (Fe_3O_4), (ii) PEG carboxylate MNPs, and (iii) RGD functionalized PEG carboxylate MNPs. (b) SEM micrograph of RGD functionalized PEG carboxylate MNPs MNPs.

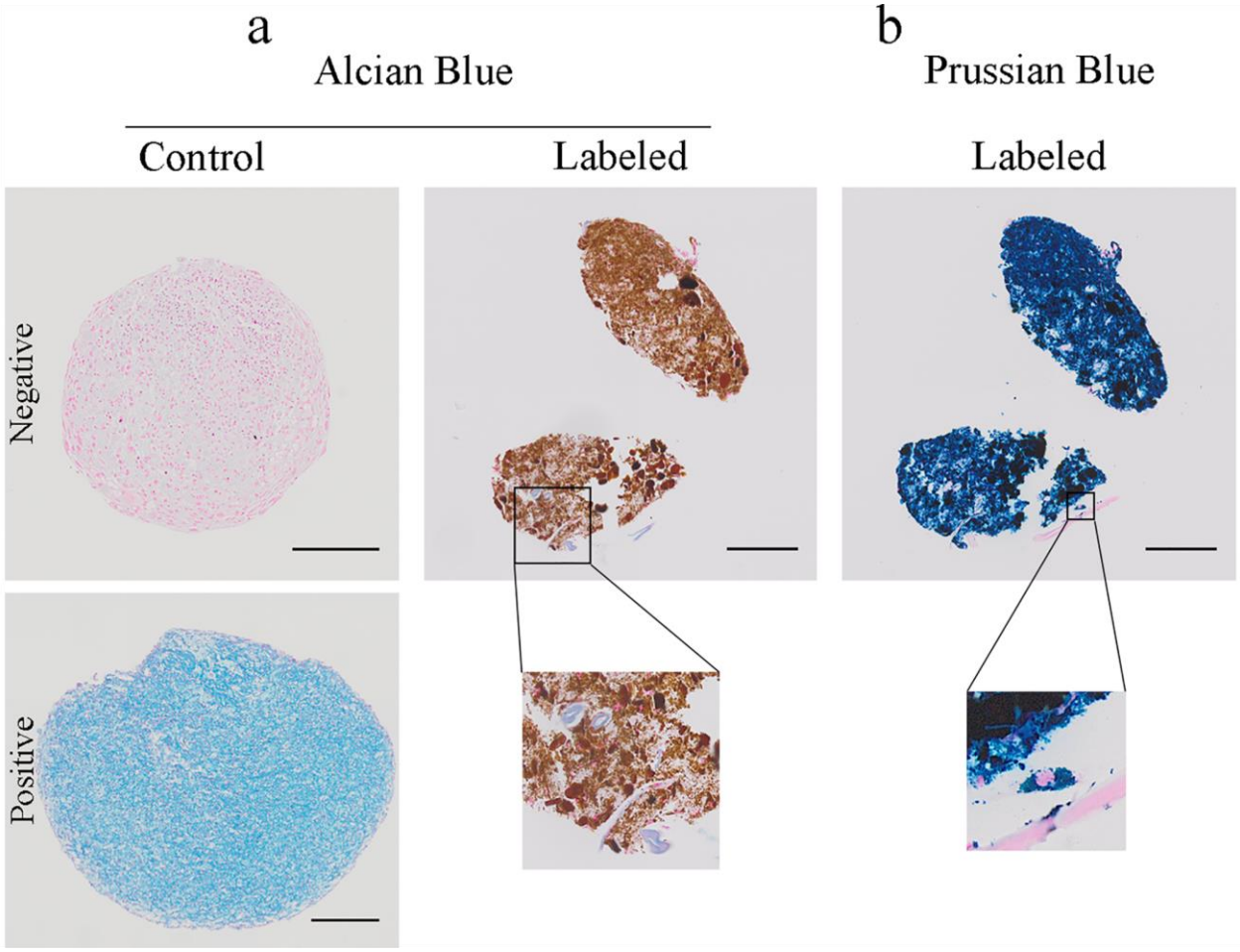

Fig. 2. Histological analyses of cell pellets for proteoglycan synthesis and iron content. (a) Alcian Blue staining was used to check proteoglycan synthesis in the control group (negative and positive control) and the magnetic nanoparticle-labeled group. In labeled cells with magnetic nanoparticles, the magnetic particles are seen brown (the typical color). (b) Prussian Blue staining reveals iron content in the cells as iron oxide stains blue. As can be seen in the magnified image, particles are seen in the cytoplasm. Cells were counterstained with Nuclear fast red that results in dark pink for cell nuclei and light pink for cytoplasm. (Scale bar = 200 μm)

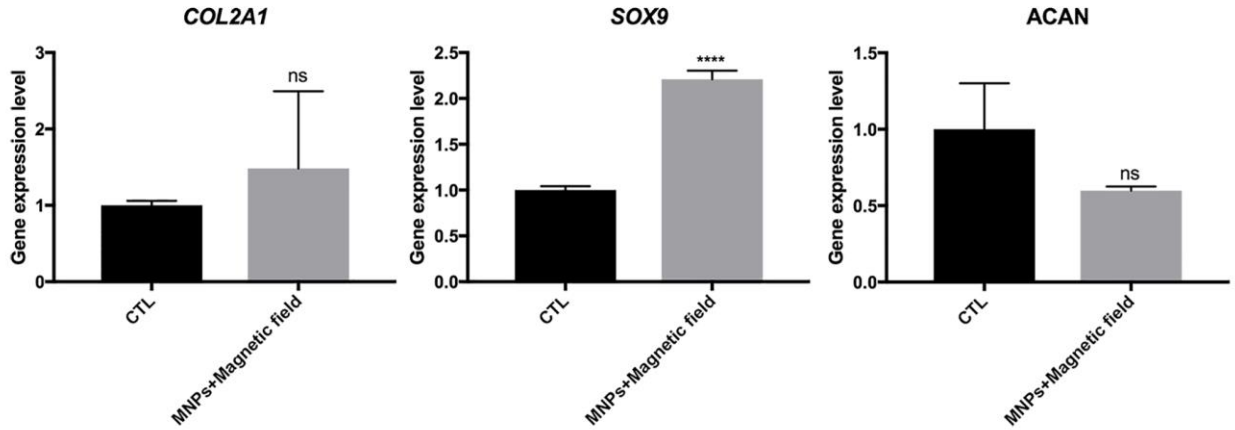

Fig. 3. Chondrogenic gene expression analysis. Gene expression was normalized to the control group (unlabeled cells cultured in basic media) and *GAPDH* was used as the housekeeping gene. ****p<0.0001; ns indicates not significant, Student's t-test.

4. Discussion

Cartilage responds to physiological strains (dynamic compression) during normal activities and post activities by the production of ECM molecules including glycosaminoglycans (GAGs) and collagen.[13] Mechanical stimulations in cartilage are transduced by mechanosensors such as the nucleus (it undergoes conformational changes in response to force), cytoplasmic proteins (such as YAP and TAZ), ion channels, and the cell membrane integrin receptors.[37], [38] Upon binding of ECM proteins to the integrins, the signaling cascades that include tyrosine and serine kinases and adaptor proteins are initiated, the activated intermediates converge on the mitogen-activated protein (MAP) kinase family and result in downstream regulation of gene transcription.[16] Integrins may also be activated through another mechanism during loadings. By considering the rupture forces per integrin-ECM bond (below 10 or even 1 pN), the applied forces disrupt most of the integrin-ECM bonds and integrins undergo conformational changes (activation) for binding to the ECM. This activation can change their affinity for cytoplasmic partners such as α-actinin and catalyze downstream cascades, which consists of tyrosine phosphorylation of different substrates (tyrosine kinases like Src and focal adhesion kinase).[17]

