Mesenchymal Stem Cells Sense Three Dimensional Type I Collagen through Discoidin Domain Receptor 1

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Abstract: The extracellular matrix provides structural and organizational cues for tissue development and defines and maintains cellular phenotype during cell fate determination. Multipotent mesenchymal stem cells use this matrix to tightly regulate the balance between their differentiation potential and self-renewal in the native niche. When understood, the mechanisms that govern cell-matrix crosstalk during differentiation will allow for efficient engineering of natural and synthetic matrices to specifically direct and maintain stem cell phenotype. This work identifies the discoidin domain receptor 1 (DDR1), a collagen activated receptor tyrosine kinase, as a potential link through which stem cells sense and respond to the 3D organization of their extracellular matrix microenvironment. DDR1 is dependent upon both the structure and proteolytic state of its collagen ligand and is specifically expressed and localized in three dimensional type I collagen culture. Inhibition of DDR1 expression results in decreased osteogenic potential, increased cell spreading, stress fiber formation and ERK1/2 phosphorylation. Additionally, loss of DDR1 activity alters the cell-mediated organization of the naïve type I collagen matrix. Taken together, these results demonstrate a role for DDR1 in the stem cell response to and interaction with three dimensional type I collagen. Dynamic changes in cell shape in 3D culture and the tuning of the local ECM microstructure, directs crosstalk between DDR1 and two dimensional mechanisms of osteogenesis that can alter their traditional roles.

INTRODUCTION

Tissue homeostasis and response to injury is maintained through a dynamic equilibrium between the extracellular matrix (ECM) and cellular components of the tissue. The ECM not only provides structural and organizational guides for tissue development but also defines and maintains cellular phenotype to drive cell fate decisions. Remodeling, the change in tissue form and function, requires both positive and negative signals from the cell and matrix components of the tissue [1]. Through changes in mechanical tension, matrix density (location and concentration) and fluid movement, remodeling affects the spacing between molecules within the extracellular space. This spacing is tunable on a cell by cell basis [1]. The formation of highly ordered multicellular structures requires this type of specific, localized matrix remodeling. For example, fibroblasts encapsulated within 3D hydrogels migrate through the fibrous meshwork *via* MMP-dependent remodeling that occurs directly at the cell surface [2]. These dynamic interactions are required during a variety of cellular behaviors and 3D hydrogel culture allows for the direct cell-mediated interaction with and remodeling of the external microenvironment.

Mesenchymal stem cells (MSC) are multipotent cells that, *in vivo*, contribute to both tissue modeling during development and the maintenance during homeostasis [3]. MSC respond to sites of injury in mature tissue, migrate and directly interact with the tissue microenvironment to induce programs of differentiation and regeneration. Cell shape, adhesion to the ECM and 3D tissue architecture are critically important in the regulation of MSC behavior both *in vitro* and *in vivo*. Exactly how MSC interact with this 3D microenvironment for specific lineage commitment, however, remains largely unknown.

Changes in cell shape, cytoskeletal tension and tissue geometry influence cell fate decisions. Cell shape controls two dimensional (2D) cell behavior through alteration of internal cytoskeletal tension [4-8]. For example, by controlling the area available for individual cell spreading, the specific lineage to which MSC differentiate is sufficiently limited; adhesive, flattened cells adopt an osteogenic phenotype and round cells tend towards adipogenesis [9]. The expression of constitutive RhoA, a cytoskeletal signaling molecule that regulates the formation of stress fibers, promotes osteogenesis irrespective of adipogenic induction but maintains a dependency on island size [9]. Controlled by the spatial architecture of the ECM, cell shape and cytoskeletal tension are primary factors in tissue patterning, morphogenesis and differentiation.

While the way cells adhere to and interact with 2D ECM is relatively well understood, there is still very little known about how structure and function are related when cells are embedded in 3D environments. The three dimensionality of tissue is essential for the maintenance of cellular function and the development of physiologically relevant structures [10,11]. Cells encapsulated within 3D matrices are able to interpenetrate and entangle themselves within collagen fibrils [12]. This is not possible on rigid, planar surfaces. It is

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still unclear, however, what cell surface receptors, cytoskeletal machinery and signaling molecules allow cells to differentially sense and respond to 3D cues.

There are three principle families of receptors involved in cell-mediated adhesions: 1. integrins (cell-matrix), 2. cadherins (cell-cell) and 3. discoidin domain receptors (cell-collagen) [1]. While these interactions are well characterized in 2D culture, the roles and expression patterns of these receptors in 3D culture may be quite different. In fact, their dynamic turnover in 3D may facilitate key changes in cellular behaviors including migration, changes in cell shape and size, and adhesion to newly deposited ECM components [1]. While a role for integrins in MSC differentiation is clear in 2D [13,14], there are discrepancies in the focal adhesion signaling centers that assemble in cells grown in 2D and those encapsulated in 3D, pliable matrices. In fact, 2D focal adhesions are exaggerated forms of the matrix adhesions found in 3D, the stress fibers associated with 2D focal adhesions are rarely observed in 3D culture [15].

Discoidin domain receptors (DDR), are collagen binding receptor tyrosine kinases that are dependent upon both the structure and proteolytic status of the ECM [16]. DDRs are expressed in a variety of tissues and upon activation regulate cell adhesion, proliferation and extracellular matrix remodeling [17]. DDRs are not activated by soluble growth factors but must specifically adhere to collagen ligands. Two receptors in this family, DDR1 and DDR2, have differential collagen specificities. DDR1 is activated by all tested collagens, while DDR2 binds only fibrillar collagens, i.e. types I and III [18,19]. Unlike most receptor tyrosine kinases, DDRs achieve maximal activation several hours following exposure to collagen and are specifically active only when in contact with collagen found in its native triple helical structure [18]. Degraded or denatured collagen fibers fail to induce kinase activity and therefore the DDR receptors are in a unique position to sense and respond to proteolytic events in the extracellular environment [17,18]. In this way, DDRs may be a critical sensor of changing spatial cues found in a 3D collagen matrix.

MSC osteogenesis is differentially regulated in 2D and 3D. In 2D culture MSC use integrins to adhere to several ECM proteins including type I collagen, vitronectin, and laminin-322. *In vitro*, 2D adhesion to these proteins is sufficient to activate focal adhesion formation and induces the upregulation of osteogenic gene markers and mineralization through mitogen activated protein kinase (MAPK), in the absence of osteogenic supplements [20,21]. When encapsulated in 3D, MSC enhance this expression of osteogenic gene markers, however, the MAPK, extracellularly regulated kinase (ERK), conversely functions to suppress that amplification [22]. Conflicting roles for ERK in MSC differentiation are reported throughout the literature and have yet to be reconciled [20,23-25]. Through this work we propose to introduce a role for DDR1 in matrix-mediated crosstalk with traditional integrin signaling pathways. The expression and cell surface localization of DDR1 is limited specifically to 3D culture and stand to play a significant role in the sensing of dimensional cues, remodeling, response to specific tuning of the ECM and pathway crosstalk for enhanced osteogenic potential over 2D.

MATERIALS AND METHODS

MSC were purchased from Lonza Group Ltd. (Allendale, NJ). MSC tissue culture media (DMEM) was purchased from Mediatech (Cellgro, Herndon VA) and penicillin G-streptomycin sulfate fungizone (FPS) from Hyclone (Fisher Scientific, Fair Lawn NJ). Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Woodland CA). Trypsin-EDTA and agarose was obtained from Sigma Chemical Co. (St. Louis MO). Purified, lyophilized bovine collagen I was purchased from MP Biomedicals (Solon OH) and solubilized collagen I from calf skin was purchased from Sigma Chemical Co. (St. Louis, MO). The Trizol reagent for RNA isolation was purchased from Invitrogen (Carlsbad CA) and QuantiTect® SYBR® Green One Step RT-PCR Kit from Qiagen (Valencia CA). Primers were ordered from Integrated DNA Technologies (Coralville IA). The phospho-ERK TiterZyme®EIA Enzyme Immunometric Assay Kit was ordered from Assay Designs (Ann Arbor, MI). Hematoxylin, Eosin and Alizarin Red S was purchased from Sigma Chemical Co (St. Louis MO). Hoechst 33258 dye was purchased from Molecular Probes Inc. (Eugene OR) and Proteinase K from Promega Inc. (Madison WI). Unless otherwise specified, the other standard reagents were obtained from Fisher Scientific (Fair Lawn NJ).

Human Mesenchymal Stem Cell Culture

MSC are provided by Lonza and are donated by healthy males and non-pregnant females between the ages of 18 and 45 years of age. All donors must have negative clinical laboratory tests for HIV, hepatitis B and hepatitis C, normal vital signs and hematology values and a negative medical history for heart disease, kidney disease, cancer, bleeding ulcers, diabetes, jaundice, liver disease, hepatitis and epilepsy. In addition, the donors must maintain a weight no more than 10% above the normal body weight for their height, have no blood or bleeding disorders and are taking no prescription medicines other than those deemed allowable. The MSC used in this study were obtained from three individual donors fitting the above criteria.

Cryopreserved MSC were grown according to manufacturer's instructions. MSC were cultured in Dulbecco's Modification of Eagle's Medium 1x (DMEM) supplemented with 10% fetal bovine serum (FBS) and fungizone/penicillin/streptomycin (FPS) [10,000 units/ml]. Medium was changed every three days and cultures were incubated at 37^{o}C in a humidified atmosphere containing 95% air and 5% CO_2. Cells were detached using trypsin-EDTA and passaged into fresh culture flasks upon reaching confluence. MSC were used between passages 6 and 8. In preparation for incorporation in 3D constructs, cells were washed with PBS, detached with trypsin-EDTA, collected, and counted using a Beckman Coulter Counter. Cells for 2D control samples were prepared in the same manner and plated in a 6-well plate at a density of $2.3x10^4$ cells/cm^2. For collagen-coated samples, solubilized collagen, 20μg/ml, was adsorbed to the surface of the

6-well plate and incubated at 37°C for at least one hour. Following adsorption of the collagen to the tissue culture plastic, the surfaces were rinsed with sterile PBS and cells plated.

