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SUBSTRATE STIFFNESS AFFECTS EARLY DIFFERENTIATION EVENTS IN EMBRYONIC STEM CELLS

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Abstract

Embryonic stem cells (ESC) are both a potential source of cells for tissue replacement therapies and an accessible tool to model early embryonic development. Chemical factors such as soluble growth factors and insoluble components of the extracellular matrix are known to affect the differentiation of murine ESCs. However, there is also evidence to suggest that undifferentiated cells can both sense the mechanical properties of their environment and differentiate accordingly. By growing ESCs on flexible polydimethylsiloxane substrates with varying stiffness, we tested the hypothesis that substrate stiffness can influence ESC differentiation. While cell attachment was unaffected by the stiffness of the growth substrate, cell spreading and cell growth were all increased as a function of substrate stiffness. Similarly, several genes expressed in the primitive streak during gastrulation and implicated in early mesendoderm differentiation, such as *Brachyury*, *Mixl1* and *Eomes*, were upregulated in cell cultures on stiffer compared to softer substrates. Finally, we demonstrated that osteogenic differentiation of ESCs was enhanced on stiff substrates compared to soft substrates, illustrating that the mechanical environment can play a role in both early and terminal ESC differentiation. Our results suggest a fundamental role for mechanosensing in mammalian development and illustrate that the mechanical environment should be taken into consideration when engineering implantable scaffolds or when producing therapeutically relevant cell populations *in vitro*.

Keywords: Embryonic stem cells; cellular mechanotransduction; gastrulation; extracellular matrix; differentiation; mammalian development.

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Introduction

Embryonic stem cells (ESCs) are pluripotent cells that can be isolated from the mammalian blastocyst and propagated in the laboratory indefinitely (Evans and Kaufman, 1981; Martin, 1981). These properties make them an exciting choice both as an accessible *in vitro* tool for studying the processes that control mammalian development and as a potential source of cells in regenerative medicine. Soluble growth factors – for example bone morphogenic proteins (Finley *et al*., 1999), activin (Sumi *et al*., 2008) and retinoic acid (Fraichard *et al*., 1995) – and insoluble proteins of the extracellular matrix (ECM) (Stevens and George, 2005; Takito and Al-Awqati, 2004) influence cell fate in both the developing embryo and ESCs, and have been used in attempts to generate clinically relevant cell populations. However, during embryogenesis cells are exposed not only to chemical signals but also to physical forces. As groups of cells divide and make morphological movements necessary for the formation of new tissue, they both generate and experience tension, compression and shear forces (Keller *et al*., 2003). Cells sense these forces through cell-cell adhesion molecules, such as cadherins, and cell-matrix adhesion molecules, such as integrins, and respond accordingly (Wang *et al*., 2009). Recent work has demonstrated that physical compression alone is sufficient to activate *Twist*, a gene involved in gastrulation in *Drosophila* embryos (Farge, 2003; Desprat *et al*., 2008).

Well-documented cellular responses to applied mechanical forces, however, only reveal a single aspect of what we are beginning to understand to be a complex system of mechanical cell sensing. Mammalian cells not only sense applied mechanical forces, but also sense the mechanical properties of their environment, such as the elasticity of the substrate on which they grow. Substrate stiffness influences how strongly cells adhere, how much force they exert and their degree of spreading. (Yeung *et al*., 2005; Goffin *et al*., 2006; Pelham and Wang, 1997; Discher *et al*., 2005). Cells also proliferate more quickly on stiff compared to soft substrates (Peyton *et al*., 2006) and DNA synthesis proceeds more rapidly in flattened, well-spread cells (Folkman and Moscona, 1978). Furthermore, substrate stiffness also has a fundamental effect on cellular differentiation. Engler *et al.* showed that mesenchymal stem cells (MSCs) plated on soft substrates (with stiffnesses comparable to brain tissue) differentiated preferentially into neurons, while those plated on substrates with stiffness similar to muscle and bone tissue differentiated into myocytes and osteoblasts respectively

(Engler *et al*., 2006). These effects may be related to the ability of a cell to spread on the substratum and form cytoskeletal stress fibres. MacBeath *et al.* have shown that MSCs prevented from spreading on small islands of ECM differentiate preferentially into adipocytes, while those allowed to spread on large islands differentiate readily to osteoblasts (McBeath *et al*., 2004). The effect of the mechanical environment on ESCs has been less well investigated, although there is evidence to suggest terminal differentiation in ESCs is affected by substrate compliance (Li *et al*., 2008). Earlier differentiation events remain uninvestigated.

