

Osteocyte differentiation is regulated by extracellular matrix stiffness and intercellular separation.

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Abstract

Osteocytes are terminally differentiated bone cells, derived from osteoblasts, which are vital for regulation of bone formation and resorption. ECM stiffness and cell seeding density have been shown to regulate osteoblast differentiation, but the precise cues that initiate osteoblast-osteocyte differentiation are not yet understood. In this study we cultured MC3T3-E1 cells on (A) substrates of different chemical composition and stiffness, as well as, (B) substrates of identical chemical composition but different stiffness. The effect of cell separation was investigated by seeding cells at different densities on each substrate. Cells were evaluated for morphology, alkaline phosphatase (ALP), matrix mineralisation, osteoblast specific genes (Type 1 collagen, Osteoblast specific factor (OSF-2)), and osteocyte specific proteins (dentin matrix protein 1 (DMP1), sclerostin (Sost)). We found that osteocyte differentiation (confirmed by dendritic morphology, mineralisation, reduced ALP, Col type 1 and OSF-2 and increased DMP1 and Sost expression) was significantly increased on soft collagen based substrates, at low seeding densities compared to cells on stiffer substrates or those plated at high seeding density. We propose that the physical nature of the ECM and the necessity for cells to establish a communication network contribute substantially to a concerted shift toward an osteocyte-like phenotype by osteoblasts in vitro.

1. Introduction

Osteocytes make up over 90% of cells in mature bone (Boukhechba et al., 2009). They play a vital role in skeletal health by acting as mechanosensors that monitor the mechanical environment within bone tissue and signalling to osteoblasts and osteoclasts to remodel the tissue so that bone strength is maintained throughout life (Klein-Nulend et al., 1995, Lanyon, 1993, Jee, 2001). Osteocytes are formed when osteoblasts undergo a dramatic phenotypic transition as they become embedded within newly deposited bone matrix. During this transition their morphology is altered from cuboidal to the dendritic shape associated with osteocytes, which is defined by a rounded cell body, a large nucleus, and long cell processes that extend from the cell body. These processes contact neighbouring cells both within and on the surface of the bone (Marotti et al., 1996, Palazzini et al., 1998, Jee, 2001), and thereby form functional syncytia by establishing gap junctional communication at these contact points (Donahue, 1998, Palazzini et al., 1998). The gene expression pattern of the cells also undergoes a dramatic change. Expression of the osteoblast marker enzyme alkaline phosphatase (ALP) is greatly reduced (Jee, 2001, Nakano et al., 2004), along with a reduction in Type I collagen (Col1T1), Bone morphogenetic protein 2 (BMP-2) and osteoblast specific factor 2 (OSF-2, also known as periostin) expression (Igarashi et al., 2002, Santos et al., 2011, Wilde et al., 2003). Expression of osteocalcin (Weinreb et al., 1990, Boivin et al., 1990) and E11 (Zhang K, 2006) is increased, while osteocyte specific markers such as PHEX (Westbroek et al., 2002), Sclerostin (Sost) (Poole et al., 2005) and dentin matrix protein 1 (DMP-1) (Feng, 2003, H.F. Rios, 2005) are either induced de-novo or dramatically upregulated.

Type 1 collagen and OSF-2 are early stage osteocyte markers, which are downregulated as osteoblasts begin to develop into osteocytes in vitro (Kato et al., 1997, Kato et al., 2001). DMP-1 is upregulated as the cells begin to extend processes and mineralise their surrounding

matrix, while Sost is a late stage osteocyte marker necessary for regulation of mineralisation (Atkins et al., 2009). The highly selective expression of molecules such as (ColT1) and OSF-2 in osteoblasts, and Sost and DMP-1 in osteocytes has led to their use as phenotypic markers in many studies (Kato et al., 2001, Gu et al., 2006, Gooi et al., 2010, Kramer et al., 2010, Krishnan et al., 2010). However, there remains very little understanding of the cues that control the phenotypic shift from osteoblasts to osteocytes.

Among the possible stimuli for the phenotypic shift from osteoblast to osteocyte are changes in the stiffness of the cell's extracellular matrix (ECM). ECM stiffness has been shown to strongly regulate a variety of cell behaviours such as migration, proliferation, and differentiation in both osteogenic and non-osteogenic cells (Pelham and Wang, 1997, Khatiwala et al., 2006b, Hsiong et al., 2008). It has been shown that MSC differentiation along different phenotypic lineages (i.e. adipogenic, myogenic, osteogenic) is dependent in part on substrate stiffness (Engler et al., 2006). In particular MSCs were shown to differentiate into osteoblast-like cells when cultured on collagen coated polyacrylamide substrates, with stiffnesses in the range of 25-40 kPa, whereas cells take on the characteristics of neurons or myoblasts when cultured on substrates with stiffness values similar to those of brain (≈ 1 kPa) and muscle tissue (≈ 11 kPa) respectively.

The separation distance of the cells, as controlled by cell seeding density, can also influence the differentiation of osteogenic cells during *in vitro* cell culture experiments. Cell seeding density plays a role in regulating osteoblast proliferation and matrix mineralisation in three-dimensional constructs *in-vitro* (Holy et al., 2000, Xiao et al., 2006), while the seeding density of human bone marrow stromal cells is an important factor for the development of cell matrix constructs for bone tissue engineering (Lode et al., 2008). Osteogenic differentiation of

MSCs in two dimensional culture, as examined by ALP and BMP-2 expression, has also been shown to be increased when cells are cultured at a low seeding density (3×10^4 cells/cm²) (Kim et al., 2009). To date, however research into the effects of both ECM stiffness and cell separation on osteogenic differentiation has focussed on osteoblast differentiation rather than the osteoblast-osteocyte transition. Osteocyte differentiation is necessary for mature bone tissue formation and as such an understanding of this stage of the differentiation pathway is crucial for the development of future tissue engineering strategies for bone.

In this study we test the hypothesis that osteocyte differentiation is regulated by both ECM stiffness and intercellular separation. To address this we employ in vitro culture of MC3T3-E1 cells to compare cellular differentiation on substrates of different chemical composition and stiffness, as well as on substrates of identical chemical composition but different stiffness. The effect of intercellular separation is investigated through the culture of cells at varied initial seeding densities on each of the chosen substrates. Osteocyte differentiation is then examined by quantifying cellular morphology, ALP activity and matrix mineralisation, as well as expression of the osteogenic genes Col1T1, OSF-2, DMP-1 and Sost.

2. Materials and Methods

Experiments were designed to test whether MC3T3-E1 (a pre-osteoblast cell line) cells would undergo phenotypic changes associated with osteoblast-osteocyte transition in vivo, under specific experimental conditions that investigated the role of ECM composition, ECM stiffness and cell seeding density. Morphological changes in MC3T3-E1 cells were assessed, paying particular attention to development of dendritic cells. ALP activity was quantified using a colorimetric assay, mineralisation was assessed using an alizarin red/cetylpyridinium chloride

assay while expression of Col Type I, OSF-2, DMP-1 and Sost gene's were quantified by RT-PCR.