3D Collagen I Gel Culture

Three-dimensional collagen I gels were prepared by mixing cells with the following reagents: DMEM (14%), FBS (10%), 5X Conc. DMEM (16%), 0.1N NaOH (10%) and 4mg.ml collagen I (50%). The final collagen concentration is 2mg/ml within each construct. Constructs of a volume of 1.0ml were made in 12-well plates and the cellular density was kept constant at $1.0x10^6$ cells per ml/ECM. The constructs were incubated at 37 °C for 30 minutes, released from the wells and incubated in DMEM. Images of the constructs were taken at days 0, 1, 3 and 7 and construct areas were recorded for analysis of compaction.

RNA Isolation and Quantitative RT-PCR

RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was isolated at day 1, 3, 7 and 14 for initial studies to determine optimal time point for gene detection. Experiments involving the inhibition of ERK were performed at day 7. Constructs were homogenized in the TRIzol reagent using the TissueRuptor power homogenizer. Isolation was performed as per manufacturer's instructions and total isolated RNA was dissolved in RNase/DNase free water and stored at -20 ºC. Total RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington DE).

Quantitative reverse transcription RT-PCR was performed using the LightCycler® 480 Real-Time PCR System (Roche, Pleasonton, CA) and the QuantiTect® SYBR® Green RT-PCR Kit with HotStar Taq DNA Polymerase. 1x QuantiTect SYBR Green, 0.5 µM Primer F and Primer R, 0.5 µl/reaction QuantiTect RT Mix were combined with sample RNA to yield a 20 µl reaction volume. Primers used for amplification of differentiation marker genes were designed using OligoPerfect™ (Invitrogen, Carlsbad, CA) and purchased through IDT Technologies (Coralville IA). RT-PCR was performed according to the following protocol defined by the manufacturer: RT 20 min at 50 ºC, 20 ºC/s ramp, PCR activation 15 min at 95 ºC, 20 ºC/s ramp, 35-55 cycles of [denaturation 15 s at 94 ºC, 20/s ramp, annealing 20-30 s at 50-60 °C, 20 °C/s ramp, extension 30 s 72 °C, 2 °C/s ramp]. The markers of cell phenotype used in this study are listed in Table **1** and represent the osteogenic, chondrogenic, adipogenic, and myogenic lineages. All samples were loaded in duplicate and normalized to total RNA content and to the performance of the housekeeping gene, GAPDH. Fold dif-

Table 1. Real Time RT-PCR Primers

Target	Primer Sequences
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward 5'-CGACCACTTTGTCAAGCTCA-3' Reverse 5'-AGGGGTCTACATGGCAACTG-3'
Bone Sialoprotein (BSP)	Forward 5'-CTGCTTCCTCACTCCAGGAC-3' Reverse 5'-GATTGCTTCCTCTGGCAGTC-3'
Integrin beta1 (ITGB1)	Forward 5'-TGAAGACTATCCCATTGACC-3' Reverse 5'-TCCTCCCTCATTTCATTCATC-3'
Integrin alpha1 (ITGA1)	Forward 5'-CATTTGCATTACACAACTGG-3' Reverse 5'-CATTCTTTCAAGAAGGTCA-3'
Integrin alpha2 (ITGA2)	Forward 5'-GGAGCAATTCAATATGCAAG-3' Reverse 5'-GTACCCAAGAACTGCTATGC-3'
Integrin alpha3 (ITGA3)	Forward 5'-ATCAACATCGTCCACAAGAC-3' Reverse 5'-GTTGTAAGCAAAGCACAGC-3'
Discoidin Domain Receptor I (DDR1)	Forward 5'-TGGCTATTCACTGAGCGATG-3' Reverse 5'-ACTGCTCTCCAACCTGCTGT-3'
Matrix Metalloproteinase 1 (MMP-1)	Forward 5'-ATGCTGAAACCCTGAAGGTG-3' Reverse 5'-CTGCTTGACCCTCAGAGACC-3'
Matrix Metalloproteinase 2 (MMP-2)	Forward 5'-ATGACAGCTGCACCACTGAG-3' Reverse 5'-ATTTGTTGCCCAGGAAAGTG-3'
Matrix Metalloproteinase 9 (MMP-9)	Forward 5'-CATCGTCATCCAGTTTGGTG-3' Reverse 5'-AGGGACCACAACTCGTCATC-3'
Matrix Metalloproteinase 10 (MMP-10)	Forward 5'-CCAGTCTGCTTGCCTATCC-3' Reverse 5'-CCAGGAAAGGAGCTGAAGTG-3'
Matrix Metalloproteinase 13 (MMP-13)	Forward 5'-AACGCCAGACAAATGTGACCC-3' Reverse 5'-TCCGCATCAACCTGCTGAGG-3'

ferences in gene expression were relative to MSC cultured on tissue culture plastic and calculated using the ΔΔCt method [26].

Nucleofected siRNA Knockdown of DDR1

DDR1 siRNA (Santa Cruz Biotechnology) and AMAXA Nucleofection technology was used to mediate the expression of DDR1 in response to type I collagen stimulus. Cells were trypsinized, counted and isolated into $6.0x10^5$ aliquots. Cells were nucleofected according to manufacturer instructions as outlined in the AMAXA Human MSC Kit and nucleofected using an AMAXA Nucleofector Device, program U-023. Scrambled control siRNA (Santa Cruz Biotechnology) and cells exposed to the AMAXA program without siRNA were used as negative controls in addition to unaffected wild type cells. Cells were immediately exposed to low calcium medium and then added to prewarmed $10cm^2$ dishes. The cells were allowed to culture overnight and RNA was isolated from a sample of the nucleofected cells while the remaining were trypsinized and encapsulated in type I collagen hydrogels as described above.

Inhibition of the MAPK Pathway

ERK activity was controlled through the inhibition of its upstream activator MEK using PD98059 dissolved in DMSO. Cells were pre-incubated with PD98059 at a concentration of 50μM for 15 min and then plated for 2D culture or encapsulated within a collagen hydrogel for 3D culture as described below. Constructs were grown in PD98059 supplemented medium for the given time points. The final DMSO concentration never exceeded 0.1% and the same amount of the DMSO vehicle was added to control samples.

Quantification of ERK

Cells were serum encapsulated in type I collagen at a density of $1x10^6/cm^3$ with and without DDR1 siRNA. Cells were extracted from the hydrogel through collagenase-2 (Sigma Chemical Co., St. Louis, MO.) digestion for one hour at 37˚C and centrifuged to obtain a cell pellet. Protein was extracted from the pellet in ice-cold RIPA buffer supplemented with a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). Samples were assayed using the TiterZyme®EIA phospho-ERK ½ Elisa Assay Kit according to manufacturer's instructions. Briefly, samples were diluted in assay buffer to normalize for cell number and incubated for one hour in a 96-well plate. Following antibody and substrate incubation, the samples were read on a plate reader at an optical density of 450 nm. The concentration of pERK (pg/μl) was quantified against a standard curve and normalized to total protein levels.

Immunofluorescence Imaging

Constructs were washed with PBS followed by 30 minute incubation at 4°C in 3% paraformaldehyde. The constructs were then washed with PBS and incubated at 4°C for 30 minutes in cell blocking solution (0.25% Tween20, 1% BSA in PBS). Collagen constructs were incubated for 45 minutes in a 1:500 dilution of phalloidin (Invitrogen) and 10 minutes at room temperature in SYTOX® Green Dye (Invitrogen) at a final concentration of 50nM in cell blocking solution. DDR and tubulin antibodies (Santa Cruz Biotechnology) were applied at a 1:200 dilution. The samples were stored at 4˚C in PBS until imaging. 3D confocal images were taken using a Zeiss LSM 51 and Image J (NIH) was used to convert the files into .tiff format for segmentation.

Second Harmonic Generation

Collagen I constructs were harvested at days 0, 0.5, 1, 2 and 3 and were second harmonic micrographs were obtained using a Zeiss LSM 510 two photon confocal microscope (Zeiss Inc., Thornwood, NY). Samples were excited by a two photon laser at 820 nm and emissions were collected at 480 nm. Images were taken at a 40x magnification. Corresponding cellular images taken as stated above and merged with the SHG images using Image J (NIH, Bethesda, MD).

Statistical Analysis

All experiments were repeated a minimum of three times, and the representing data are presented as mean ±SEM. Statistical analyses were performed using Student's unpaired t-test, and a p-value ≤ 0.05 was considered significant.

RESULTS

The 3D Expression and Localization of Discoidin Domain Receptor 1, Integrins and Matrix Metalloproteinases

3D culture affects the phenotype and behavior of many cell types when compared to traditional 2D culture. This alteration in behavior is presumably mediated by changes in cell shape, cell signaling and cellular interaction with the 3D orientation of the matrix. Previous work established opposite roles for integrin mediated osteoinduction in MSC cultured in 2D and 3D [22]. Therefore, a quantitative increase in integrin signaling does not explain the enhancement of osteogenic gene expression in 3D. For this reason, we looked for an alternative receptor that might function to inhibit or alter integrin signals, specifically in the presence of 3D collagen fibers. Discoidin domain receptor 1 (DDR1) plays a critical role in not only binding to collagens but also in sensing their structure and proteolytic state. When cultured in 3D, as compared to 2D controls, both tissue culture plastic (TCP) and type I collagen coated plates, MSC upregulated their expression of DDR1 as early as day 1, (Fig. **1D**), and localize into punctate adhesions by day 3, (Fig. **1C**). DDR1 in 2D cultures, in the presence or absence of adsorbed type I collagen, remains diffuse and localized to the cytoplasm, (Fig. **1A,B**).

Though the signals propagated *via* integrin adhesion to type I collagen seem to play a different role in 3D matrices, their presence will remain critical to MSC survival, adhesion and behavior. We tested for the presence of key collagen binding integrins in 3D culture as compared to 2D controls, both tissue culture plastic (TCP) and collagen coated plates (COL). MSC were cultured for three and seven days within these different environments. Integrin subunits β1 and α1 showed no significant change in expression when cultured in 3D. Integrins α2 and α3, however, showed trends of increase, though they do not reach statistical significance given wide variance in the extent of transcript upregulation, (Fig. **2A-D**).