In this study, we hypothesised that we could influence early differentiation events in ESCs by modulating substrate stiffness. We chose to pay particular attention to germ layer specification at gastrulation. Gastrulation is perhaps the first major morphological rearrangement of the early embryo and involves significant cell migration and tissue movement, and so we considered a role for cellular mechanosensing particularly important at this time. We synthesized substrates with varying stiffnesses using polydimethylsiloxane (PDMS), allowed ESCs to grow on them, and measured the expression of genes known to be involved in the process of gastrulation and germ layer formation. We also tested whether increasing stiffness of the substrate enhanced the terminal osteogenic differentiation of ESCs in the presence of osteogenic supplements.

Materials and Methods

Material synthesis

PDMS substrates of variable stiffnesses were fabricated using a SYLGARD 184 silicone elastomer kit (Dow Corning, Barry, UK) as per the manufacturer's instructions with crosslinker concentrations of 1, 3, 9, 17 and 23% (w/w). Mixed, degassed solutions were then poured into 6 well plates to a depth of at least 1 mm and cured at 70ºC for 24 hours. Plates were rinsed in 70% ethanol, air dried and treated with ammonia plasma for 2 minutes at 50 W using a Plasma Prep 5 plasma machine (GaLa Gabler Labor Instrumente, Bad Schwalbach, Germany). Type I collagen was covalently linked to the PDMS surface using N-Sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (Sulpho-SANPAH; Thermo Fisher Scientific, Loughborough, UK) by the method of Pelham and Wang (Pelham and Wang, 1997). Sulfo-SANPAH was first dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/µl, and then diluted in 50 mM HEPES (Sigma Aldrich, Dorset, U.K.), pH 8.5, to a final concentration of 0.5 mg/ml. Sulpho-SANPAH solution was used to cover the surface of each well in sterile conditions. Plates were then exposed to UV light from a transilluminator for 10 minutes from a distance of 10-30 cm. Excess sulpho-SANPAH solution was then removed and plates were subjected to a further 10 minutes exposure to UV light. Wells were then washed three times in sterile PBS and 1ml of a 50 µg/ml solution of type I collagen (BD Biosciences, Oxford, U.K.) was added to each well. Wells were incubated at 4ºC overnight. Collagen solution was then aspirated, each well was washed twice with sterile PBS and plates were stored at 4ºC for up to 2 weeks.

Substrate surface contact angle measurements

Static water contact angle of the PDMS substrate surface was measured with an Easydrop Drop Shape Analysis System (Krüss Surface Science, Hamburg, Germany) before and after the collagen coating. A 5 µl drop of pure water was placed on the substrate surface and photographed. The shape of the drop was then analysed using a sessile drop fitting model. For each PDMS substrate, the measurements were performed on five different areas of the surface and the values were averaged.

Substrate surface Young's modulus measurements

The elastic properties of PDMS surfaces and mouse calvarium and liver (control adult tissues) were measured by atomic force microscopy (AFM). A PicoForce AFM with a NanoV controller (Veeco Instruments Inc., Cambridge, UK) was used for force measurement. A silicon probe (FESP type, also from Veeco) with cantilever spring constant of 4.5 N/m for PDMS substrates, and 78 N/m and 0.35 N/m for calvarium and liver samples respectively, was modified by attachment of a 20.3 ± 1.4 µm diameter glass sphere (Borosilicate Glass Microspheres, Duke Scientific Co., Palo Alto, CA, USA) to the end of a cantilever with Loctite (Henkel, Düsseldorf, Germany) 350 UV adhesive. Force measurements on each PDMS sample were conducted as a 5 × 5 point matrix with an even separation of 10 µm. The Young's modulus, E, was calculated by averaging all 25 retracting force curves based on the Hertz model with the assumption that the glass sphere possesses an infinite E in comparison to the sample surfaces.

Cell culture and tissue isolation

TG2α E14 embryonic stem cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 2 mM L-glutamine (Invitrogen, Paisley, UK), 100 µM β-mercaptoethanol (Sigma, Dorset, UK) and 1000 U/ml leukaemia inhibitory factor (LIF), a growth factor used for the propagation of undifferentiated ESCs (Chemicon, Chandler's Ford, UK). Cells were fed every day and were passaged every 3-4 days at a density of 3000-6000/cm^2. For differentiation experiments, undifferentiated ESCs were dissociated to single cells, and plated at a density of 30 000 cells/cm^2 (unless stated otherwise) in alpha minimal essential medium (αMEM) supplemented with 15% (v/v) FBS without LIF. Cells were fed at day 2, then twice each day from day 3 to day 6 and once per day thereafter up to 10 days. Embryoid bodies (EBs) were made by partially dissociating ESCs to clumps containing around 15-30 cells and transferring them to non-adhesive bacteria-grade petri dishes. EBs were fed every 2 days.