Mechanical stimulations are believed to promote chondrogenesis, but direct mechanical stimulation of cells only became possible as nanotechnology progressed. Mechanotransduction studies on integrins (possibly α5β1) via collagen-magnetic beads showed that well-defined forces induce an immediate (< 1 s) calcium influx and actin assembly induced by mechanical perturbation is dependent on calcium and tyrosine phosphorylation and actin, in turn, regulates calcium signaling in cells.[39], [40] When magnetic tweezers and a twisting device were used to transfer force directly from the integrins to the local cytoskeleton, focal adhesion proteins were deformed and cytoskeletal rearrangements and mechanotransduction at multiple distant sites within the cell were observed.[37] Compared to the mechanical stimulation of cells cultured in 2D or 3D scaffolds, magnetic nanoparticles allow a controllable and direct stimulation of cells. The force excreted on a nanoparticle can range from 10^{-12} to 10^{-9} N, which lies in the range of *in vivo* forces experienced by cells.[41]

In this study, we evaluated magneto-mechanical actuation of integrin receptors as a potential stimulation for cartilage tissue engineering. FTIR analysis confirmed the surface modification of MNPs with RGD (Fig 1). Histological results showed that cells internalized the MNPs during the treatment period (Fig 2). The internalization mechanism of MNPs might be due to the same mechanism suggested by Sethi and colleagues.[42] They explained that the binding of integrins to the RGD-peptide elicits integrin-microfilament assembly, which in turn drags the non-mechanically attached RGD-peptides into the cell. Moreover, endocytosis might be in charge of the turnover and the distribution of integrins. The variation in the endocytic process controls the internalization of specific integrin subtypes and influences the dynamics and localization of integrins and may contribute to the specificity of mechanotransduction.[43] As a result, the internalization of MNPs makes mechanical stimulation of the cell membrane mechanoreceptors questionable. However, it is important to note that MNPs not only affect cell differentiation under an external magnetic field but nanoparticle aggregation and cellular uptake alone can efficiently effect stem cell differentiation. Fayol and colleagues investigated the impact of dose-dependent uptake of nanoparticles on osteogenesis, adipogenesis, and chondrogenesis of MSCs.[44] Their results showed that adipogenesis and osteogenesis were not affected by MNPs labeling as adipogenic and osteogenic markers were positively expressed. By contrast, chondrogenic differentiation showed a dose-dependent behavior and the study groups with an intracellular iron dose beyond 9 pg showed negative for standard chondrogenic staining. Lima and colleagues suggest that incorporation of magnetic nanoparticles and magnetic fields show osteogenic and chondrogenic potentials, however, their results indicate a greater impact on osteogenesis.[36] The difference between the osteogenic and chondrogenic potentials of MNPs might be related to the study conducted by Fayol and colleagues as discussed above.

We identified a significant upregulation of *SOX9* and insignificant expression levels for *COL2A1* and *ACAN* transcript compared to the control group (cells treated in basic media) (Fig 3). Similar gene expression pattern has been reported from MNP labeled MSCs that were induced for chondrogenic differentiation. Fayol and colleagues discuss that high doses of internalized MNPs have an inhibitory effect of chondrogenesis.[44] Expression of aggrecan and type II collagen were also not detected in Chang and colleagues' experiment on amine-surface-modified SPIO labeled MSCs.[45] In contrast, when chondrocytes were utilized instead of MSCs, the MNP labeled chondrocytes were successful in maintaining the phenotypic stability and incorporating into cartilage tissue engineering systems.[46] The results suggest that, although weak, magneto-mechanical stimulations might only partially support chondrogenesis in magnetically labeled pellet cell culture. In other words, although mechanical stimulation of cell surface receptors is in favor of chondrogenesis, the internalization effect should also be considered.