Cell-mediated reorganization of the ECM requires adhesion, secretion, degradation and assembly of the proteins within that matrix. DDR1 and integrins are responsible for

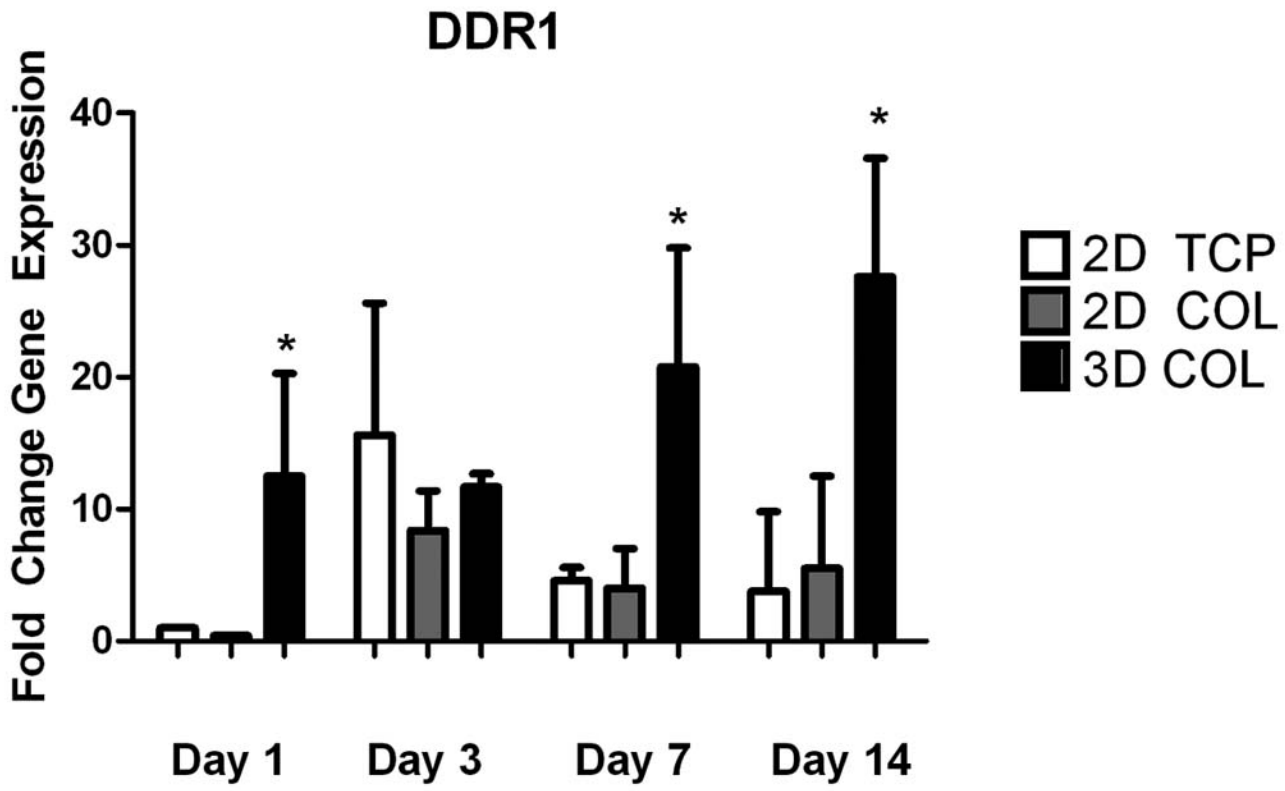

Fig. (1). DDR1 expression and localization is upregulated in 3D culture. Immunofluorescence demonstrates signficiant changes in DDR1 localization in 2D and 3D environments. A. MSC on glass and B. MSC on type I collagen coated coverslips show diffuse, cytoplasmic staining of the DDR1 receptor (red). Nucleus (blue). C. Encapsulated in 3D, this changes to specific localized puncta scattered within 3D space (red). Nucleus (blue), actin (green). One optical slice within a z-stack shown with xy, xz and yz planes, representative images, scale bar = 20μm. D. One optical slice through stack stained with secondary only as a negative control shows no punctuate staining as seen with the staining of the DDR1 receptor. E. Expression of DDR1, detected by qRT-PCR, is significantly upregulated immediately upon encapsulation within a 3D environment and high levels of expression are sustained through two weeks in culture as compared to 2D tissue culture plastic (TCP) and collagen coated plates (COL). All samples were normalized to the housekeeping gene, GAPDH, and expressed here as a fold change over TCP at day one. *Statistical significance $p \leq 0.05$, n=3.

the upregulation of matrix metalloproteinases (MMPs) in response to collagen adhesion that mediate the cellular remodeling response. Therefore, we assessed the expression of MMPs in MSC encapsulated in type I collagen at days 1, 3, 7 and 14. Of the MMPs assayed, three showed significant increases over 2D controls, TCP and COL (Fig. **3A**). These MMPs, MMP-1, MMP-9 and MMP-13, were sustained at high levels of expression through two weeks of culture in 3D, (Fig. **3B-D**).

siRNA Mediated Knockdown of DDR1 in MSC

In order to better understand the role played by DDR1 in mediating both remodeling and osteogenic differentiation in type I collagen gels we used siRNA to knockdown DDR1 mRNA levels. A cocktail of 3-5 siRNA specific to the DDR1 mRNA transcript was introduced to MSC in culture using Amaxa Nucleofection technology one day prior to encapsulation within type I collagen gels. Transcript levels of DDR1 were quantified in 2D after nucleofection, just prior to encapsulation, and following seven days of 3D culture. At both time points DDR1 was significantly downregulated by 80%, consistent with Amaxa's reported nucleofection efficiency in MSC, (Fig. **4**). Negative controls used were untreated MSC (WT-), MSC exposed to the nucleofection program without siRNA (WT+) and MSC nucleofected with scrambled, negative control siRNA (Neg). A positive GAPDH siRNA control was used during optimization of the nucleofection protocol but was not used in the following experimental runs (data not shown). Control samples showed no significant change in DDR1 levels as assessed by qRT-PCR. Additionally, DDR2 and GAPDH mRNA levels remained unaffected by the DDR1-specific siRNA treatment (data not shown). Following nucleofection, MSC were encapsulated within 3D

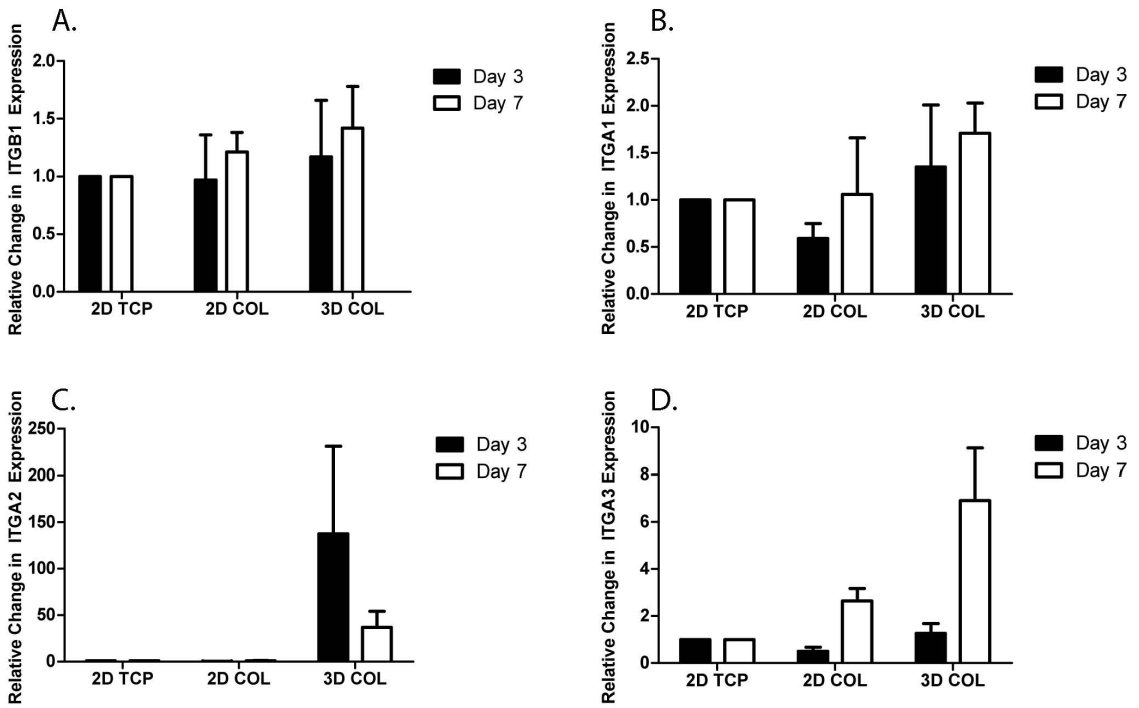

Fig. (2). MSC maintain levels of integrin expression comparable to 2D culture with (COL) and without collagen (TCP). MSC were cultured in 2D and 3D environments for 3 and 7 days and integrin expression assessed using qRT-PCR. Three collagen binding integrin subunits and one fibronectin subunit were tested, A. ITGβ1, B. ITGα1, C. ITGα2 and D. ITGα3. Though upward trends are observed for the α2 and α3 subunits in 3D they do not reach significance as compared to 2D controls (TCP and COL). All samples were normalized to the housekeeping gene, GAPDH, and expressed here as a fold change on TCP. *Statistical significance $p \leq 0.05$, n=3.