Calvarium and liver samples were harvested from a 6-week-old FVB/N female mouse, which was sacrificed in accordance with Imperial College London ethical guidelines. A scalpel blade was used to gently scrape away

as much soft tissue as possible from the surface of harvested calvarium. Fresh liver and calvarium were frozen slowly in cell culture medium supplemented with FBS and DMSO. Samples were thawed at room temperature prior to testing.

Adhesion and measurements of cell perimeter

Cells were plated at 100 000 cells/well, in 12-well plates. 24 hours post-plating cells were washed twice in PBS, fixed for 20 minutes with 70% (v/v) ethanol, and then washed twice in PBS. Fixed cells were stained with 0.5% (w/v) crystal violet for 10 minutes and dye was then extracted from cells with 0.1M citric acid. Absorbance was measured at 550 nm on an absorbance spectrometer (Anthos Labtec Instruments, Wals/Salzburg, Austria). Standard curves were created for known numbers of cells on fibronectin coated plates.

For measurements of cell perimeter, cultures of cells growing on each of the substrates were imaged and captured using an inverted phase contrast microscope (Olympus IX51, London, U.K.) equipped with DP Controller software. Three separate images of representative areas of each well were examined using ImageJ software (Freeware, available at http://rsb.info.nih.gov/ij/) and single cells, identified by definite cell boundaries and single nuclei, were identified. Cell perimeter was measured by tracing the border of ten cells per image and measuring the border using the 'Perimeter' function of ImageJ.

Cellular DNA quantification

At indicated time-points ESCs were pelleted and incubated overnight with shaking at 56°C in 50 μg/ml Proteinase K solution (Sigma). After enzyme inactivation at 90°C for 10 minutes, Hoechst 33258 (Sigma) was added to a final concentration of 1 μg/ml and fluorescence emission was then measured at 450 nm under excitation at 350 nm on a fluorescence plate reader (MFX, Dynex Technologies, Chantilly, VA, USA). Aliquots containing known numbers of cells were pelleted and treated as above to obtain a standard curve for calculation of cell number from absorbance values. Measurements were performed in triplicate.

Phalloidin staining of cells

At 2 and 24 hours following seeding, ESCs were fixed for 20 minutes with 4% (w/v) paraformaldehyde. Cells were then washed and incubated with 0.2% (v/v) Triton X-100 (Sigma) for 45 minutes. Cells were washed twice in PBS and then incubated for 30 minutes in 0.1% (w/v) BSA. Cells were then covered with alexa-488-conjugated phalloidin (Invitrogen) at a concentration of 5 U/ml in PBS containing 0.1% (w/v) BSA for 20 minutes according to the manufacturer's instructions. Cells were then washed twice in PBS and imaged with an upright fluorescence microscope (Olympus BX-60).

Determination of cellular proteins using Western blot

Cells were seeded on substrates at 600 000 cells/cm^2 in growth medium and were incubated for 2.5 hours. The cells were lysed for 10 minutes on ice in Radio Immunoprecipitation Buffer (RIPA; Sigma) supplemented with phosphatase (Sigma) and protease (BD Biosciences) inhibitors. Lysates were centrifuged at 12 000 g for 10 minutes and the soluble fraction was stored. 20 μg total protein were separated in a 10% (w/v) SDS-polyacrylamide gel and electroblotted onto a polyvinylidiene difluoride membrane (Bio-Rad, Hertfordshire, UK). The membrane was blocked in 5% (w/v) BSA for one hour at room temperature and then incubated in 1/1000 dilutions of primary antibodies (anti-focal adhesion kinase (FAK) or anti-phosphorylated FAK (pFAK), Abcam, Cambridge, U.K.) overnight at 4°C with gentle agitation. The membranes were washed and then incubated in secondary antibodies conjugated with horseradish peroxidise (Bio-Rad) for 1 hour. Chemiluminescence substrate (Thermo Fisher Scientific, Loughborough, UK) was applied and protein was visualised with Syngene Dyversity CCD image analyse (Syngene, Cambridge, U.K.). Alpha-tubulin (Active Motif, Rixensart, Belgium) was used as a control at 1/5000 dilution. Western blot was repeated on three independent samples.