2.1 Experimental Design: Substrate stiffness and seeding density experiments

Preliminary studies investigated the effect of a range of different substrates and seeding densities on MC3T3-E1 morphology. The results of these studies were used to select the experimental conditions outlined here. MC3T3-E1 cells were plated at seeding densities of 10^3 or 10^4 cells/cm² and cultured on: (a) NaOH neutralised collagen (Col), (b) NaOH neutralised collagen crosslinked with *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC) at 2.5 mM (ColEDAC1), (c) NaOH neutralised collagen crosslinked with EDAC at 12.5 mM (ColEDAC2), (d) Acetic acid neutralised collagen (ColAA), (e) Thin matrigel (MatThin), (d) Thick matrigel (MatThick) and (f) Uncoated tissue culture plastic (TC plastic). Cells were allowed to grow on all substrates for 1, 4, 9, 14 or 21 days respectively. Triplicate wells were analysed for each condition to quantify cell morphology, ALP expression, ECM mineralisation and gene expression. MLO-Y4 cells were cultured for 4 days on acetic acid neutralised collagen (ColAA) as a positive control (Kato et al., 1997).

2.2 Cell culture

Two bone cell lines were used in this study. MC3T3-E1 cells are a murine derived osteoblast cell line, which can express high amounts of ALP, type 1 collagen and OSF-2 (Hurley et al., 1993, Oshima et al., 2002). The cells are capable of differentiating into osteocytes and mineralising their surrounding matrix (Sudo et al., 1983). They are considered to be a good model of primary osteoblasts (Quarles et al., 1992). For these studies MC3T3-E1 cells were maintained in Alpha Modified Eagle's Medium (α -MEM) supplemented with 10% foetal bovine serum, 100 U/mL penicillin streptomycin and 100 ug/mL L-glutamine (all Sigma Aldrich) prior to all experiments.

MLO-Y4 cells are a murine derived cell line which share numerous characteristics with primary osteocytes such as high production of osteocalcin and E11 and low expression of alkaline phosphatase and OSF-2, as well as the extension of numerous dendritic processes per cell (Kato et al., 1997, Bonewald, 1999). MLO-Y4 cells were cultured at 5×10^3 cells/cm² on type 1 collagen at 0.15 mg/mL in acetic acid and cultured in Dulbecco's MEM supplemented with 5% foetal bovine serum, 5% foetal calf serum (Sigma Aldrich), 100 U/mL penicillin streptomycin and 100 ug/mL L-glutamine as recommended by Kato et al (Kato et al., 1997).

2.3 Preparation of ECM substrates

Type 1 rat tail collagen (Life Technologies) was neutralised with NaOH (Sigma Aldrich) at 18.4 μ M /g collagen, and diluted with 10% Phosphate Buffered Saline (PBS) (all Sigma Aldrich) and 68% distilled H₂O. The mixture was then pipetted in 150 μ L volumes onto 13 mm diameter coverslips (Sarstedt) and incubated for 30 minutes at 37°C, before being double rinsed with sterile PBS. This resulted in the formation of a soft, thick, gel like coating on the coverslips (Col). To produce substrates of different mechanical stiffness but identical ligand density, substrates were crosslinked with EDAC (Sigma Aldrich) by incubating at 19 μ M/mg collagen (ColEDAC1) or 95 μ M/mg collagen (ColEDAC2) EDAC for 3.5 hours at room temperature as described previously (Haugh et al., 2011). Substrates were then rinsed with PBS and incubated in fresh PBS for 3 hours at room temperature to remove any remaining EDAC before being washed twice with sterile distilled H₂O.

Type 1 rat tail collagen was also neutralized with acetic acid (Sigma Aldrich) at 2.34 μ M /mg collagen and incubated for 1 hour at room temperature to create a thin collagen coating (ColAA) of identical ligand density to the NaOH neutralized collagen substrates described

above. This mixture was pipetted onto 13 mm diameter coverslips in 150 μL volumes and allowed to incubate at room temperature for 60 minutes. Excess liquid was then removed and substrates were double rinsed with sterile PBS prior to cell plating.

Matrigel (Sigma Aldrich) was diluted 1:3 in α -MEM and plated on 13 mm coverslips in volumes of 200 μL . The coverslips were then incubated for either 30 minutes at 37°C or 1 minute at room temperature to create thick (1.5 mm - MatThick) and thin (40 μm - MatThin) substrates. Again substrates were washed twice with sterile PBS prior to cell plating.

2.4 Substrate stiffness measurement by Atomic Force Microscopy (AFM)

Substrate stiffness measurements were conducted using an Agilent 5500 Atomic Force Microscope (AFM). A pyrex-nitride pyramidal tip of face angle 30° was used. Force-distance curves were obtained from each of the following substrates; Col, ColEDAC1, ColEDAC2, ColAA and TC. These curves were then used to calculate the material stiffness according to equation 1,

$$F = \frac{E}{1-\nu^2} \frac{\tan \alpha}{\sqrt{2}} \delta^2 \quad (1)$$

where E is the Young's Modulus, ν is the Poisson's ratio, α is the face angle of the tip, δ is the penetration depth of the tip and F is the force measured at the AFM control. F is calculated through equation 2,

$$x = \frac{F}{k} \quad (2)$$

where k is the spring constant of the cantilever and x is the measured piezo movement. The spring constant, k , was calculated by finding the resonant frequency of the cantilever during testing (52 N/m). Using this equation substrate stiffness was measured five times at three

different locations on each substrate and the values averaged to generate a stiffness value for each measured substrate. The accuracy of the AFM methods used in this work was confirmed by testing a silicon sample of known stiffness of 1 MPa (shown in figure 2).

2.5 Morphological analysis of cell phenotype

Cultures were fixed using 4% paraformaldehyde (Fluka) in piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer (Sigma Aldrich) after 1, 4, 9 or 14 days of culture. Cells were permeabilised with Triton-X100 (Sigma Aldrich), diluted to 0.05% in PBS, and then incubated in phalloidin-TRITC according to the manufacturer's protocol (Life technologies) to stain the actin cytoskeleton. Cells were then counterstained with DAPI dilactate (Sigma Aldrich) and rinsed with PBS prior to being mounted in DPX mounting media (Sigma Aldrich) for imaging.

Images were taken using an Olympus IX50 inverted fluorescence microscope at different locations on the coverslips at 10X magnification, giving a 0.62 cm² image area. In total 15 images were taken at each time-point, for each condition (5 on each replicate coverslip). Cell processes were defined as cellular features located at the cell membrane that had a cross sectional diameter of no more than 1 µm and extended for a distance of at least 5 µm. Using this classification cell morphologies were quantified according as follows: (1) *Spread* cells had no cell processes and exhibited a spread morphology, (2) *Aligned* cells had no cell processes but exhibited preferential alignment in a particular direction (defined by a short axis to long axis ratio of less than 0.5), (3) *Dendritic* cells exhibited the small cell body and long thin cell processes associated with osteocytes (4) *Transitional* cells showed a spread morphology with processes extending from the cell body. Figure 1 shows an example of each assigned

morphological description. MLO-Y4 cells cultured for 4 days on acetic acid neutralized collagen were analysed according to the same classification system and used as a positive control.

2.6 Quantification of Cell number

Cell number was quantified through a Hoechst 33258 assay kit according to the manufacturer's protocol (Abcam). Hoechst fluorescently labels double-stranded DNA which can then be detected using a plate reader (Perkin Elmer 2030).