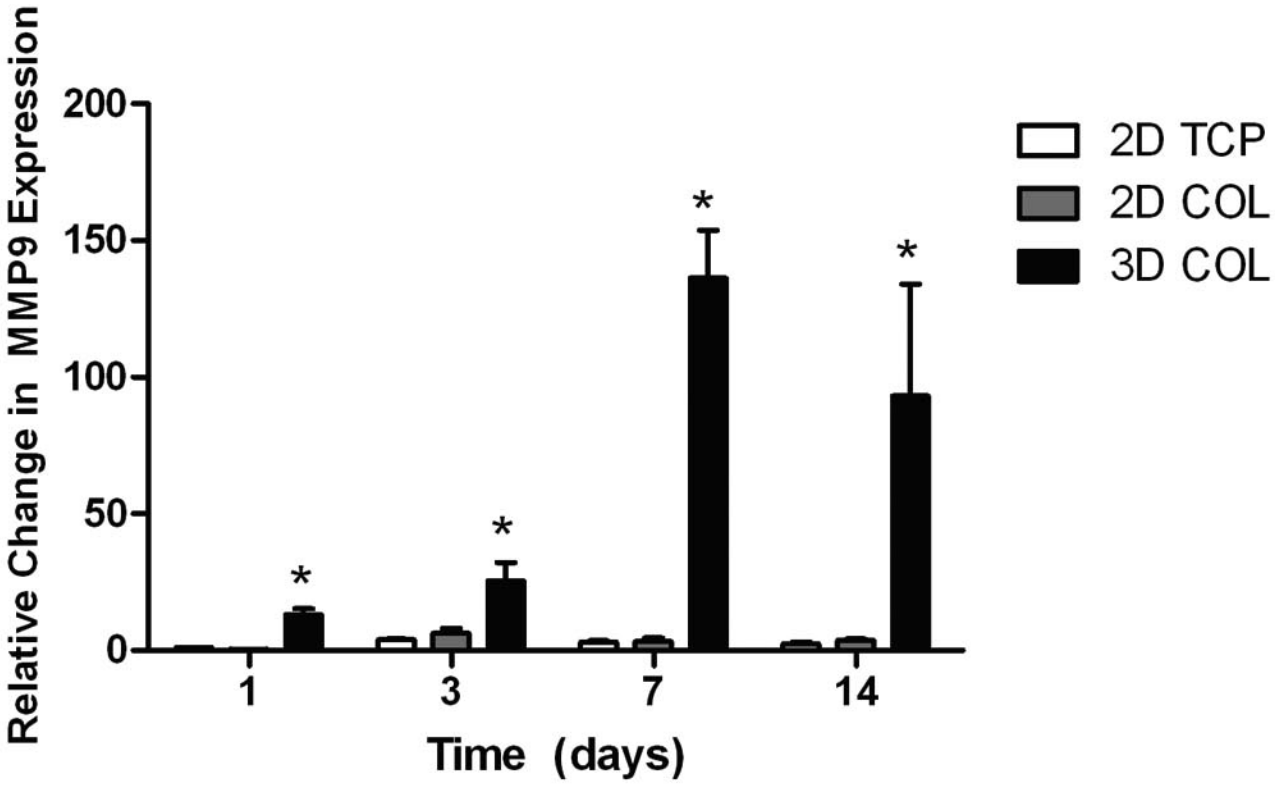

Fig. (3). MMP expression is upregulated in 3D type I collagen culture. A. MMP 1, 2, 9, 10 and 13 expression was assessed using qRT-PCR in 3D type I collagen culture as compared to 2D controls (TCP and COL). MMPs 1, 9 and 13 were significantly upregulated in 3D. B. MMP1, C. MMP9 and D. MMP13 sustained these high levels of expression over two weeks in culture. All samples were normalized to the housekeeping gene, GAPDH and expressed here as a fold change over TCP. *Statistical significance $p \leq 0.05$, n=3.

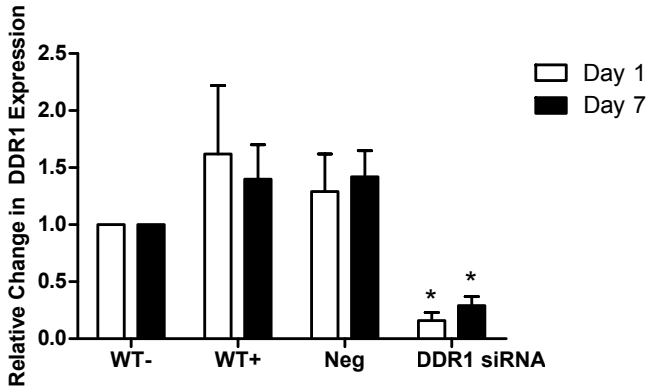

Fig. (4). DDR1 mRNA was significantly knocked down in MSC using siRNA nucleofection technology. Controls used were untreated MSC (WT-), MSC nucleofected without siRNA (WT+) and MSC nucleofected with a negative scrambled siRNA control (Neg). Both prior to encapsulation (one day after nucleofection) and after 7 days in 3D culture, MSC showed significant knockdown of the DDR1 transcript. All samples were normalized to the housekeeping gene, GAPDH and expressed here as a fold change over TCP. *Statistical significance $p \leq 0.05$, $n=3$).

type I collagen microenvironments. Within these environments, MSC compact their matrix to levels comparable with control gels, (Fig. **5**). MSC were cultured in these 3D environments for one week and imaged at days 1, 3 and 7 to determine changes in construct volume. Additionally, viability was assessed in these constructs at day 7 using Live/Dead (calcein-AM and ethidium homodimer) staining. No significant cell death was observed with the knockdown of DDR1 (data not shown).

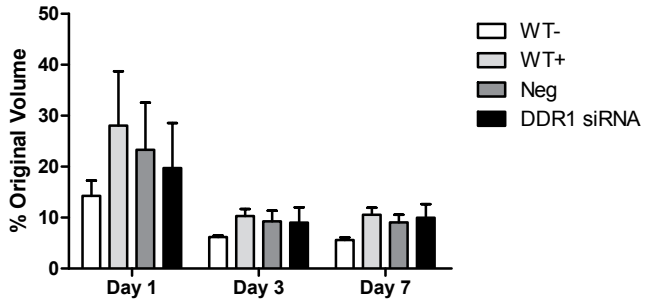

Fig. (5). MSC are able to compact their 3D environment in the absence of DDR1. There was no significant change in either the final volume or rate of compaction observed with the knockdown of DDR1 expression in 3D type I collagen hydrogel microenvironments. *Statistical significance $p \leq 0.05$, $n \geq 3$.

Loss of DDR1 Affects Cell Shape in 3D

Fibroblasts cultured in 3D type I collagen gels exhibit dendritic extensions that contain a microtubule core with actin tips [27]. The inhibition of actin polymerization in 2D cultures of fibroblasts results in the recapitulation of these dendritic extensions. In pre-stressed 3D gels, which mimic the rigidity in 2D culture, these cells show well established stress fibers and lamellipodia [27]. Thus, the cytoskeleton plays an important role in the regulation of cell spreading and the tension state in response to dimensional cues. MSC in 3D exhibit the morphology observed in fibroblasts with tubulin enrichment of 3D dendritic extensions, (Fig. **6**). Knockdown of DDR1 inhibited the formation of a microtubule core in dendritic extensions characteristic of 2D culture, (Fig. **6**). Additionally, the generation of well defined stress fibers, typical of 2D culture, was induced with the inhibition of DDR1 expression, so that without prestressing of the collagen gels 2D morphology is recapitulated in 3D, (Fig. **7A, B**). MSC that lack DDR1 expression are significantly larger and more spread than their wildtype counterparts within the same 3D environment as quantified by total area and volume, (Fig. **7C**). No significant changes in cell shape were observed in 2D culture (data not shown).

DDR1 Suppresses ERK Phosphorylation in 3D

Our previous work showed that integrin mediated signaling required for 2D induction of osteogenic differentiation played a suppressive role in 3D [22]. As DDR1 is preferentially expressed by MSC cultured in 3D, DDR1 signaling player may affect the traditional transduction of integrin mediated signals to enhance osteogenesis in 3D matrices. Inhibition of ERK activity using the MEK inhibitor, PD98059 [28], resulted in a loss of DDR1 localization, demonstrating interaction between these two intracellular signaling pathways. MSC were cultured in 3D type I collagen gels with and without PD98059 and imaged at day 1. The distinct punctate staining observed in 3D culture was decreased with the inhibition of ERK at this early timepoint, (Fig. **8A,B**). Following 7 days in culture there was no significant change in DDR1 expression in the presence of PD98059, (Fig. **8C**). To demonstrate interaction with MAPK downstream of DDR1 activation, phosphorylated ERK was quantified by ELISA one day after encapsulation. ERK phosphorylation in the absence of DDR1 was enhanced over controls, though it does not reach statistical significance over WT- ($p=0.056$), (Fig. **9**).

Loss of DDR1 Decrease MSC Osteogenic Potential

The intimate and dynamic relationship between a stem cell and its extracellular environment is required for fate determination. MSC encapsulated in 3D type I collagen significantly upregulate their osteogenic potential when compared to a 2D presentation of the same osteoinductive stimulus. Therefore, the cells' ability to sense the orientation and dimension of type I collagen may be a critical component in their commitment to an osteogenic lineage. MSC encapsulated in 3D type I collagen gels were cultured for 7 days and assayed for their osteogenic gene expression. Bone sialoprotein (BSP) showed a significant decrease in expression with the knockdown of DDR1 after 7 days in culture as detected by qRT-PCR indicating a loss of osteogenic potential in the absence of DDR1, (Fig. **10**). The osteogenic gene markers osteocalcin (BGLAP), runt-related transcription factor 2 (RUNX2), osterix(OSX) and collagen I (COLI) showed no significant change from controls (data not shown).