Determination of cellular gene expression using quantitative PCR

At indicated time-points, cultures of ESCs were pelleted and snap frozen in liquid nitrogen. RNA was isolated from cell pellets with an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sensimix OneStep kit (Quantace, London, U.K.) was used to perform reverse transcription and PCR on RNA in a single step, using SYBR green as a fluorescent dye to detect amplified double-stranded DNA. Thermal cycling and fluorescence detection was performed using a Corbett Rotorgene 6000 (Qiagen). Primers to *Gapdh, Nanog*, *Fgf5*, *Brachyury*, *Foxa2*, *Sox1*, *Eomes*, *Mixl1*, *Twist1, Gata6, Cdh1*, *Cdh2, Runx2 and Spp1* were designed using Primer Bank (http://pga.mgh.harvard.edu/primerbank/) and sequences and cycling conditions are listed in Supplementary Table 1. Serial 1:5 dilutions of isolated RNA were used to plot standard curves for each primer pair and therefore calculate efficiency. Quantification of gene expression was performed only in the linear range of each primer pair. The ΔΔCT method (Livak and Schmittgen, 2001) was used to quantify changes in the expression of each gene of interest between samples, using the housekeeping gene *Gapdh* as the normaliser.

Osteogenic differentiation

Undifferentiated ESCs were plated as described in 'Cell Culture' but were incubated in αMEM supplemented with 15% (v/v) FBS, 280 μM ascorbate, 10 mM β-glycerophosphate and 1 μM dexamethasone (Sigma). Medium was changed every day up to day 6 and every 2-3 days thereafter for a period of 28 days. At day 28, cultures were fixed for 20 minutes in 10% (v/v) formalin buffered saline. Cells were then washed twice in PBS and 0.5 ml 1% (w/v) alizarin red S (Sigma), pH 4.2 was added to each well for 10 minutes. Cultures were then washed thoroughly in running tap water until no further red stain

was released from the cell layers, and were then left to air dry. Fixed cultures were then examined with an inverted fluorescence microscope (510-560 nm excitation, >590 nm emission). Representative images were taken and discrete, fluorescing areas were counted manually in each field of view.

To confirm the presence of calcium, differentiated cultures of ESCs were also stained for calcium using the von Kossa method. Briefly, cultures were washed in PBS, covered with a solution of 0.3 M $AgNO_3$ and exposed to natural light for 1 hour. Cells were then washed twice in distilled water and covered with 0.33 M $Na_2S_2O_3$ for 5 minutes. Cultures were washed again and stained with nuclear fast red. Cultures were imaged under an inverted light microscope.

Statistical analysis

Results are expressed as mean ± standard deviation. For each result, four separate, independent experiments were performed for each group unless otherwise stated (n=4). All results were compared using a Student's *t*-test or one-way ANOVA with a post-hoc Tukey test. Pearson's correlation was used to test for significant linear relationships between substrate stiffness and gene expression. Significance was assumed when $p < 0.05$.

Results

Surface characterisation

We created substrates suitable for cell culture with a range of stiffnesses using PDMS. Prior to surface treatment all PDMS surfaces were hydrophobic, with water droplet contact angles of around 120º. Following plasma treatment and covalent linkage of collagen to the surfaces, water droplet contact angle was reduced to < 90º in all cases (Fig. 1A). There was no significant difference in contact angle between any of the surfaces, but contact angle was more variable on the softest PDMS substrate. Surfaces stored for up to 2 weeks did not show any change in hydrophilicity. There was no significant difference between the amount of collagen crosslinked onto each PDMS surface, but the amount of collagen on TCP was significantly higher ($p < 0.05$; Supplementary Fig. 1). Indentation measurements of collagen-coated PDMS substrates by AFM yielded values of 0.041 ± 0.005; 0.26 ± 0.06; 1.86 ± 0.14; 2.7 ± 0.6; and 2.33 ± 0.15 MPa for 1%, 3%, 9%, 17% and 23% concentrations of crosslinker respectively (Fig. 1B). All groups were significantly different from each other with $p < 0.01$. Substrates will be referred to hereafter by their measured Young's moduli (0.041 MPa, 0.26 MPa, 1.9 MPa, 2.3 MPa and 2.7 MPa). Surface Young's moduli of calvaria and liver were measured at 0.15 ± 0.04 MPa and 0.018 ± 0.015 MPa respectively. Growth media incubated either with PDMS substrates or TCP contained less than 2 ppm Si, as measured by ICP, indicating negligible leaching of elastomer or crosslinker into the growth medium (not shown).