On the mechanical aspect, experiments have mainly focused on stimulations with dynamic loadings due to the dynamic nature of physiological movements in cartilage. In a study conducted by Lucchinetti and colleagues, an increase in the integrin content showed that chondrocytes response to cyclic compressive stress and that integrin α5β1 acts as a mechanical transducer between the ECM and the cells.[47] Furthermore, compressive loadings in the presence of RGD peptides with affinity to the fibronectin binding site of integrin α5β1 blocked the catabolic effect of dynamic loading in intervertebral discs and suggest that mechanical stresses are recognized through RGD integrins.[48] A study on the chondrogenic induction of cyclic compressive loadings showed an increased expression of type II collagen, aggrecan, and TGF-β1 genes.[49] Their results further revealed that compressive loading could promote chondrogenesis by inducing the synthesis of TGF-β1. Furthermore, the hypothesis that static compressive forces are transduced to molecular signaling during early chondrogenesis has also been

tested. The results from the static compressive force (1 kPa) on embryonic limb buds caused upregulation of the positive regulator (SOX9) and a downregulation in the negative regulator (IL-1β) of chondrogenesis followed by a significant increase in type II collagen and aggrecan expression.[50]

In the context of static magnetic fields, it should be noted that the magnetic interaction between a cell and an external magnetic field is very weak as the susceptibilities of all human tissues, at both the organ and cellular levels, are close to that of water. Water is the predominant compound of most tissues and the tissue concentration of paramagnetic molecules (O_2 or iron ($Fe2^+$ or Fe^{3+})) are too small to overcome the dominant diamagnetism.[51] Amin and colleagues studied the effect of static magnetic fields (0.1, 0.2, 0.4 or 0.6 T) on the chondrogenesis of BM-MSCS pellets.[52] Their results showed no significant changes in sGAG levels, however, the 0.4 T static magnetic field elicited a strong terminal chondrogenic response in the presence of chondrogenic media.

Results from our study indicate that although mechanical stimulation of cells does take place during magnetic stimulation the prolonged effect of cell membrane stimulation should be neglected as the RGD-conjugated nanoparticles are taken upon by cells. While there have been prior studies where they report positive effects of mechanical actuation of MNPs on chondrogenic differentiation of MSCs, our results are different from the previously published findings. In fact, the role of mechanical stimulation on chondrogenic differentiation is undeniable and not new as there are numerous publications on it. However, the role of MNPs in cellular function is still undetermined. The MNPs change size under the application of an external magnetic field and the internalization process greatly influences the theory of mechanical stimulation of cell surface receptors by an external magnetic field. As discussed, the concentration of the internalized nanoparticles has a key role in promoting cell differentiation. In the field of receptor magneto-mechanical actuation, the stress is laid on receptor targeting. However, one should also consider the internalization process for long-term cellular analyses. Upon the current observations, it is crucial to extend the understanding of the interactions between nanoparticles and intracellular machinery. Although it seems like that the surface state of MNPs influences the inhibition of chondrogenic differentiation but Andreas and colleagues suggest that the chondrogenic inhibition is a result of cytoskeleton disorganization induced by nanoparticles.[53]

5. Conclusion

MSCs are used as an alternative cell source for cartilage regenerative therapies. Biomechanical factors regulate cartilage homeostasis as cartilage is exposed to different physical factors. Nanomagnetic actuation technique, which uses MNPs as an active part during stimulations, is regarded as a useful platform for remotely controlled mechanotransduction studies. This work evaluated the effect of mechanical stimulation of integrin receptors on chondrogenesis of MSCs in cell pellets. We show that magneto-mechanical stimulation of MNPs (internalized) did not enhance chondrogenesis. Although cellular uptake is a prerequisite process in several applications, the internalization of MNPs attached to cell surface receptors for long-term mechanical stimulation purposes makes cell studies complex. Additional studies may be necessary to elucidate the relation between internalized MNPs and differentiation process.