2.7 Quantification of ALP activity

ALP activity was analysed in cells plated on Col, CoEDAC1, CoEDAC2, and TC plastic. Col and CoEDAC1 were chosen as they had the highest percentage of dendritic cells by morphological analysis. CoEDAC2 was chosen as it is chemically identical to CoEDAC1. This allowed for the effect of substrate stiffness to be examined independent of chemical differences in the substrates. TC plastic was used as a control substrate. Intracellular ALP activity was measured using a colorimetric ALP assay (Sigma Aldrich) as described previously (Birmingham et al., 2012), at all four time-points, for all conditions. Briefly, cells were lysed by freezing at -80°C, thawing at room temperature and mechanically scraped from the substrate. The ALP assay uses *p*-nitrophenyl phosphate (*p*NPP), which changes its emission wavelength when dephosphorylated by ALP. The change in emission wavelength was measured at 540 nm on a plate reader (Perkin Elmer 2030). Results were then normalized to cell number as determined through the Hoescht assay outlined above. Cell culture media from the same substrates (Col, CoEDAC1, CoEDAC2, and TC plastic) was also analysed to quantify extracellular ALP production. Media was changed 24 hours before cell culture media was harvested and analysed

using the p-nitrophenyl phosphate assay described above. Blank media was used as a background control, to ensure that any phosphate or calcium in the media (140 mg/L sodium phosphate and 200 mg/L calcium chloride in α -MEM) did not influence the overall differences between groups. These experiments sought to analyse changes in ALP production over time as an indicator of osteoblast to osteocyte transition. It is known that immature MC3T3-E1 cells initially produce low levels of ALP, but as these cells differentiate into mature osteoblasts they upregulate ALP production and finally as they differentiate into osteocytes they decrease ALP production (Mikuni-Takagaki et al., 1995).

2.8 Mineralisation

Mineralisation of the extracellular matrix by MC3T3-E1 cells was analysed after 4, 9 and 14 days using an alizarin red/cetylpyridinium chloride assay (Sigma Aldrich), as described previously (Birmingham et al., 2012, Brennan et al., 2012, Stanford et al., 1995), on specific substrates (Co1, Co1EDAC1, Co1EDAC2, TC plastic). Media was removed and cells were rinsed twice in PBS before being incubated with a 2% alizarin red solution (Sigma Aldrich) for 20 minutes on an orbital rocker. The solution was removed and the substrates were washed three times in deionised H₂O to remove any unbound alizarin red. Cells were then incubated with 10% cetylpyridinium chloride solution (Sigma Aldrich) on an orbital rocker for 20 minutes at room temperature to disintegrate bound alizarin red. The absorbance of these samples at 562 nm was measured using a plate reader (Perkin Elmer 2030) to determine calcium deposition. MC3T3-E1 media was used as a control to ensure reported levels of calcium were due to cellular activity rather than media components (200 mg/L Calcium chloride in α -MEM).

2.9 Gene expression

Expression of osteoblast specific (Type 1 collagen, OSF-2) and osteocyte specific (DMP1, Sost) gene's was analysed by RT-PCR on specific substrates (Col, CoEDAC1, CoEDAC2) after 14 and 21 days of culture. These substrates were chosen based on the results of the morphological studies, as cells cultured on Col and CoEDAC1 at 10^3 cells/cm² had the highest levels of dendrite formation indicative of osteocyte differentiation, whereas cells cultured on CoEDAC2 and/or plated at 10^4 cells/cm² were predominantly spread indicative of osteoblast differentiation.

Cells were lysed using 1 mL TRI reagent before phase separation by the introduction of 200 μ L chloroform. RNA was precipitated using 70% ethanol and then washed using an ENZA RNA isolation kit according to the manufacturer's protocol (Omega Bio-tek) and dissolved in 30 μ L of RNase free water (Qiagen). The quality of the RNA was measured using a Nanodrop spectrometer (Thermo-scientific) before being converted to cDNA using a cDNA synthesis kit (Omega Biosciences) and Gene Amp 9700 A Thermal cycler (Applied Biosystems).

RT-PCR was performed on a Step-One plus analyser (Applied Biosystems) using Taqman probes (Applied Biosystems) for Type 1 collagen (Mm00801666_g1) and OSF-2 (Mm00450111_m1) on the cells harvested after 14 days of culture and DMP-1 (Mm00803833_g1) and Sost (Mm04208528_m1) on cells harvested after 21 days of culture. RT-PCR data was analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with GAPDH (Mm03302249_g1) as a housekeeping gene and all reactions were conducted in biological and technical triplicates.

2.10 Statistical Analysis

All experiments were conducted in biological triplicate (N=3). Cell morphologies were compared to the positive control (MLO-Y4 cells cultured on ColAA) and a negative control

(MC3T3-E1 cells cultured on TC plastic). Statistical significance of the effects of substrate stiffness and seeding density on cell morphology was determined by using a Welch's t-test to find the p-values of the difference between each mean. Pierce's criterion was used to determine statistical outliers in ALP and mineralisation data, while Student's t-test was used to determine statistical difference in ALP, mineralisation and gene expression experiments.

3. Results

3.1 AFM mechanical properties

TC plastic had a stiffness of 80.1 ± 4.8 MPa, while silicon, tested as a control of known stiffness, was measured as 1.18 ± 0.2 MPa. Col was the softest substrate with a stiffness of 9.7 ± 0.3 Pa. As expected substrate stiffness increased as a function of the concentration of crosslinking agent. CoEDAC1 and CoEDAC2 had stiffnesses of 286 ± 22.1 Pa and 957 ± 12.0 Pa respectively. ColAA had a similar stiffness to CoEDAC2 at 921 ± 9.6 Pa. All substrates had significantly different stiffnesses from one another with the exception of the CoEDAC2 and ColAA substrates, which did not show a significant difference ($p < 0.05$). These results are summarised in Figure 2. Matrigel is liquid at room temperature and so the stiffness of these two substrate could not be measured.

3.2 Morphological analysis of cell phenotype

After 14 days, 70.1% of MC3T3-E1 cells cultured on the TC plastic control were classed as spread with 24.5% extending cell processes. Cells cultured on the stiff (CoEDAC2) or intermediate (CoEDAC1) stiffness collagen based substrates at high initial seeding density showed a similar morphological pattern with approximately 56% of cells classed as spread and

31% exhibiting cell processes at both culture conditions. A lower percentage of spread cells (46.0%) was observed on the softest substrate at the higher seeding density of 10^4 cells/cm², while 36.8% of cells on these substrates extended cell processes. Cells cultured at the lower seeding density of 10^3 cells/cm² on CoIEDAC2 or TC plastic also predominantly exhibited a spread morphology with over 63% of cells being classed as spread and less than 28% extending cell processes under both culture conditions. In contrast, cells cultured on the softest (Col) or intermediate stiffness (CoIEDAC1) substrates at 10^3 cells/cm² were predominantly osteocyte-like with over 50% extending cell processes under both culture conditions, while less than 37% of the cells cultured under these conditions were classed as spread. 47% of the MLO-Y4 control cells were classed as spread while 48.6% extended cell processes. Sample images of MC3T3-E1 cells on Col and TC plastic are shown in Figure 5. MLO-Y4 cells were cultured for 4 days on ColAA as a positive control with 50.3 ± 2.7 % of these cells classed as dendritic, see Figure 3.

3.3 ALP activity of cells

Both intracellular and extracellular alkaline phosphatase activity increased continuously in MC3T3-E1 cells cultured on TC plastic and CoIEDAC2 for the duration of culture for both seeding densities, see Figures 5 and 6. All cells cultured at the higher seeding density of 10^4 cells/cm² on all substrates also showed an increase in ALP activity for the duration of culture. Similarly ALP expression increased significantly up to 9 days of culture in MC3T3-E1 cells cultured at a low seeding density (10^3 cells/cm²) on the two softest substrates (Col and CoIEDAC1). Expression in cells on these two substrates at the lower seeding density of 10^3 cells/cm² was then downregulated after 14 days of culture (although this change was not statistically significant).