Loss of DDR1 Inhibits the Progression of MSC-Mediated Organization of the Type I Collagen Microstructure

Although MSC showed no significant difference in the extent to which they compacted their matrix when compared to controls, the way in which they reorganized the type I

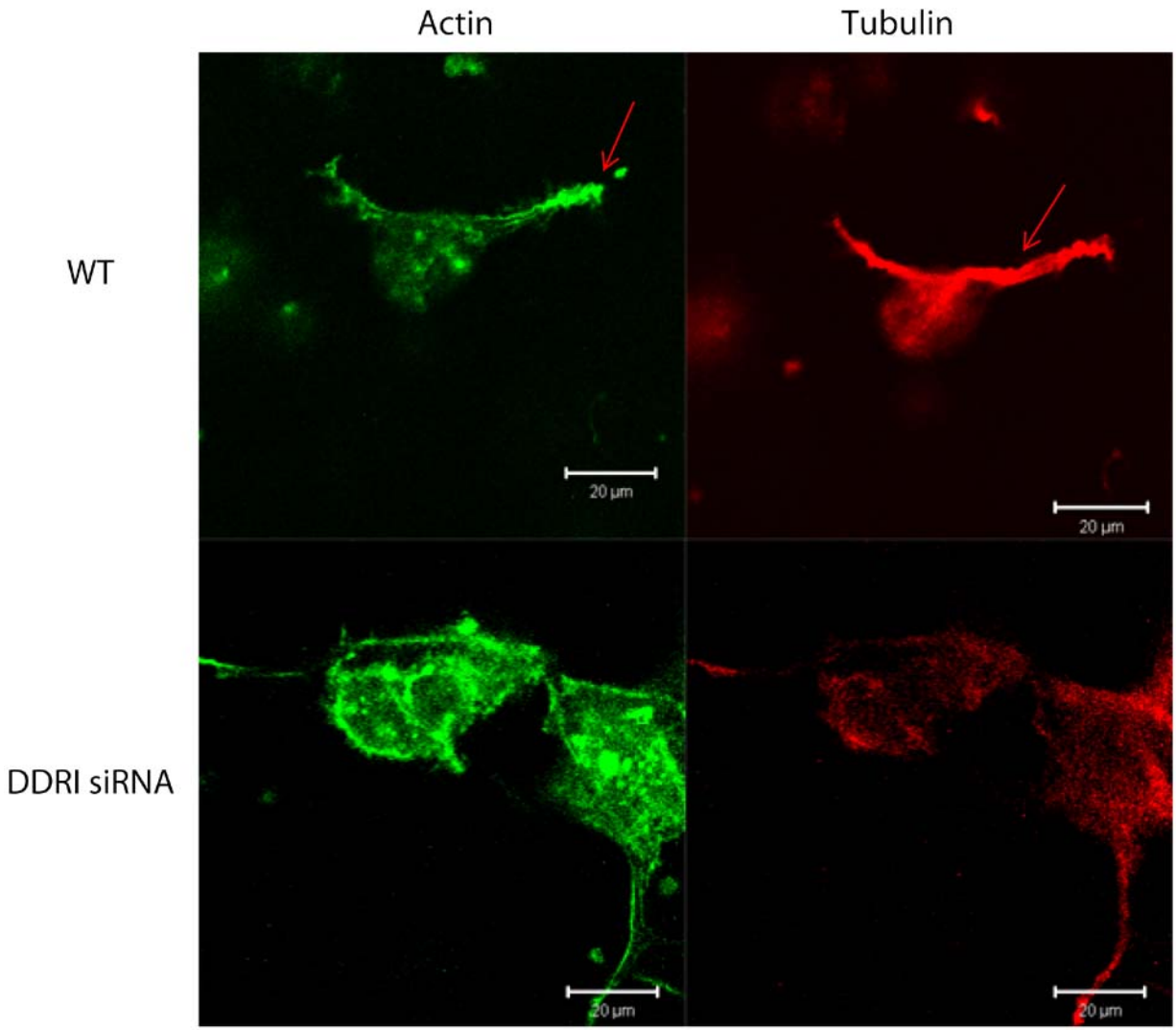

Fig. (6). MSC dendritic extensions in 3D type I collagen matrices are enriched with a microtubule core. Suppression of DDR1 in MSC culture results in a loss of this microtubule enrichment. MSC were cultured in type I collagen gels for one day and imaged. Actin (green) and tubulin (red). Arrows indicate dendritic extensions with tubulin enrichment and actin tip. One optical slice of z-stack, representative images, scale bar = 20µm.

collagen fibers, at the microstructural level, is strikingly different. Wild type gels exhibited the characteristic entanglement of fibers that reorganize around the cell with time creating intense areas of type I collagen consolidation at the cell interface. With the inhibition of DDR1 expression, SHG images at day 1 and 3 reveal significant alignment of fibers between adjacent cells but no apparent reorganization of those fibers around the cell interface, (Fig. **11**).

DISCUSSION

Sensing the dimensionality of the extracellular matrix (ECM) facilitates the differentiation and morphogenesis of progenitor and stem cells. This work presents a mechanism through which human mesenchymal stem cells (MSC) interact with and respond to the dimension of their type I collagen matrix to enhance their osteogenic potential. 3D culture upregulates the expression of the discoidin domain receptor 1 (DDR1). Expression and activation of this collagen activated receptor tyrosine kinase did not affect hydrogel compaction, but enhanced cell spreading in 3D, stress fiber generation, suppressed ERK phosphorylation, osteogenic differentiation and matrix remodeling. DDR1 expression, therefore promotes MSC sensitivity to the fine spatial tuning of the ECM during organizational dynamics and remodeling.

DDR1 is widely expressed during development and present in a variety of adult tissues[17]. High levels of DDR1 expression are observed in breast, ovarian, lung and brain tumors [29-31] and overexpression correlates with increased cell survival and invasiveness in hepatocellular carcinomas, pituitary adenoma and prostate cancer [32-34]. Pulmonary fibrosis and inflammation is attenuated by DDR1 suppression [35-37]. DDR1 also plays an essential role in mammary gland development, lactogenesis and arterial wound repair [38-40]. Key to all of these events is the regulation of collagen turnover, invasion into and organization of the ECM.

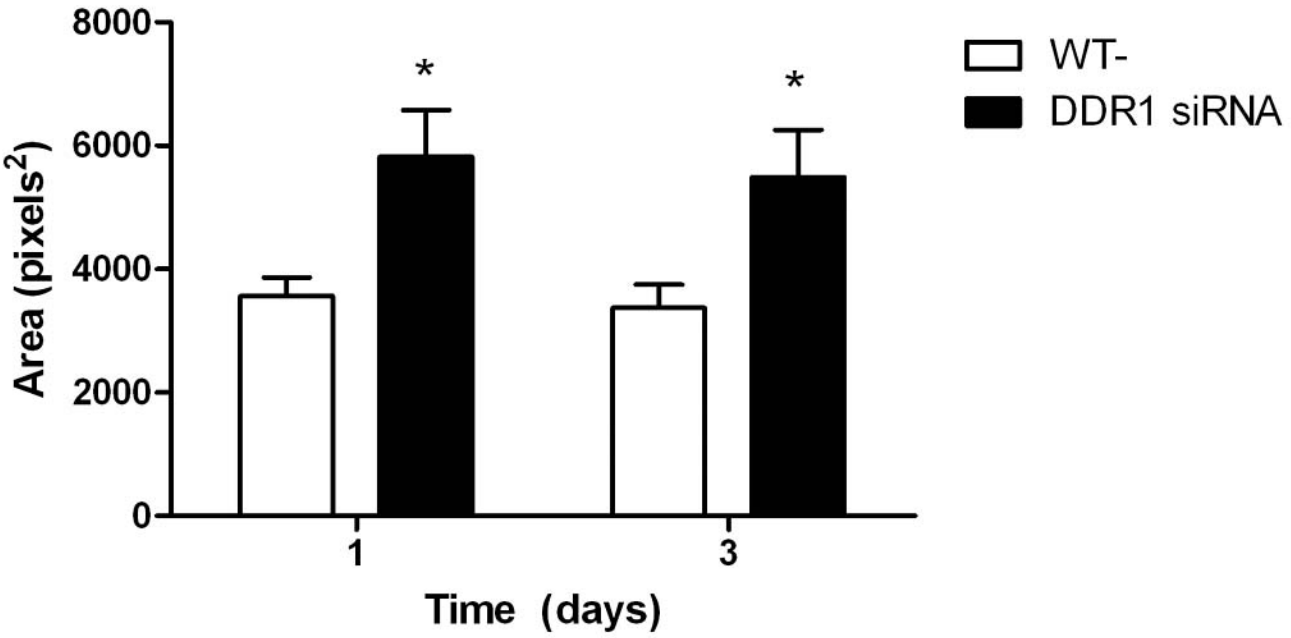

Fig. (7). Knockdown of DDR1 enhances cell spreading in 3D. A. Wildtype cells in 3D show dendritic extensions and lack distinct stress fibers. B. MSC without DDR1 showed enhanced spreading within this same environment with strikingly prominent actin stress fibers. Nucleus (red) and actin (green). 2D projections of 3D confocal images, representative images, scale bar = 50μm. C. Quantification of these images showed a significant increase in cell spreading with the inhibition of DDR1 expression, n≥20.

3D type I collagen enhances the osteogenic potential of MSC over 2D controls [22]. Analysis of the role played by ERK in collagen induced osteogenesis demonstrates that integrin signaling is not the sole mechanism through which MSC interact with their matrix during differentiation. We demonstrate here that 3D MSC culture upregulates both DDR1 expression and localization. When transitioned into 3D culture, MSC do not, however, upregulate the expression of the collagen activated integrin receptors. The β1 and α1 integrin subunits exhibit no significant change in expression when compared to 2D controls while integrins α2 and α3 showed trends of increase but did not reach significance. Our previous work demonstrated that MSC use the α2β1 integrin to bind to type I collagen in culture and these results are consistent with that finding [41].

The α2 integrin plays a critical role in tissue contractility, MMP expression and matrix remodeling, therefore, its upward trend may represent a response to matrix compliance [42]. Tissue contraction is a hallmark of hydrogel culture. No significant change in volume over time was observed with the knockdown of DDR1 as compared to controls indicating that the primary role for DDR1 in collagen organization is not these initial contractile events. The upward trend in expression of α2 may therefore be the primary mechanism for the contractile response to 3D type I collagen. The laminin receptor α3 also shows trends of upregulation in 3D. Laminin localizes to developing and mature bone tissue and induces osteogenic differentiation in 2D culture of MSC [21]. It is clear that integrins remain important in 3D culture and presumably maintain their roles in promoting cellular adhesion, survival and migration. Integrins, however seem to be less sensitive to dimensional cues than DDR1.