References

[1] A. J. Sophia Fox, A. Bedi, and S. A. Rodeo, "The basic science of articular cartilage: structure, composition, and function," *Sports health*, vol. 1, no. 6, pp. 461–468, 2009.

[2] D. Chen, J. Shen, W. Zhao, T. Wang, L. Han, J. L. Hamilton, and H.-J. Im, "Osteoarthritis: toward a comprehensive understanding of pathological mechanism," *Bone research*, vol. 5, p. 16044, 2017.

[3] P. D. Tatman, W. Gerull, S. Sweeney-Easter, J. I. Davis, A. O. Gee, and D.-H. Kim, "Multiscale biofabrication of articular cartilage: bioinspired and biomimetic approaches," *Tissue Engineering Part B: Reviews*, vol. 21, no. 6, pp. 543–559, 2015.

[4] E. B. Hunziker, "Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects," *Osteoarthritis and cartilage*, vol. 10, no. 6, pp. 432–463, 2002.

[5] H. K. Heywood, G. Nalesso, D. A. Lee, and F. Dell'Accio, "Culture expansion in low-glucose conditions preserves chondrocyte differentiation and enhances their subsequent capacity to form cartilage tissue in three-dimensional culture," *BioResearch open access*, vol. 3, no. 1, pp. 9–18, 2014.

[6] A. I. Caplan, "Mesenchymal stem cells," *Journal of orthopaedic research*, vol. 9, no. 5, pp. 641–650, 1991.

[7] H. Koga, L. Engebretsen, J. E. Brinchmann, T. Muneta, and I. Sekiya, "Mesenchymal stem cell-based therapy for cartilage repair: a review," *Knee Surgery, Sports Traumatology, Arthroscopy*, vol. 17, no. 11, pp. 1289–1297, 2009.

[8] A. O. Oseni, C. Crowley, M. Z. Boland, P. E. Butler, and A. M. Seifalian, "Cartilage tissue engineering: the application of nanomaterials and stem cell technology," in *Tissue engineering for tissue and organ regeneration*, InTech, 2011.

[9] N. Fahy, M. Alini, and M. J. Stoddart, "Mechanical stimulation of mesenchymal stem cells: Implications for cartilage tissue engineering," *Journal of Orthopaedic Research®*, vol. 36, no. 1, pp. 52–63, 2018.

[10] L. A. McMahon, F. J. O'Brien, and P. J. Prendergast, "Biomechanics and mechanobiology in osteochondral tissues," 2008.

[11] C. Vinatier, D. Mrugala, C. Jorgensen, J. Guicheux, and D. Noël, "Cartilage engineering: a crucial combination of cells, biomaterials and biofactors," *Trends in biotechnology*, vol. 27, no. 5, pp. 307–314, 2009.

[12] G. Musumeci, "The effect of mechanical loading on articular cartilage." Multidisciplinary Digital Publishing Institute, 2016.

[13] J. Sanchez-Adams, H. A. Leddy, A. L. McNulty, C. J. O'Conor, and F. Guilak, "The mechanobiology of articular cartilage: bearing the burden of osteoarthritis," *Current rheumatology reports*, vol. 16, no. 10, pp. 451, 2014.

[14] S. J. Millward-Sadler and D. M. Salter, "Integrin-dependent signal cascades in chondrocyte mechanotransduction," *Annals of biomedical engineering*, vol. 32, no. 3, pp. 435–446, 2004.

[15] A. N. Gasparski and K. A. Beningo, "Mechanoreception at the cell membrane: more than the integrins," *Archives of biochemistry and biophysics*, vol. 586, pp. 20–26, 2015.

[16] R. F. Loeser, "Integrins and chondrocyte–matrix interactions in articular cartilage," *Matrix Biology*, vol. 39, pp. 11–16, 2014.