3.4 Mineralisation

The results of the mineralization of the ECM over time caused by MC3T3's cultured on the different substrates are presented in Figure 7. No increase in mineral production over time by MC3T3-E1 cells on tissue culture plastic at either 10^3 or 10^4 cells/cm² was observed. Similarly, on the stiffer collagen substrate (CoEDAC2) with a seeding density of 10^3 cells/cm², no significant change in mineralisation over time was observed. However a significant increase was observed from day 4 to day 9 on this substrate with a seeding density of 10^4 cells/cm². The highest levels of mineralisation were observed on Col and CoEDAC1 when cells were cultured at 10^3 cells/cm². An increase in mineralisation over time was also observed in these culture conditions. An increase in mineralisation over time was also observed on CoEDAC1 with a seeding density of 10^4 cells/cm². However, levels of mineralisation under this condition were significantly lower than in either Col or CoEDAC1 with a seeding density of 10^3 cells/cm².

3.5 Gene Expression

The results of the gene expression of MC3T3-E1 cells are shown in Figure 8. MC3T3-E1 cells cultured on CoEDAC1 at 10^3 cells/cm² were chosen as the control from which to measure the relative expression of all genes examined. Type 1 collagen expression was lowest in cells cultured on CoEDAC1 at the lower seeding density. Low levels of type 1 collagen expression were also observed in cells cultured on Col and CoEDAC2 at the higher seeding density of 10^4 cells/cm², see Figure 8. OSF-2 was lowest in cells cultured on CoEDAC1 at 10^3 cells/cm², with a low level of expression also observed in cells cultured on the softest substrate (Col) at 10^3 cells/cm². DMP-1 expression after 21 days of culture was highest in cells cultured on

CoLEDAC1 at the 10^3 cells/cm², while Sost was detected in trace amounts in cells cultured on CoLEDAC1 at both seeding densities, but not in cells cultured on any other substrates.

4. Discussion

The results of this study show that MC3T3-E1 cells progress along the osteogenic lineage to become osteocyte-like cells when cultured on soft collagen substrates at low seeding density (10^3 cells/cm²), as indicated by phenotypic changes associated with early osteocytic differentiation (dendritic morphology, reduction in ALP expression, ECM mineralisation). Furthermore, downregulation of the osteoblast specific genes Col type 1 and OSF-2 as well as increased expression of DMP1, a gene upregulated during osteoblast to osteocyte differentiation, and the late stage osteocyte marker Sost can be induced in MC3T3-E1 cells through culture on collagen based substrates of approximately 286 Pa at an initial seeding density of 10^3 cells/cm². In contrast, cells cultured on stiffer collagen based substrates, or at a high initial cell seeding density proliferated and displayed the spread morphology, continually high ALP expression, low levels of ECM mineralisation, and high levels of Type 1 collagen and OSF-2 expression associated with the osteoblast phenotype. As a whole these findings show that the mechanical and compositional properties of the ECM, as well as the necessity for the cells to establish a communication network, contribute greatly to osteocyte differentiation.

The use of the cell lines MLO-Y4 and MC3T3-E1 is a possible limitation to this study. However, both cell lines have been shown to be excellent representatives of primary osteocytes and osteoblasts respectively (Sudo et al., 1983, Kato et al., 1997, Quarles et al., 1992, Bonewald, 1999). MLO-Y4 cells exhibit low levels of ALP activity, which does not change over time (Kato et al., 1997). Primary osteoblasts/osteocytes were not used because of difficulties in cell isolation

and characterisation (Hentunen, 2010) and the necessity for large cell numbers to satisfy the numerous experimental conditions and time points in our study design. Furthermore expression of Sost and DMP-1 are downregulated in primary osteocytes when cultured on non-mineralised collagen or fibronectin matrices (Yang, 2009), and for this reason they were deemed inappropriate for our collagen-based substrate experiments.

It should be noted that these cells did not express significant levels of Sost, which is a late stage osteocyte marker, involved in the regulation of bone formation. However, Sost expression has only previously been induced in vitro in the MC3T3-E1 cell line through the addition of osteogenic growth factors (D Mattinzoli, 2012, Uchihashi et al., 2013), and in vivo it is not expressed until after mineralisation has begun (Poole et al., 2005). Our results show trace amounts of Sost expression in cells cultured on the CoEDAC1 substrate, but not on any other substrate. Furthermore, increased DMP-1 expression has not previously been reported without the addition of osteogenic growth factors or direct application of mechanical strain (Krishnan et al., 2010, D Mattinzoli, 2012). The results presented here show a significant increase in DMP-1 expression and trace amounts of Sost when cells are cultured on soft collagen based substrates (286 Pa) at low initial seeding density (10^3 cells/cm²). These findings highlight the importance of substrate mechanical properties even in the absence of commonly used osteogenic factors.

Osteocyte differentiation has been induced in MC3T3-E1 cells through treatment with fibroblast growth factor 2 (FGF-2) (Gupta et al., 2010), or by culture of the cells on collagen based substrates in the presence of osteogenic factors (Uchihashi et al., 2013). Cell migration, focal adhesion formation as well as calcium deposition by MC3T3-E1 cells have been shown to depend on substrate stiffness (Khatiwala et al., 2006a). Furthermore, mineralisation by embryonic stem cells has been shown to increase on stiffer substrates (Evans et al., 2009), while

perhaps most interestingly, culture of pluripotent mesenchymal cells on a relatively rigid ECM substrate (40 kPa) favored osteoblastic differentiation, whereas culture on a more flexible substrate (1 kPa) led to a neural differentiation pathway (Engler et al., 2006). However, previous studies have only examined stem cell/osteoprogenitor to osteoblast differentiation and no study has monitored osteocyte differentiation as a function of ECM stiffness. In the current study we show for the first time the importance of ECM stiffness for regulating pre-osteoblast to early osteocyte transition. While osteoblast differentiation has previously been shown to be enhanced on stiffer substrates (20-40 kPa (Engler et al., 2006, Kong et al., 2005)), our studies suggest that early osteocyte differentiation is in fact governed by softer substrates (<300 Pa). In vivo osteocytes are formed when certain osteoblasts become embedded in newly formed osteoid, which is a soft, non-mineralised collagen matrix (Franz-Odenaal et al., 2006, Dallas and Bonewald, 2010), and simultaneously undergo the morphological changes associated with osteocyte differentiation (Doty, 1981). The cells begin to downregulate certain osteoblast specific genes, such as Type 1 collagen and OSF-2, while upregulation occurs in DMP-1 (Yang, 2004, H.F. Rios, 2005), and the osteocyte specific gene Sost (Yang, 2009, Winkler et al., 2003). It is interesting to note from the results of our study that the cells which displayed the traits of osteocyte differentiation were cultured on soft collagen based substrates. We propose that this substrate provides a similar extracellular mechanical environment to osteoid (Engler et al., 2006), and that this mechanical environment is necessary for osteoblast to osteocyte transition in vivo. However the precise mechanical properties of osteoid are unknown, as the tissue represents a thin layer approximately 350 nm deep (Engler et al., 2006), and as such there are significant challenges with obtaining samples for ex vivo mechanical testing. Applying methods such as nanoindentation to assess mechanical properties *in situ*, would be inappropriate as the stiffness of

a thin sample will be affected by the surrounding mineralised bone tissue (Oliver and Pharr, 1992).