Though no significant changes were observed in the expression of β1, α1, α2 and α3 integrin subunits, striking changes were seen in cell morphology. The generation of distinct stress fibers is atypical of 3D culture. Fibroblasts grown in 3D lose their spindle like morphology and extend microtubule rich dendritic arms into the extracellular space [27]; this morphology was recapitulated by MSC in 3D type I collagen culture. Knockdown of DDR1 generates a distinctly 2D-like morphology irrespective of the compliant nature of the type I collagen hydrogel. Consistent with this,

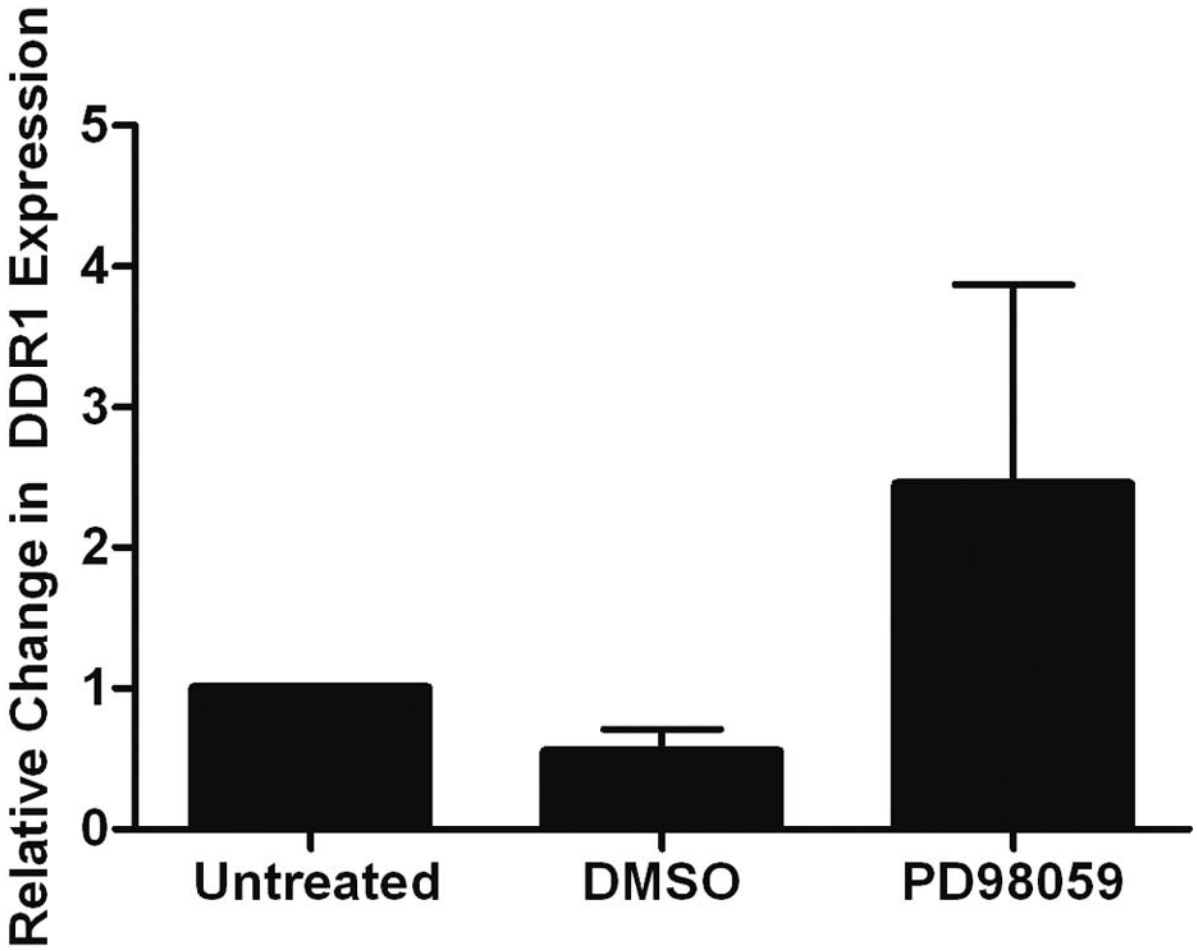

Fig. (8). Interaction between MAPK signaling and DDR1 localization. A. Untreated gels show distinct punctate staining of the DDR1 receptor. B. Inhibition of ERK with PD98059 resulted in a dramatic loss of DDR1 localization within the 3D gel. Nucleus (blue), actin (green) and DDR1 (red). 2D projections of 3D confocal images, representative images, scale bar = 50μm. C. Expression levels of DDR1 after 7 days of 3D culture in the presence of PD98059 shows no significant change from untreated and DMSO controls. All samples were normalized to the housekeeping gene, GAPDH and expressed here as a fold change over TCP. *Statistical significance p≤0.05, n=3.

inhibition DDR1 in MDCK cells enhances spreading in 2D. DDR1 activation leads to the suppression of α2β1 integrin activation of Cdc42 and subsequent downstream cytoskeletal dynamics [43]. With the elimination of DDR1 and its downstream signaling effects, static adhesions were formed as the

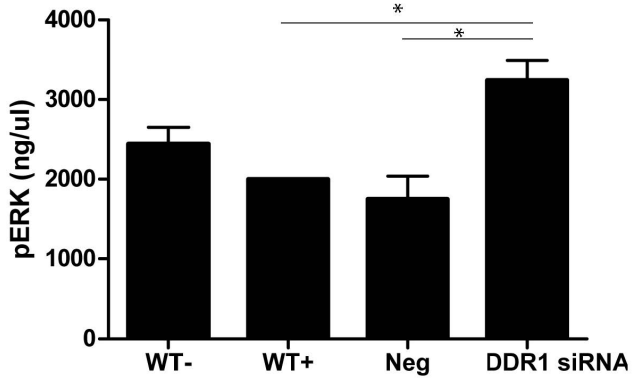

Fig. (9). Phosphorylated ERK increased with the suppression of DDR1 expression. MSC were cultured in 3D type I collagen for 1 day after nucleofection and protein isolated for ELISA detection of phosphorylated ERK. ERK phosphorylation trends towards an increase over WT-, WT+ and Neg controls but does not reach statistical significance over WT-. *Statistical significance p≤0.05, n=3.

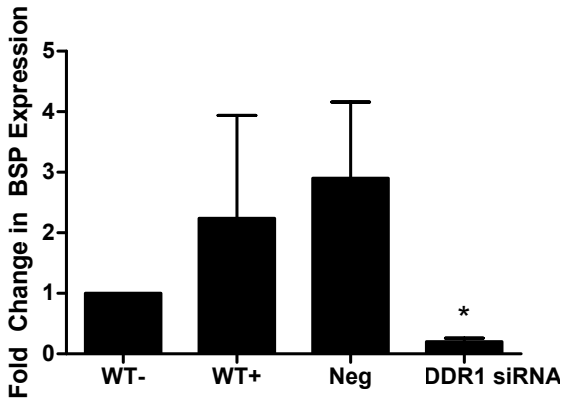

Fig. (10). Knockdown of DDR1 results in a loss of osteogenic potential. MSC nucleofected with DDR1 siRNA and encapsulated in 3D type I collagen gels for seven days showed significant loss in their osteogenic potential, detected by bone sialoprotein (BSP) expression, as compared to control 3D gels (WT-, WT+, Neg). *Statistical significance p≤0.05, n≥3.

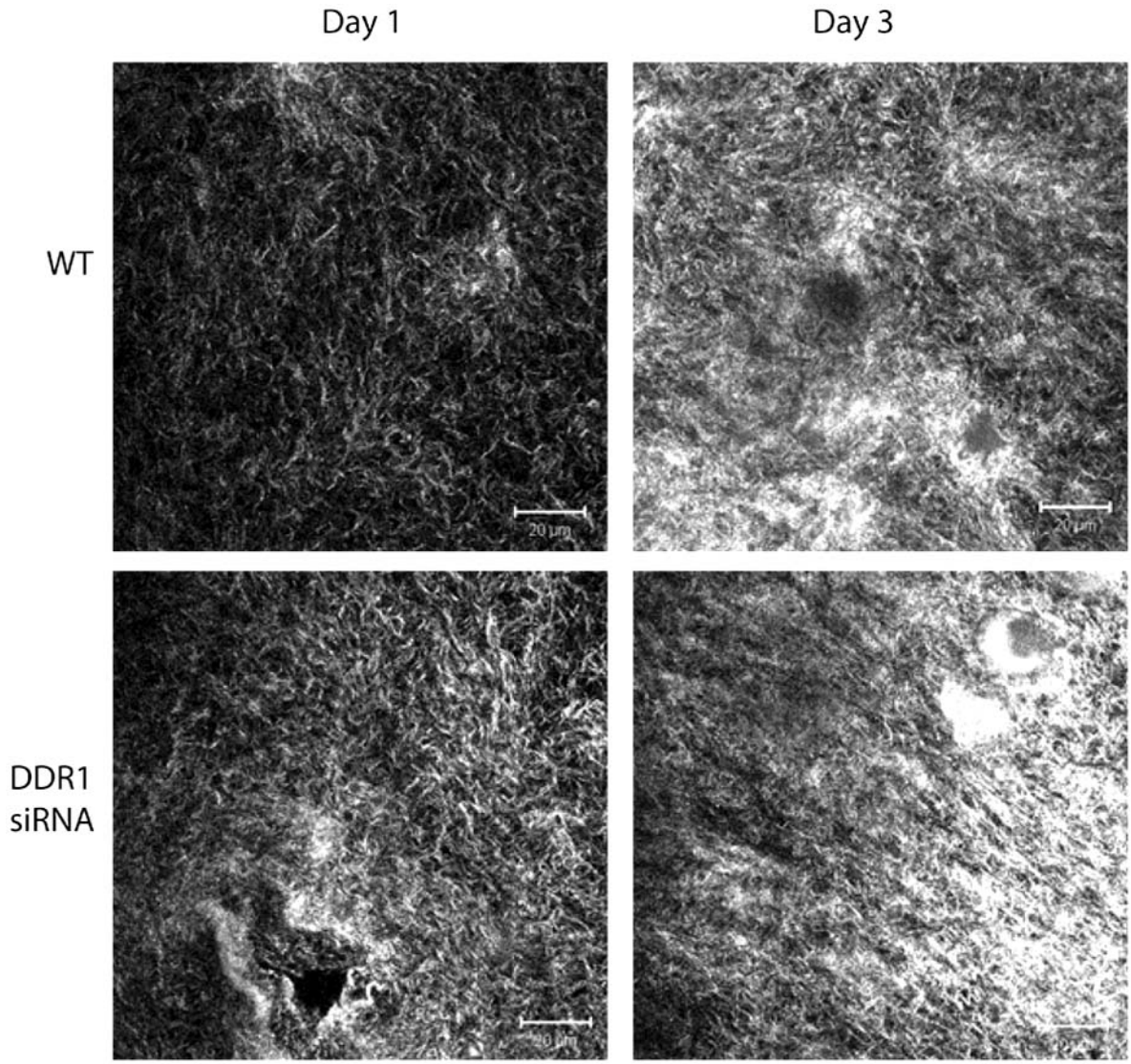

Fig. (11). Loss of DDR1 results in changes in the microstructure of the type I collagen matrix. MSC encapsulated in 3D type I collagen gels were imaged at day 1 and 3 using SHG to image the collagen microstructure (white). Within these gels the MSC compacted their matrix equivalent to controls but were unable to reorganize and remodel the matrix around the cell interface. Single optical slices of z-stack shown, representative images, scale bar = 20μm.

actin cytoskeleton was decoupled from the dynamic, mechanical nature of the environment.