[17] P. Roca-Cusachs, T. Iskratsch, and M. P. Sheetz, "Finding the weakest link–exploring integrin-mediated mechanical molecular pathways," *J Cell Sci*, vol. 125, no. 13, pp. 3025–3038, 2012.

[18] J. Cores, T. G. Caranasos, and K. Cheng, "Magnetically Targeted Stem Cell Delivery for Regenerative Medicine," *Journal of functional biomaterials*, vol. 6, no. 3, pp. 526–546, 2015.

[19] Y. Gao, J. Lim, S.-H. Teoh, and C. Xu, "Emerging translational research on magnetic nanoparticles for regenerative medicine," *Chemical Society reviews*, vol. 44, no. 17, pp. 6306–6329, 2015.

[20] H.-C. Kim, E. Kim, S. W. Jeong, T.-L. Ha, S.-I. Park, S. G. Lee, S. J. Lee, and S. W. Lee, "Magnetic nanoparticle-conjugated polymeric micelles for combined hyperthermia and chemotherapy," *Nanoscale*, vol. 7, no. 39, pp. 16470–16480, 2015.

[21] M. H. Cho, E. J. Lee, M. Son, J.-H. Lee, D. Yoo, J. Kim, S. W. Park, J.-S. Shin, and J. Cheon, "A magnetic switch for the control of cell death signalling in in vitro and in vivo systems," *Nature materials*, vol. 11, no. 12, p. 1038, 2012.

[22] J. W. M. Bulte, T. Douglas, B. Witwer, S.-C. Zhang, E. Strable, B. K. Lewis, H. Zywicke, B. Miller, P. van Gelderen, and B. M. Moskowitz, "Magnetodendrimers allow endosomal magnetic labeling and in vivo tracking of stem cells," *Nature biotechnology*, vol. 19, no. 12, p. 1141, 2001.

[23] J. M. Kanczler, H. S. Sura, J. Magnay, D. Green, R. O. C. Oreffo, J. P. Dobson, and A. J. El Haj, "Controlled differentiation of human bone marrow stromal cells using magnetic nanoparticle technology," *Tissue Engineering Part A*, vol. 16, no. 10, pp. 3241–3250, 2010.

[24] C. Monzel, C. Vicario, J. Piehler, M. Coppey, and M. Dahan, "Magnetic control of cellular processes using biofunctional nanoparticles," *Chemical science*, vol. 8, no. 11, pp. 7330–7338, 2017.

[25] J. R. Henstock, M. Rotherham, H. Rashidi, K. M. Shakesheff, and A. J. El Haj, "Remotely Activated Mechanotransduction via Magnetic Nanoparticles Promotes Mineralization Synergistically With Bone Morphogenetic Protein 2: Applications for Injectable Cell Therapy," *Stem cells translational medicine*, p. sctm-2014, 2014.

[26] A. D. Dikina, B. P. Lai, M. Cao, M. Zborowski, and E. Alsberg, "Magnetic field application or mechanical stimulation via magnetic microparticles does not enhance chondrogenesis in mesenchymal stem cell sheets," *Biomaterials science*, vol. 5, no. 7, pp. 1241–1245, 2017.

[27] S. H. Cartmell, J. Dobson, S. B. Verschueren, and A. J. El Haj, "Development of magnetic particle techniques for long-term culture of bone cells with intermittent mechanical activation," *IEEE Transactions on NanoBioscience*, vol. 99, no. 2, pp. 92–97, 2002.

[28] S. Hughes, J. Dobson, and A. J. El Haj, "Magnetic targeting of mechanosensors in bone cells for tissue engineering applications," *Journal of biomechanics*, vol. 40, pp. S96–S104, 2007.

[29] B. Son, H. D. Kim, M. Kim, J. A. Kim, J. Lee, H. Shin, N. S. Hwang, and T. H. Park, "Physical Stimuli-Induced Chondrogenic Differentiation of Mesenchymal Stem Cells Using Magnetic Nanoparticles," *Advanced healthcare materials*, vol. 4, no. 9, pp. 1339–1347, 2015.