Previous studies have established the importance of seeding density for differentiation of BMSCs into osteoblasts; ALP activity in rat bone marrow stromal cells was higher when cells were seeded at a lower initial seeding density of 3×10^4 per cm^2 , compared to 14.9×10^4 per cm^2 (Kim et al., 2009). Bone formation on implanted HA scaffolds, plated with goat bone marrow cells, was shown to be increased when cells were plated at higher seeding densities up to 47.8×10^6 cells/ cm^3 in osteogenic media for 7 days prior to implantation (Wilson et al., 2002). MG-63 osteosarcoma cells cultured at a low initial seeding densities in 3D collagen scaffolds showed significantly higher rates of ALP expression than those at higher seeding densities by 2 days (Bitar et al., 2007). The results presented here show similar changes in ALP expression over time due to initial cell seeding density, albeit that these previous studies were performed with different cell phenotypes, and also substrate stiffness and seeding densities were an order of magnitude larger than those used in this study.

It has already been widely hypothesised that osteocytes extend processes to facilitate intercellular communication (Donahue, 1998, Palazzini et al., 1998). It is intriguing to speculate that the combined effect of substrate stiffness and cell seeding density observed here is driven by the need for osteocytes to establish a communication network. The osteocyte network is akin to that of neurites and it has previously been reported that neurite formation increases on substrates in the range of 250-500 Pa (Georges, 2006, Estell, 2012). Therefore, we propose that the formation of dendrites is driven by ECM stiffness and intercellular separation across a range of phenotypes. Osteoblastic cells on stiffer substrates find it easier to proliferate allowing the formation of gap junctions between cell bodies without the need to establish cell processes.

(Yamaguchi et al., 1994) (Yellowley et al., 2000). However cells on softer substrates might be prevented from proliferating sufficiently due to the mechanics of the substrate, and after a time might resort to extending processes to establish a communication network with neighbouring cells.

Several candidate molecules are known to be involved in the osteoblast-osteocyte transition, in particular small GTPases including Rac, Rho and CDC42, which govern cellular spreading, migration and the extension of cellular processes (Huvneers and Danen, 2009)(Tanaka-Kamioka et al., 1998, Kamioka et al., 2007, McNamara et al., 2009). Loss of ALP activity, as observed in this study, involves not only a change in gene expression but also loss or inactivation of pre-existing enzyme in osteoblasts. Such losses could occur through shedding of membrane-derived vesicles or by enzymatic cleavage of the enzyme itself (Dean et al., 1996, Xie, 1995). Finally, diffusible signals may also play important roles in the osteoblast-osteocyte transition; it has recently been demonstrated that sustained stimulation of osteoblastic cells with the oncostatin-M, a member of the IL6 family, could induce a broad range of gene expression changes consistent with osteocyte formation in cultured osteoblasts (Brounais et al., 2008).

5. Conclusion

These experiments represent the first investigation into the effects of both substrate stiffness and cell seeding density on osteoblast-osteocyte transition and shed light on the external cues (substrate stiffness and intercellular separation) necessary for osteocyte differentiation. For the first time, pre-osteoblast MC3T3 cells have been induced to undergo osteocyte differentiation, without the addition of growth factors or application of mechanical loading, when cultured on

soft collagen based substrates provided intercellular separation necessitates process formation. Furthermore, for the cells to differentiate further along the osteogenic pathway, to the level of a early osteocyte, an optimal stiffness of approximately 286 Pa must be used. We propose that this simulates the in-vivo environment of the cells, where osteoblasts develop on osteoid, a soft collagen based matrix, and subsequently differentiate when they become embedded within this matrix. Knowing how the mechanical environment affects osteocyte development is a vital step in the recreation of viable bone tissue for implantation.

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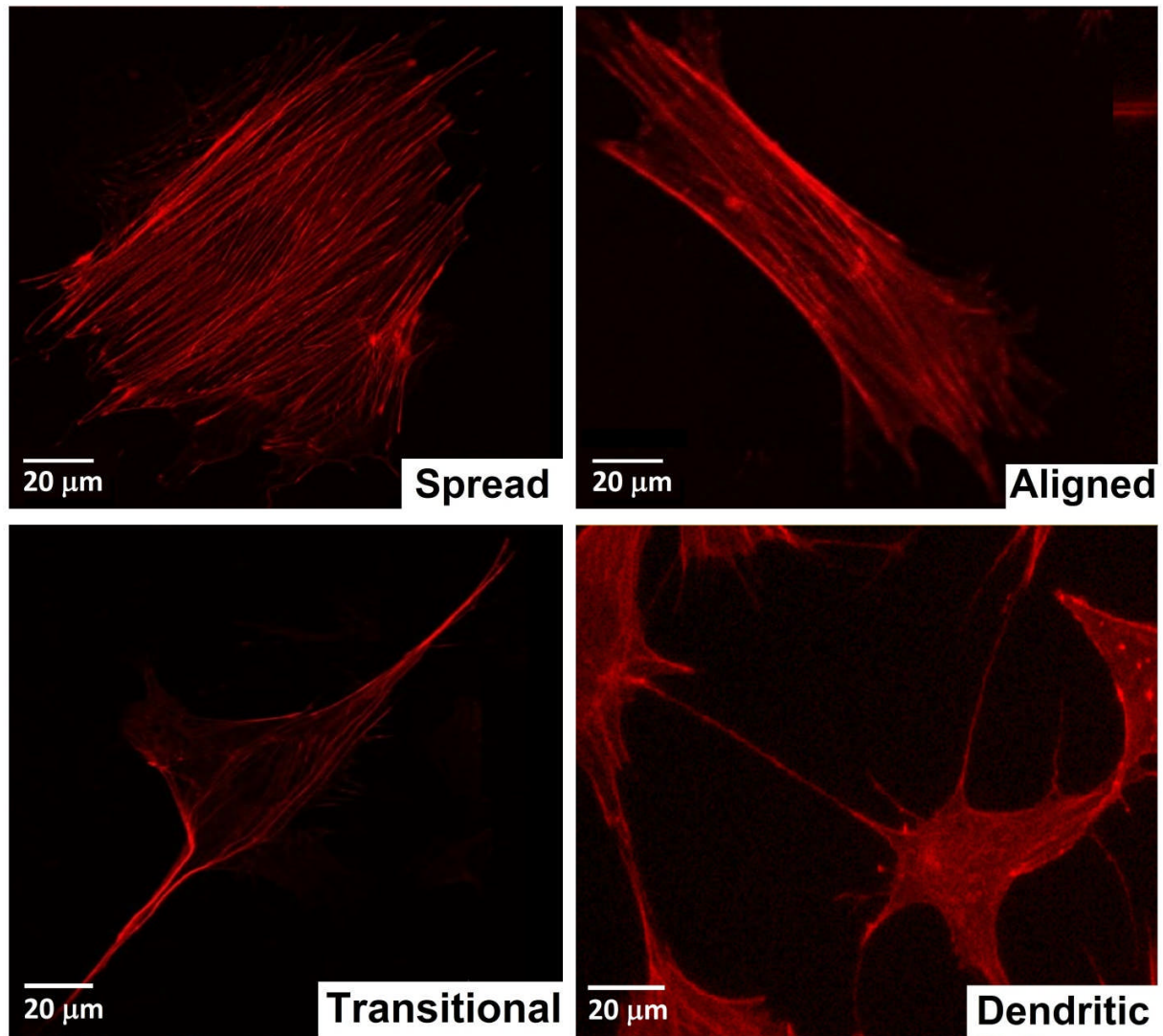


Figure 1: Examples of each morphology used to perform morphological analysis of the cells on each substrate. All cells were stained with rhodamine-phalloidin.