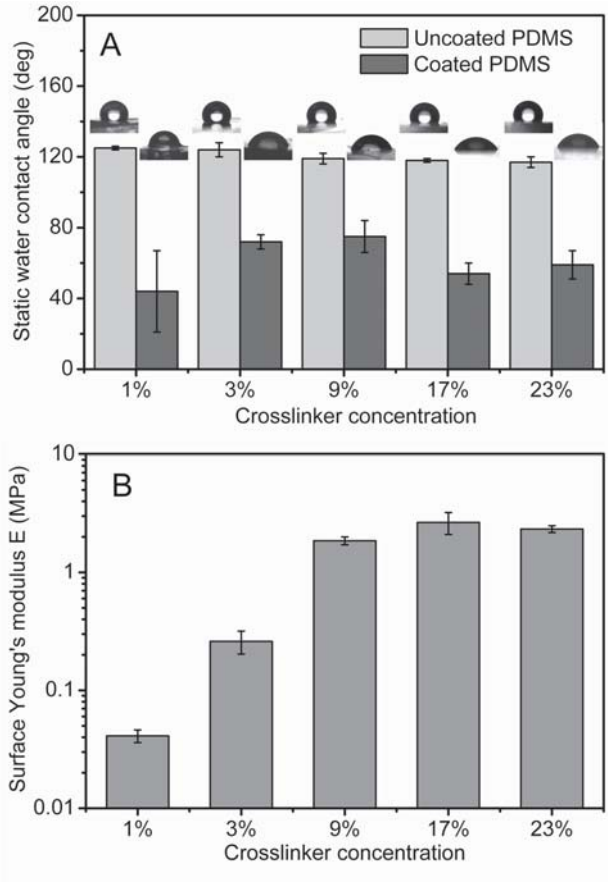

Figure 1. Surface characterization of PDMS substrates. (A) Covalent binding of type I collagen to PDMS substrates significantly increased surface wettability of all substrates, while there were no significant differences between substrates, as measured by static water contact angle. (B) Surface Young's moduli (*E*) measured using AFM demonstrated that 1% PDMS < 3% < 9% < 23% < 17% for *E*.

Cell attachment and cell proliferation

We next compared cell attachment and cell spreading on all surfaces using crystal violet and phalloidin staining, respectively. After 24 hours, cell attachment was greatest on the softest PDMS (0.041 MPa), although this was only significant compared to on 1.86 MPa PDMS ($p = 0.03$) (Fig. 2A). Cells exhibited similar morphology on all surfaces, with a mixture of stellate, rounded and bipolar morphologies (Fig. 2B). Quantitative measurements of cell perimeter showed no significant differences between the 3 stiffest substrates (1.9-2.7 MPa) and TCP. However, cell perimeter on the softest PDMS (0.041 MPa) was significantly lower than on the second softest PDMS (0.26 MPa). Furthermore, cell perimeter values on the two softest substrates were significantly lower than on all other surfaces ($p < 0.01$) (Fig. 2C).

Phalloidin staining of cytoskeletal actin after 2 hours showed plated cells had a poorly defined actin cytoskeleton on all substrates, with little evidence of stress fibre formation (Fig. 2D). Most staining was evident at the periphery of the cells. Cells appeared marginally more well spread on stiffer compared to soft substrates (Fig. 2D). At 24 hours, cytoskeletal stress fibres were more prominent