[30] L. Li, K. Y. Mak, J. Shi, C. H. Leung, C. M. Wong, C. W. Leung, C. S. K. Mak, K. Y. Chan, N. M. M. Chan, and E. X. Wu, "Sterilization on dextran-coated iron oxide nanoparticles: Effects of autoclaving, filtration, UV irradiation, and ethanol treatment," *Microelectronic engineering*, vol. 111, pp. 310–313, 2013.

[31] B. Johnstone, T. M. Hering, A. I. Caplan, V. M. Goldberg, and J. U. Yoo, "< i> In Vitro</i> Chondrogenesis of Bone Marrow-Derived Mesenchymal Progenitor Cells," *Experimental cell research*, vol. 238, no. 1, pp. 265–272, 1998.

[32] W. Wang, B. Li, J. Yang, L. Xin, Y. Li, H. Yin, Y. Qi, Y. Jiang, H. Ouyang, and C. Gao, "The restoration of full-thickness cartilage defects with BMSCs and TGF-beta 1 loaded PLGA/fibrin gel constructs," *Biomaterials*, vol. 31, no. 34, pp. 8964–8973, 2010.

[33] T. D. Schmittgen and K. J. Livak, "Analyzing real-time PCR data by the comparative C T method," *Nature protocols*, vol. 3, no. 6, p. 1101, 2008.

[34] G. Yuan, Y. Yuan, K. Xu, and Q. Luo, "Biocompatible PEGylated Fe3O4 nanoparticles as photothermal agents for near-infrared light modulated cancer therapy," *International journal of molecular sciences*, vol. 15, no. 10, pp. 18776–18788, 2014.

[35] M. Jackson, P. H. Watson, W. C. Halliday, and H. H. Mantsch, "Beware of connective tissue proteins: assignment and implications of collagen absorptions in infrared spectra of human tissues," *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, vol. 1270, no. 1, pp. 1–6, 1995.

[36] J. Lima, A. I. Gonçalves, M. T. Rodrigues, R. L. Reis, and M. E. Gomes, "The effect of magnetic stimulation on the osteogenic and chondrogenic differentiation of human stem cells derived from the adipose tissue (hASCs)," *Journal of Magnetism and Magnetic Materials*, vol. 393, pp. 526–536, 2015.

[37] J. L. Alonso and W. H. Goldmann, "Cellular mechanotransduction," *transport*, vol. 1, p. 7, 2016.

[38] B. C. Low, C. Q. Pan, G. V Shivashankar, A. Bershadsky, M. Sudol, and M. Sheetz, "YAP/TAZ as mechanosensors and mechanotransducers in regulating organ size and tumor growth," *FEBS letters*, vol. 588, no. 16, pp. 2663–2670, 2014.

[39] M. Glogauer, P. Arora, G. Yao, I. Sokholov, J. Ferrier, and C. A. McCulloch, "Calcium ions and tyrosine phosphorylation interact coordinately with actin to regulate cytoprotective responses to stretching," *Journal of Cell Science*, vol. 110, no. 1, pp. 11–21, 1997.

[40] M. Glogauer, J. Ferrier, and C. A. McCulloch, "Magnetic fields applied to collagen-coated ferric oxide beads induce stretch-activated Ca2+ flux in fibroblasts," *American Journal of Physiology-Cell Physiology*, vol. 269, no. 5, pp. C1093–C1104, 1995.

[41] N. J. Sniadecki, "Minireview: a tiny touch: activation of cell signaling pathways with magnetic nanoparticles," *Endocrinology*, vol. 151, no. 2, pp. 451–457, 2010.