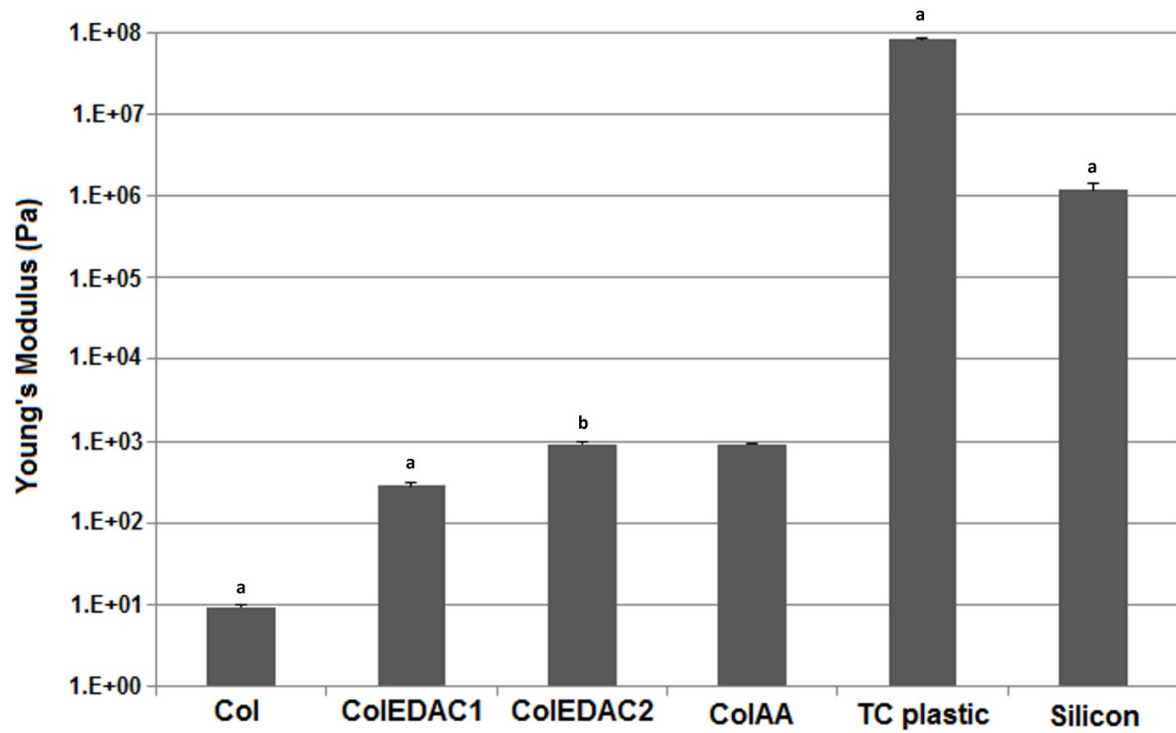


Figure 2: Substrate stiffness measurements by Atomic Force microscopy. Error bars indicate standard deviation of repeat measurements. **a** indicates statistical difference from all other values. **b** indicates statistical difference from Col, CoIEDAC1, TC plastic and Silicon.

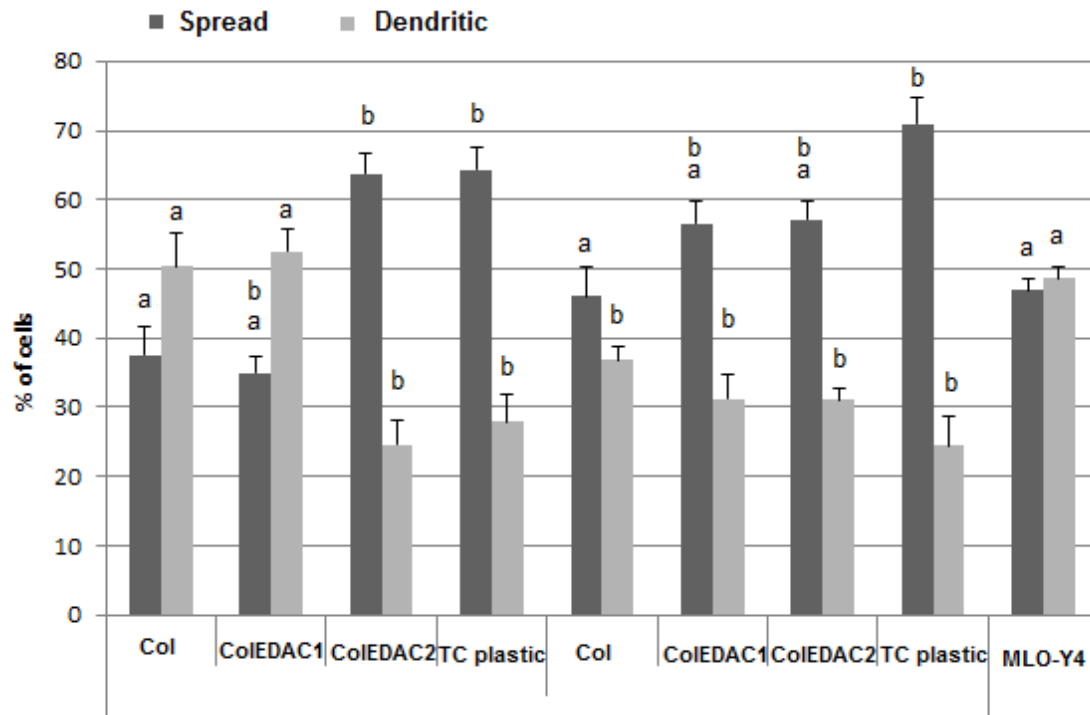


Figure 3: Percentage of cells displaying the dendritic morphology typical of osteocytes after 14 days of culture on each substrate at an initial seeding density of either 10^3 cells/cm² or 10^4 cells/cm². Error bars indicate standard deviation of repeat wells. **a** indicates statistical difference with negative control (MC3T3's on TC plastic at 10^4 cells/cm², $p < 0.01$). **b** indicates statistical difference with positive control (MLO-Y4's on ColAA at 10^4 cells/cm², $p < 0.01$).

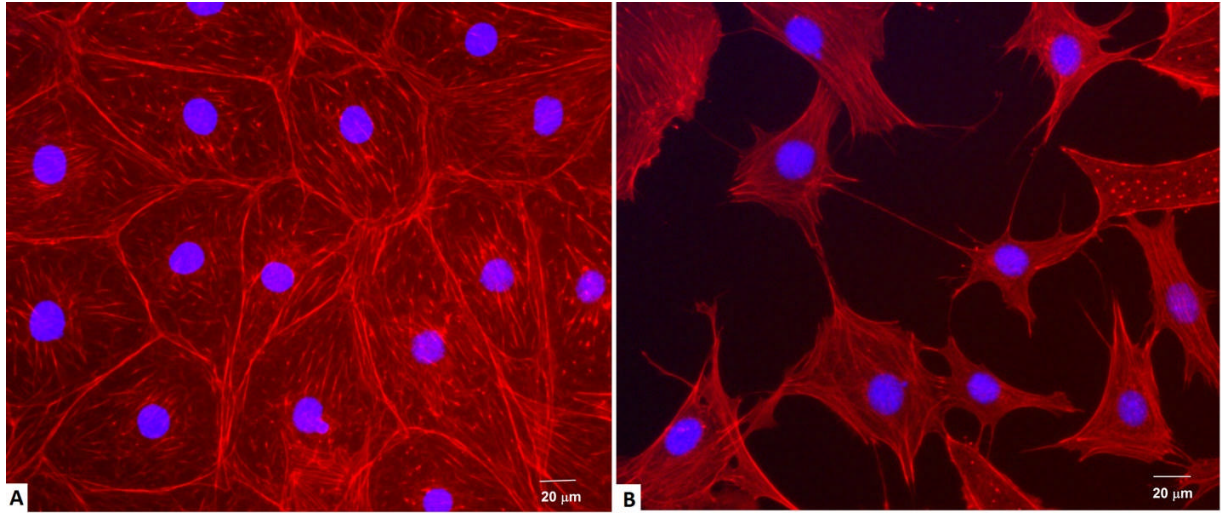


Figure 4: Sample morphologies from A) TC plastic at 10^4 cells/cm² for 14 days, B) Col at 10^3 cells/cm² for 14 days.