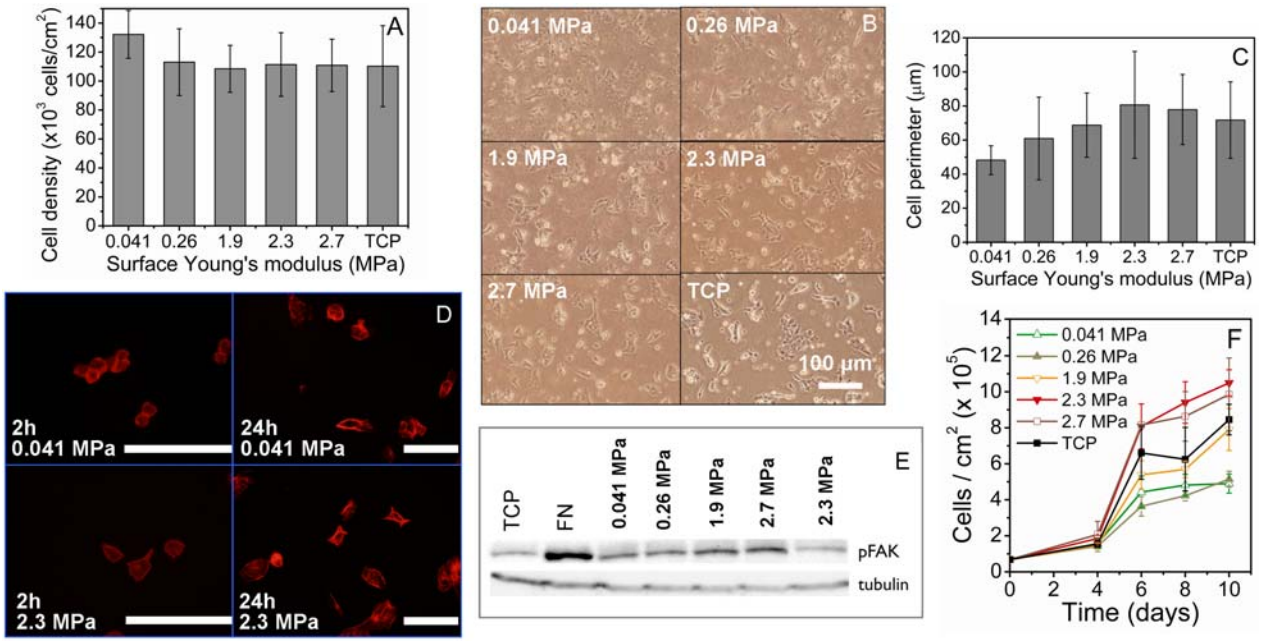

Figure 2. Effects of PDMS substrate stiffness on cell attachment and cell spreading. **(A)** Cell attachment measured after 24 hours in the presence of serum on PDMS substrates and TCP. Results are expressed as means of 3 experiments. Attachment was only significantly different between 0.041 MPa and 1.9 MPa PDMS ($p = 0.03$). **(B)** Phase contrast micrographs of cells on PDMS substrates and TCP after 24 hours. **(C)** Cell perimeter of cells adherent on PDMS and TCP surfaces after 24 hours. Results are representative of 3 experiments, with 10 single cells measured in 3 separate representative images. Cell perimeter on 0.041 MPa PDMS was significantly lower than on 0.26 MPa PDMS, and was lower on the two softest substrates than on any other substrate ($p < 0.01$). There were no significant differences between other substrates. **(D)** Phalloidin staining of intracellular actin (red) in cells cultured for 2 and 24 hours on 0.041 and 2.3 MPa PDMS substrates. White bars illustrate 100 µm. **(E)** Western blots for pFAK in lysates of cells adherent on PDMS substrates, fibronectin (FN) and TCP after 1 hour. α-tubulin was also measured as a housekeeping control. (F) Total cell number per well in cultures of differentiating ESCs as a function of time was estimated by measurement of DNA concentration in protease-digested cell isolates.

on all surfaces, with the greatest visible formation of stress fibres on 2.3 MPa PDMS and TCP (Fig. 2D).

Measurement of pFAK by Western blot revealed that levels of this protein were similar on all PDMS substrates, but with slightly more protein detected in cells grown on substrates with Young's moduli of 1.9 and 2.7 MPa than in cells grown on substrates with Young's moduli of 0.04, 0.26 and 2.3 MPa, or TCP (Fig. 2E). pFAK was highest on fibronectin, an ECM protein that promotes cell attachment. We next investigated whether there were any substrate-dependent differences in cell proliferation rates as measured by total DNA quantification. By day 4, there was no significant difference in cell number between any of the surfaces, but by day 6 there were significantly more cells on stiffer substrates (1.9 – 2.7 MPa) than on soft substrates (0.041 and 0.26 MPa; $p < 0.01$; Fig. 2F).