Manipulation of 2D matrix compliance alters cell spreading, stress fiber formation and MSC differentiation [44]. Additionally, restricting cell spreading through controlled adhesive islands restricts development of cytoskeletal tension and the differentiation potential of MSC [9]. Uncoupling can be achieved by introducing constitutively active actin regulators to drive specific differentiation [9]. 3D matrices can then regulate cell shape through the complex restriction of cellular volume and temporal changes in matrix density and compliance. These dynamic changes in cell shape and matrix mechanics could then significantly alter MSC differentiation and disruption of the interplay between cell shape and its matrix could play a suppressive role. DDR1 may integrate with traditional integrin signaling to mediate the behaviors upregulated in response to extracellular cues.

Consistent with this idea, we demonstrated an interaction between traditional MAPK signaling and DDR1 activation and localization. The decreased localization of DDR1 in response to ERK inhibition by PD98059 is consistent with previous work that implicates a positive feedback loop between DDR1 and ERK [45]. Interestingly, however, with the knockdown of DDR1 we observed an upward trend in ERK activation, that while not significant, indicates a possible suppressive role for DDR1 in mediating the MAPK pathway. The collagenase-2 processing required for extraction of cells from the type I collagen gels for ELISA analysis may dephosphorylate ERK and therefore distort absolute quantification of ERK activation. However, given the simultaneous processing of each 3D sample we would expect equivalent levels of de-phosphorylation to occur in each sample thereby maintaining relative changes in pERK. ERK, therefore, may promote its own inhibition, inducing DDR1 activation in response to integrin activation of the MAPK pathway effec-

tively coupling these two signals. Similar negative feedback loops have been demonstrated in ERK signaling [46]. This presents a mechanism similar to that demonstrated above in 2D where the introduction of DDR1, as an additional collagen sensor, acts to suppress the propagation of integrin mediated signals [43].

In 3D, ERK suppresses osteogenic differentiation potentially through inhibition of Smad signaling [47-49]. DDR1's role in the control of ERK activation may therefore represent a potential mechanism of osteogenic induction in 3D. Knockdown of DDR1 inhibited the osteogenic potential of MSC within 3D type I collagen culture as determined by BSP expression. BSP was significantly downregulated after seven days of culture in MSC nucleofected with DDR1 siRNA as compared to controls. Bimodal activation of BSP is required for osteogenic differentiation and therefore it has consistently been used as a responsive marker at early time points during osteogenic induction. No change was observed for the other markers testing, BGLAP, COL I and the transcription factors RUNX2 and OSX. Specific temporal regulation of these markers is required for osteogenesis and patterns of expression vary. RUNX2 and OSX are largely regulated by phosphorylation events in addition to the upregulation of their mRNA expression. BGLAP is typically a late marker of osteogenesis and COL I is consistently downregulated in the presence of a type I collagen matrix as activation of the $\alpha1\beta1$ integrin inhibits collagen synthesis [42].

The receptor tyrosine kinase inhibitor and cancer therapeutic, imatinib (Gleevac©), potently inhibits both DDR1 and DDR2 [50]. Long term use of imatinib in the treatment of chronic myeloid leukemia inhibits bone tissue remodeling in patients [49]. Application of imatinib *in vitro* to cultures of bone marrow derived MSC has produced interesting but inconsistent affects on osteoblastogenesis. While some reports demonstrate an inductive role for imatinib in osteogenesis, others indicate a suppression of proliferation and a shift in the differentiation potential from osteogenesis to adipogenesis [49,51,52]. Imatinib's general specificity to all receptor tyrosine kinases complicates the interpretation of this data but lends support to the potential role for DDR1 and DDR2 in bone turnover and remodeling. The role for DDR1 may lie in the organization and remodeling of the matrix around the developing osteoblast to guide differentiation. The cell source and culture system will greatly affect the role for and importance of DDR1; cultures that allow for a dynamic interplay to exist between cells and their environment or the presentation of a disorganized matrix to differentiating cells (as might be seen in a fracture) may enhance the role and need for DDR1.

The intimate relationship between developing stem and progenitor cells and their ECM is required for morphogenesis. Knockdown of DDR1 in 3D type I collagen culture of MSC successfully inhibited the organization of the matrix. Fibrillogenesis drives cell and tissue morphogenesis through the fine tuning of the structure and mechanics of the ECM. Inhibition of fibronectin fibrillogenesis prevents neovessel formation by endothelial cells encapsulated within 3D matrices [53]. The behavior of these same cells plated in 2D is independent of fibronectin fibrillogenesis. Inhibition of fibrillogenesis in 3D prevents endothelial cells from spreading within their matrix, proliferating and forming higher order structure [53]. During chondrogenesis a progression of cellular adhesions must occur to propagate the necessary signals and behavior. In late stages of chondrogenesis, adhesion *via* integrins and DDR2 to the matrix links the ECM to the actin cytoskeleton and drives terminal differentiation [54]. Additionally, in airway remodeling and development, intimate interactions between developing cells and the ECM not only aid in repair by regulating cell migration and proliferation but also promote the differentiation of the repairing cells to fully restore the wounded tissue [55].

DDR1 knockdown does not affect macroscopic compaction of the type I collagen hydrogel as compared to controls, however, significant changes were observed in the microstructural organization of type I collagen fibers. This implicates DDR1 in the organization of the disorganized collagen matrix around the cell interface as opposed to cooperative 'pulling', mechanical generation of traction forces between cells probably performed by the $\alpha2\beta1$ integrin [42]. The mechanical properties of the matrix that result from this cell/matrix interplay play an important role in the regulation of the actin cytoskeleton and promotion/inhibition of osteogenic signals [44,56-59]. The composition and structural properties of the ECM can be tuned on a cell by cell basis, uniquely biasing cells in close neighborhoods. In addition, the ECM can be tuned around an individual cell by the localized secretion of MMPs, degradation and assembly events. These specifically localized changes in the mechanical and structural nature of the ECM may drive the dynamic activation and deactivation of DDR1, changes in cell shape and the balance in signal transduction from various adhesion points. Geometric control of cell shape by changing ECM dynamics alters intracellular distances [60]. Spatial restriction, in this way, can force crosstalk between pathways that would not cross in a planar cell. DDR1 activation may therefore be a mechanism of signal balance. Given the added complexity in 3D and the non-linear nature of signaling networks, absolute quantities of signal activation may be less relevant than the dynamic integration and balance of multiple pathways. In this way the cell senses and integrates cues in a spatial and temporal manner to maintain and affect the critical balance in signals required for specific cellular function.

CONCLUSION

This work presents DDR1 as a potential mediator of MSC crosstalk with its 3D type I collagen matrix. Conflicting roles for downstream signaling molecules during differentiation in 3D and 2D environments implicates a role for unique mechanisms of signal transduction in response to matrix dynamics and mechanical forces. DDR1 binds to and organizes type I collagen fibers and in so doing activates a series of signals that regulate diverse cell behaviors from matrix remodeling, proliferation, migration and differentiation. Here we demonstrate that DDR1 is significantly upregulated in MSC encapsulated within a 3D type I collagen microenvironment and through crosstalk with traditional integrin mediated signaling, upregulates the osteogenic potential of MSC. DDR1, therefore, may play a significant role in the ability of MSC to sense and therefore respond to the dimensionality of their microenvironment enabling the development of physiological function. A mechanistic understanding of the interactions that occur at the cell surface interface, in 3D environments, is a prerequisite for biomaterial

development and the directed differentiation of MSC in engineered tissue constructs.

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REFERENCES

[1] Goldsmith EC, Borg TK. The dynamic interaction of the extracellular matrix in cardiac remodeling. J Card Fail 2002; 8(6 Suppl): S314-S318.

[2] Raeber GP, Lutolf MP, Hubbell JA. Mechanisms of 3-D migration and matrix remodeling of fibroblasts within artificial ECMs. Acta Biomater 2007; 3(5): 615-29.

[3] Caplan AI. Mesenchymal stem cells. J Orthop Res 1991; 9(5): 641-50.

[4] Dike LE, Chen CS, Mrksich M, *et al.* Geometric control of switching between growth, apoptosis, and differentiation during angiogenesis using micropatterned substrates. *In Vitro* Cell Dev Biol Anim 1999; 35(8): 441-8.

[5] Parker KK, Brock AL, Brangwynne C, *et al.*Directional control of lamellipodia extension by constraining cell shape and orienting cell tractional forces. FASEB J 2002; 16(10): 1195-204.

[6] Jiang X, Bruzewicz DA, Wong AP, *et al.* Directing cell migration with asymmetric micropatterns. Proc Natl Acad Sci USA 2005; 102(4): 975-8.