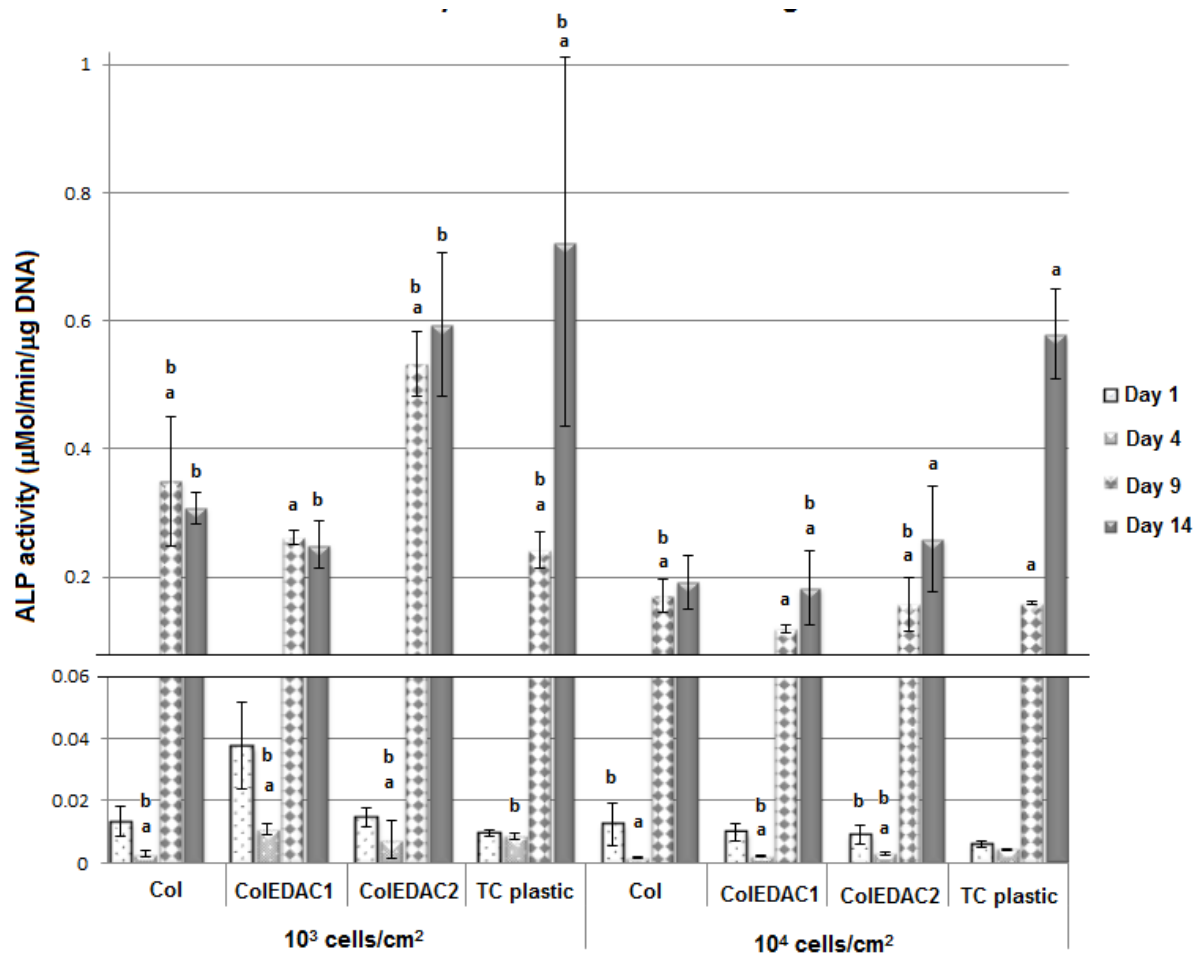


Figure 5: Extracellular ALP activity over time of MC3T3's on: Col, CoIEDAC1, CoIEDAC2, TC plastic, measured from media extracted from wells upon harvest. Error bars indicate standard deviation of repeat wells. **a** indicates statistical difference from previous timepoint of same condition ($p < 0.01$). **b** indicates statistical difference from control (MC3T3's on tissue culture plastic at 10^4 cells/cm²) and same timepoint ($p < 0.01$).

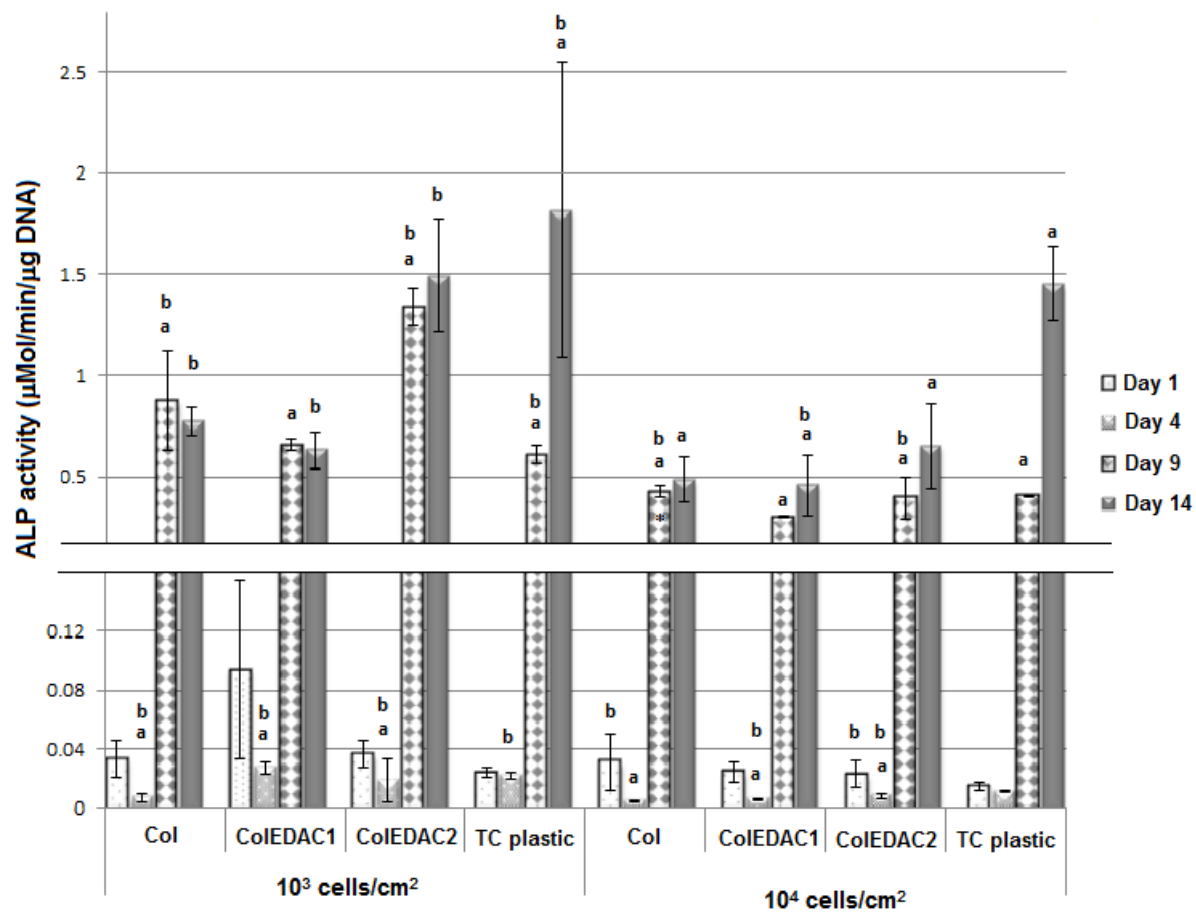


Figure 6: Intracellular ALP activity over time of MC3T3's on: Col, CoIEDAC1, CoIEDAC2, TC plastic. Error bars indicate standard deviation of repeat wells. * indicates statistical outlier. **a** indicates statistical difference from previous timepoint of same condition ($p < 0.01$). **b** indicates statistical difference from control (MC3T3's on tissue culture plastic at 10^4 cells/cm²) and same timepoint ($p < 0.01$).