Time course of gene expression

We next investigated the time course of gene expression of several genes expressed in early mammalian development. As expected, the expression of *Nanog* (a marker of the inner cell mass and undifferentiated embryonic stem cells) decreased following withdrawal of LIF and remained low on all substrates. Expression levels did not differ significantly between substrates, but *Nanog* expression was significantly lower in EBs compared to on any substrate at day 8 and day 10 ($p < 0.01$) (Fig. 3A). The expression of *Fgf5*, a gene that is expressed in the primitive ectoderm of the developing mouse embryo but not in the inner cell mass or ESCs (Haub and Goldfarb, 1991) increased significantly by day 4, peaking at this time-point in cells on softer compared to stiffer PDMS substrates. *Fgf5* expression in cells on PDMS substrates with Young's moduli of 2.3 and 2.7 MPa and TCP was significantly higher than on other substrates and was sustained for a longer time period, with similar expression levels at day 8, before a decline. In EBs *Fgf5* expression peaked at day 6 and was significantly higher at this time-point than in cells plated on any other substrate ($p < 0.01$; Fig. 3B). Expression of *Brachyury*, a gene involved in gastrulation and the formation of posterior mesoderm (Beddington *et al*., 1992; Wilson *et al*., 1995), peaked at day 6 in cells on all substrates and in EBs. *Brachyury* expression at this time point was lower on the softest PDMS than on any other substrate ($p < 0.01$) and increased in relation to substrate stiffness, with the highest expression on TCP. *Brachyury* expression however, was greatest in EBs, being a factor of 2.6 above that on TCP ($p < 0.001$; Fig. 3C). Expression of *Foxa2*, a marker expressed in the anterior primitive streak during gastrulation, and predominantly in definitive endoderm (Sasaki and Hogan, 1993), increased in cells on all substrates from before day 4 to day 10. Expression