[7] Thery M, Racine V, Pepin A, Piel M, Chen Y, Sibarita JB, *et al*. The extracellular matrix guides the orientation of the cell division axis. Nat Cell Biol 2005;7(10):947-53.

[8] Ruiz SA, Chen CS. Emergence of patterned stem cell differentiation within multicellular structures. Stem Cells 2008; 26(11): 2921-7.

[9] McBeath R, Pirone DM, Nelson CM, *et al.* Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell 2004; 6(4): 483-95.

[10] Emerman JT, Pitelka DR. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In Vitro* 1977; 13(5): 316-28.

[11] Pedersen JA, Swartz MA. Mechanobiology in the third dimension. Ann Biomed Eng 2005; 33(11): 1469-90.

[12] Rhee S, Grinnell F. Fibroblast mechanics in 3D collagen matrices. Adv Drug Deliv Rev 2007 10; 59(13):1299-305.

[13] Salasznyk RM, Klees RF, Williams WA, *et al.* Focal adhesion kinase signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells. Exp Cell Res 2007 1; 313(1): 22-37.

[14] Martino MM, Mochizuki M, Rothenfluh DA, *et al*. Controlling integrin specificity and stem cell differentiation in 2D and 3D environments through regulation of fibronectin domain stability. Biomaterials 2009; 30(6): 1089-97.

[15] Cukierman E, Pankov R, Stevens DR, *et al*. Taking cell-matrix adhesions to the third dimension. Science 2001 23; 294(5547): 1708-12.

[16] Lukashev ME, Werb Z. ECM signalling: orchestrating cell behaviour and misbehaviour. Trends Cell Biol 1998; 8(11): 437-41.

[17] Vogel WF, Abdulhussein R, Ford CE. Sensing extracellular matrix: an update on discoidin domain receptor function. Cell Signal 2006; 18(8): 1108-16.

[18] Vogel W, Gish GD, Alves F, *et al.* The discoidin domain receptor tyrosine kinases are activated by collagen. Mol Cell 1997; 1(1): 13-23.

[19] Shrivastava A, Radziejewski C, Campbell E, *et al*. An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors. Mol Cell 1997; 1(1): 25-34.

[20] Salasznyk RM, Klees RF, Hughlock MK, *et al*. ERK signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells on collagen I and vitronectin. Cell Commun Adhes 2004; 11(5-6):137-53.

[21] Klees RF, Salasznyk RM, Kingsley K, *et al*. Laminin-5 induces osteogenic gene expression in human mesenchymal stem cells through an ERK-dependent pathway. Mol Biol Cell 2005; 16(2): 881-90.

[22] Lund AW, Stegemann JP, Plopper GE. Inhibition of ERK promotes collagen gel compaction and fibrillogenesis to amplify the osteogenesis of human mesenchymal stem cells in three dimensional, collagen I culture. Stem Cells Dev 2008.

[23] Chen TH, Chen WM, Hsu KH, *et al*. Sodium butyrate activates ERK to regulate differentiation of mesenchymal stem cells. Biochem Biophys Res Commun 2007; 355(4): 913-8.

[24] Constant VA, Gagnon A, Yarmo M, *et al*. The antiadipogenic effect of macrophage-conditioned medium depends on ERK1/2 activation. Metabolism 2008; 57(4): 465-72.

[25] Liao QC, Li YL, Qin YF, *et al*. Inhibition of adipocyte differentiation by phytoestrogen genistein through a potential downregulation of extracellular signal-regulated kinases 1/2 activity. J Cell Biochem 2008.

[26] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25(4): 402-8.

[27] Rhee S, Jiang H, Ho CH, *et al*. Microtubule function in fibroblast spreading is modulated according to the tension state of cell-matrix interactions. Proc Natl Acad Sci USA 2007; 104(13): 5425-30.

[28] Dudley DT, Pang L, Decker SJ, *et al*. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 1995; 92(17): 7686-9.

[29] Barker KT, Martindale JE, Mitchell PJ, *et al*. Expression patterns of the novel receptor-like tyrosine kinase, DDR, in human breast tumours. Oncogene 1995; 10(3): 569-75.

[30] Nemoto T, Ohashi K, Akashi T, *et al*. Overexpression of protein tyrosine kinases in human esophageal cancer. Pathobiology 1997; 65(4):195-203.

[31] Weiner HL, Huang H, Zagzag D, *et al*. Consistent and selective expression of the discoidin domain receptor-1 tyrosine kinase in human brain tumors. Neurosurgery 2000; 47(6): 1400-9.

[32] Park HS, Kim KR, Lee HJ, *et al*. Overexpression of discoidin domain receptor 1 increases the migration and invasion of hepatocellular carcinoma cells in association with matrix metalloproteinase. Oncol Rep 2007; 18(6):1435-41.

[33] Yoshida D, Teramoto A. Enhancement of pituitary adenoma cell invasion and adhesion is mediated by discoidin domain receptor-1. J Neurooncol 2007; 82(1): 29-40.

[34] Shimada K, Nakamura M, Ishida E, *et al.* Prostate cancer antigen-1 contributes to cell survival and invasion though discoidin receptor 1 in human prostate cancer. Cancer Sci 2008; 99(1): 39-45.

[35] Matsuyama W, Watanabe M, Shirahama Y, *et al*. Activation of discoidin domain receptor 1 on CD14-positive bronchoalveolar lavage fluid cells induces chemokine production in idiopathic pulmonary fibrosis. J Immunol 2005; 174(10): 6490-8.

[36] Matsuyama W, Watanabe M, Shirahama Y, *et al.* Suppression of discoidin domain receptor 1 by RNA interference attenuates lung inflammation. J Immunol 2006; 176(3):1928-36.

[37] Avivi-Green C, Singal M, Vogel WF. Discoidin domain receptor 1-deficient mice are resistant to bleomycin-induced lung fibrosis. Am J Respir Crit Care Med 2006; 174(4): 420-7.

[38] Hou G, Vogel W, Bendeck MP. The discoidin domain receptor tyrosine kinase DDR1 in arterial wound repair. J Clin Invest 2001; 107(6): 727-35.

[39] Faraci-Orf E, McFadden C, Vogel WF. DDR1 signaling is essential to sustain Stat5 function during lactogenesis. J Cell Biochem 2006; 97(1): 109-21.

[40] Vogel WF, Aszodi A, Alves F, *et al.* Discoidin domain receptor 1 tyrosine kinase has an essential role in mammary gland development. Mol Cell Biol 2001; 21(8): 2906-17.

[41] Salasznyk RM, Williams WA, Boskey A, *et al.* Adhesion to Vitronectin and Collagen I Promotes Osteogenic Differentiation of Human Mesenchymal Stem Cells. J Biomed Biotechnol 2004; 2004(1): 24-34.

[42] Heino J. The collagen receptor integrins have distinct ligand recognition and signaling functions. Matrix Biol 2000; 19(4): 319-23.

[43] Yeh YC, Wang CZ, Tang MJ. Discoidin domain receptor 1 activation suppresses alpha2beta1 integrin-dependent cell spreading through inhibition of Cdc42 activity. J Cell Physiol 2009; 218(1): 146-56.

[44] Engler AJ, Sen S, Sweeney HL, *et al.* Matrix elasticity directs stem cell lineage specification. Cell 2006; 126(4): 677-89.

[45] Hilton HN, Stanford PM, Harris J, *et al*. KIBRA interacts with discoidin domain receptor 1 to modulate collagen-induced signalling. Biochim Biophys Acta 2008; 1783(3): 383-93.

[46] Sundaram MV. RTK/Ras/MAPK Signaling. WormBook 2006; 1-19.

[47] Kretzschmar M, Liu F, Hata A, Doody J, *et al*. The TGF-beta family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. Genes Dev 1997; 11(8): 984-95.

[48] Aubin J, Davy A, Soriano P. *In vivo* convergence of BMP and MAPK signaling pathways: impact of differential Smad1 phosphorylation on development and homeostasis. Genes Dev 2004; 18(12):1482-94.

[49] Tibullo D, Giallongo C, La CP, *et al*. Effects of imatinib mesylate in osteoblastogenesis. Exp Hematol 2009; 37(4): 461-8.

[50] Day E, Waters B, Spiegel K, *et al*. Inhibition of collagen-induced discoidin domain receptor 1 and 2 activation by imatinib, nilotinib and dasatinib. Eur J Pharmacol 2008; 599(1-3): 44-53.

[51] Dewar AL, Farrugia AN, Condina MR, *et al*. Imatinib as a potential antiresorptive therapy for bone disease. Blood 2006; 107(11): 4334-7.

[52] Fierro F, Illmer T, Jing D, *et al*. Inhibition of platelet-derived growth factor receptorbeta by imatinib mesylate suppresses proliferation and alters differentiation of human mesenchymal stem cells *in vitro*. Cell Prolif 2007; 40(3): 355-66.

[53] Zhou X, Rowe RG, Hiraoka N, *et al*. Fibronectin fibrillogenesis regulates three-dimensional neovessel formation. Genes Dev 2008; 22(9):1231-43.

[54] Woods A, Wang G, Beier F. Regulation of chondrocyte differentiation by the actin cytoskeleton and adhesive interactions. J Cell Physiol 2007; 213(1):1-8.

[55] Coraux C, Roux J, Jolly T, *et al*. Epithelial cell-extracellular matrix interactions and stem cells in airway epithelial regeneration. Proc Am Thorac Soc 2008; 5(6): 689-94.

[56] Hinz B, Celetta G, Tomasek JJ, *et al*. Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. Mol Biol Cell 2001; 12(9): 2730-41.

[57] Tomasek JJ, Gabbiani G, Hinz B, *et al*. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 2002; 3(5): 349-63.

[58] Nakagawa S, Pawelek P, Grinnell F. Extracellular matrix organization modulates fibroblast growth and growth factor responsiveness. Exp Cell Res 1989; 182(2): 572-82.

[59] Wozniak MA, Desai R, Solski PA, *et al*. ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix. J Cell Biol 2003; 163(3): 583-95.

[60] Wang F, Weaver VM, Petersen OW, *et al*. Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. Proc Natl Acad Sci USA 1998; 95(25): 14821-6.

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