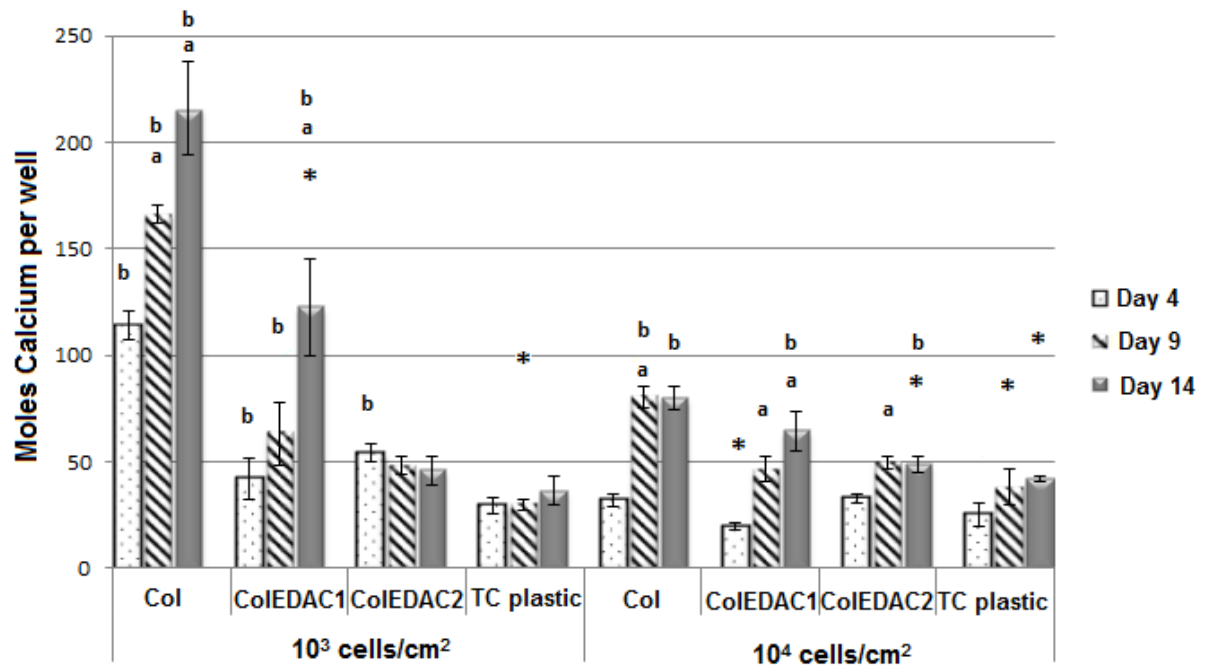


Figure 7: Mineralisation of ECM over time caused by MC3T3's cultured on: Col, CoIEDAC1, CoIEDAC2, TC. Error bars indicate standard deviation of repeat wells. * indicates outlier from statistical data. **a** indicates statistical difference from previous timepoint ($p < 0.05$). **b** indicates statistical difference from control (MC3T3's on tissue culture plastic at 10^4 cells/cm²) at same timepoint ($p < 0.05$).

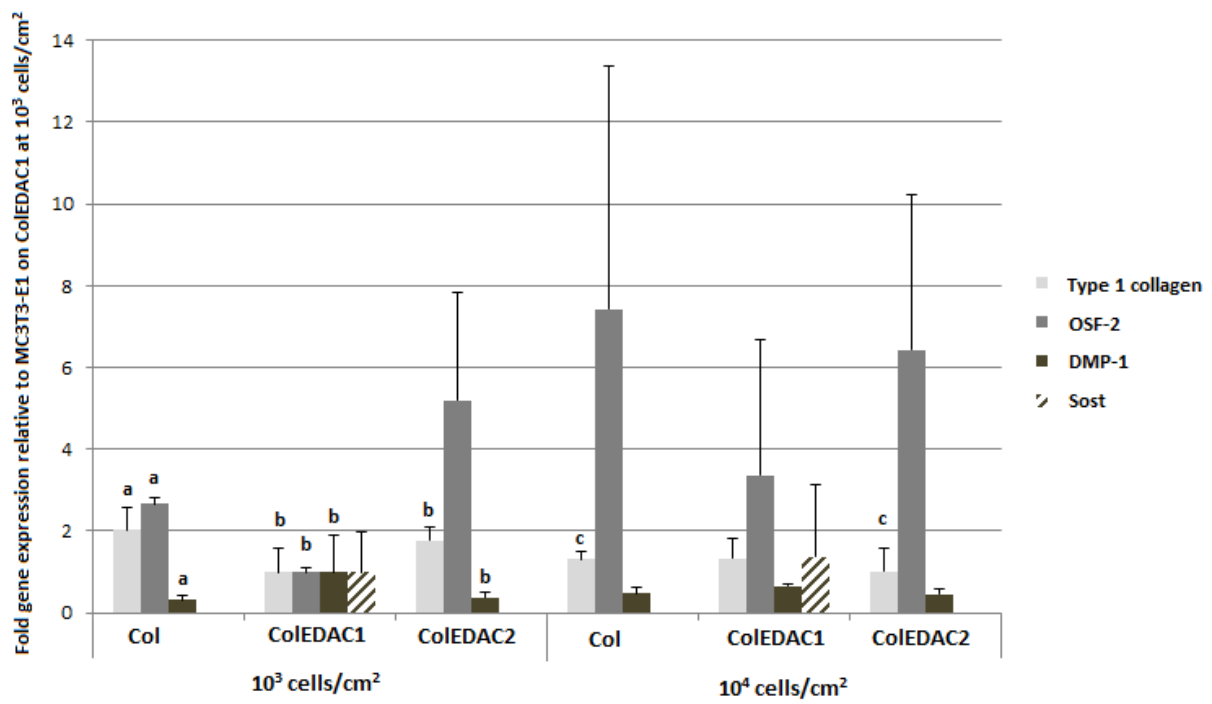


Figure 8: Gene expression in MC3T3's cultured on: Col, CoEDAC1, CoEDAC2 for 14 (type 1 collagen, OSF-2) or 21 (DMP-1, Sost) days. Error bars indicate standard deviation. **a** indicates statistical difference from Col at the same seeding density. **b** indicates statistical difference from CoEDAC1 at the same seeding density. **c** indicates statistical difference from 10³ cells/cm² seeding density on the same substrate ($p < 0.05$).

Highlights

- MC3T3-E1 cells cultured on collagen based substrates of different stiffness.
- Cells developed towards an osteocyte phenotype on soft collagen based substrates.
- Cells developed towards an osteocyte phenotype at low initial seeding density.