[42] K. K. Sethi, V. Mudera, R. Sutterlin, W. Baschong, and R. A. Brown, "Contraction-mediated pinocytosis of RGD-peptide by dermal fibroblasts: Inhibition of matrix attachment blocks contraction and disrupts microfilament organisation," *Cytoskeleton*, vol. 52, no. 4, pp. 231–241, 2002.

[43] S. Seetharaman and S. Etienne-Manneville, "Integrin diversity brings specificity in mechanotransduction," *Biology of the Cell*, vol. 110, no. 3, pp. 49–64, 2018.

[44] D. Fayol, N. Luciani, L. Lartigue, F. Gazeau, and C. Wilhelm, "Managing magnetic nanoparticle aggregation and cellular uptake: a precondition for efficient stem-cell differentiation and MRI tracking," *Advanced healthcare materials*, vol. 2, no. 2, pp. 313–325, 2013.

[45] Y. Chang, Y. Liu, J. H. Ho, S. Hsu, and O. K. Lee, "Amine-surface-modified superparamagnetic iron oxide nanoparticles interfere with differentiation of human mesenchymal stem cells," *Journal of Orthopaedic Research*, vol. 30, no. 9, pp. 1499–1506, 2012.

[46] J.-Y. Su, S.-H. Chen, Y.-P. Chen, and W.-C. Chen, "Evaluation of Magnetic Nanoparticle-Labeled Chondrocytes Cultivated on a Type II Collagen–Chitosan/Poly (Lactic-co-Glycolic) Acid Biphasic Scaffold," *International journal of molecular sciences*, vol. 18, no. 1, p. 87, 2017.

[47] E. Lucchinetti, M. M. Bhargava, and P. A. Torzilli, "The effect of mechanical load on integrin subunits α5 and β1 in chondrocytes from mature and immature cartilage explants," *Cell and tissue research*, vol. 315, no. 3, pp. 385–391, 2004.

[48] T. Kurakawa, K. Kakutani, Y. Morita, Y. Kato, T. Yurube, H. Hirata, S. Miyazaki, Y. Terashima, K. Maeno, and T. Takada, "Functional impact of integrin α5β1 on the homeostasis of intervertebral discs: a study of mechanotransduction pathways using a novel dynamic loading organ culture system," *The Spine Journal*, vol. 15, no. 3, pp. 417–426, 2015.

[49] C. Huang, Y. Charles, K. L. Hagar, L. E. Frost, Y. Sun, and H. S. Cheung, "Effects of cyclic compressive loading on chondrogenesis of rabbit bone-marrow derived mesenchymal stem cells," *Stem cells*, vol. 22, no. 3, pp. 313–323, 2004.

[50] I. Takahashi, G. H. Nuckolls, K. Takahashi, O. Tanaka, I. Semba, R. Dashner, L. Shum, and H. C. Slavkin, "Compressive force promotes sox9, type II collagen and aggrecan and inhibits IL-1beta expression resulting in chondrogenesis in mouse embryonic limb bud mesenchymal cells," *Journal of cell science*, vol. 111, no. 14, pp. 2067–2076, 1998.

[51] J. F. Schenck, "The role of magnetic susceptibility in magnetic resonance imaging: MRI magnetic compatibility of the first and second kinds," *Medical physics*, vol. 23, no. 6, pp. 815–850, 1996.

[52] H. D. Amin, M. A. Brady, J.-P. St-Pierre, M. M. Stevens, D. R. Overby, and C. R. Ethier, "Stimulation of Chondrogenic Differentiation of Adult Human Bone Marrow-Derived Stromal Cells by a Moderate-Strength Static Magnetic Field," *Tissue Engineering Part A*, 2014.

[53] K. Andreas, R. Georgieva, M. Ladwig, S. Mueller, M. Notter, M. Sittinger, and J. Ringe, "Highly efficient magnetic stem cell labeling with citrate-coated superparamagnetic iron oxide nanoparticles for MRI tracking," *Biomaterials*, vol. 33, no. 18, pp. 4515–4525, 2012.