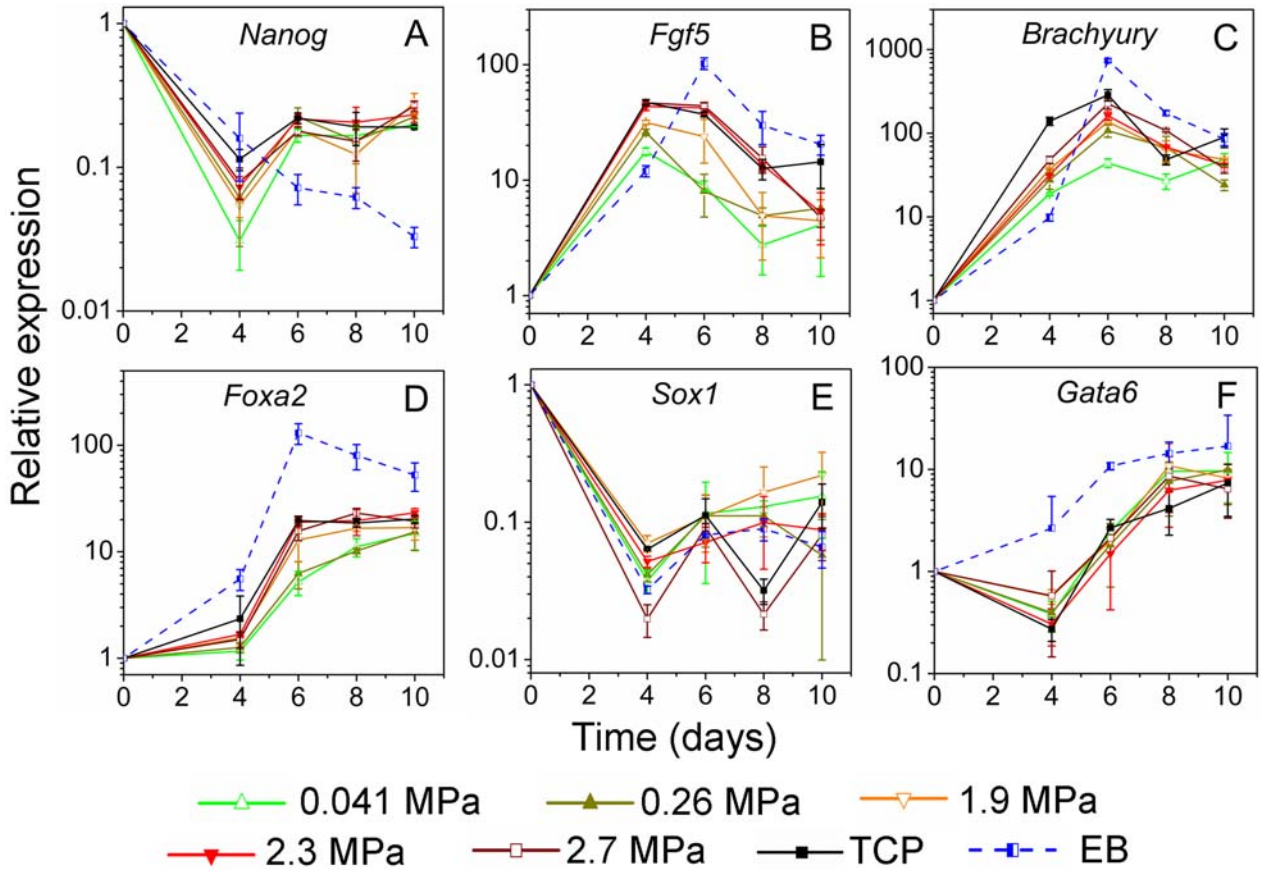

Figure 3. Time-course relative expression of genes involved in pluripotency (*Nanog* **[A]**), primitive ectoderm differentiation (*Fgf5* **[B]**), posterior primitive streak and mesendoderm (*Brachyury* **[C]**), anterior primitive streak and mesendoderm (*Foxa2* **[D]**), neuroepithelium (*Sox1* **[E])**, and primitive endoderm (*Gata6* **[F]**). Expression of all genes was normalised to the housekeeper, *Gapdh*, and results are expressed as relative increase compared to day 0 ESCs.

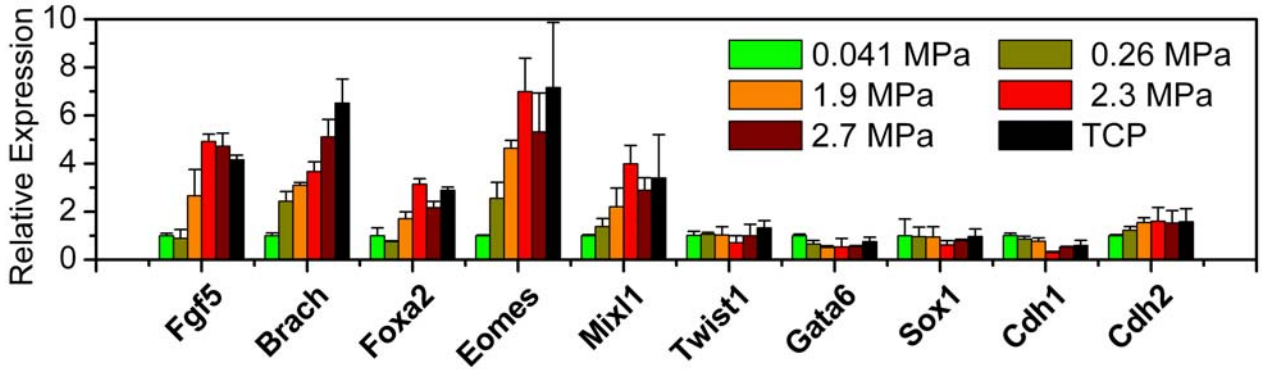

Figure 4. Relative expression of putative markers of the primitive ectoderm (*Fgf5*), primitive streak and mesendoderm precursors (*Brachyury*, *Foxa2*, *Eomes*, *Mixl1*), anterior mesendoderm (*Twist1*), neuroepithelium (*Sox1*), primitive endoderm (*Gata6*) and cadherins 1 and 2 (E- and N-cadherin; *Cdh1* and *Cdh2*) in cultures of ESCs 6 days after plating. Expression of all genes was normalised to the housekeeper, *Gapdh*, and results are expressed as relative increase compared to expression in cells on the softest PDMS at day 6.

of *Foxa2* increased and peaked earlier in cells on stiffer substrates compared to those on softer substrates, with, like *Brachyury*, a progressive increase in expression with increasing substrate stiffness at day 6 ($p < 0.05$ between all groups except between cells on 2.7 MPa PDMS and TCP). In EBs *Foxa2* expression was significantly higher than on any other substrate ($p<0.001$; Figure 3D). Expression of *Sox1*, a gene involved in the differentiation of neurectoderm from primitive ectoderm (Pevny *et al*., 1998) declined significantly in all cells and did not differ significantly between cells on any substrate or in EBs (Fig. 3E). Expression of *Gata6*, a gene involved in the differentiation of primitive endoderm and the extraembryonic tissues (Chazaud *et al*., 2006), initially decreased in cells on all substrates at day 4, but then rose (Fig. 3F). There were no significant differences between substrates, but expression in EBs was significantly higher than on substrates at all other time points ($p < 0.01$).

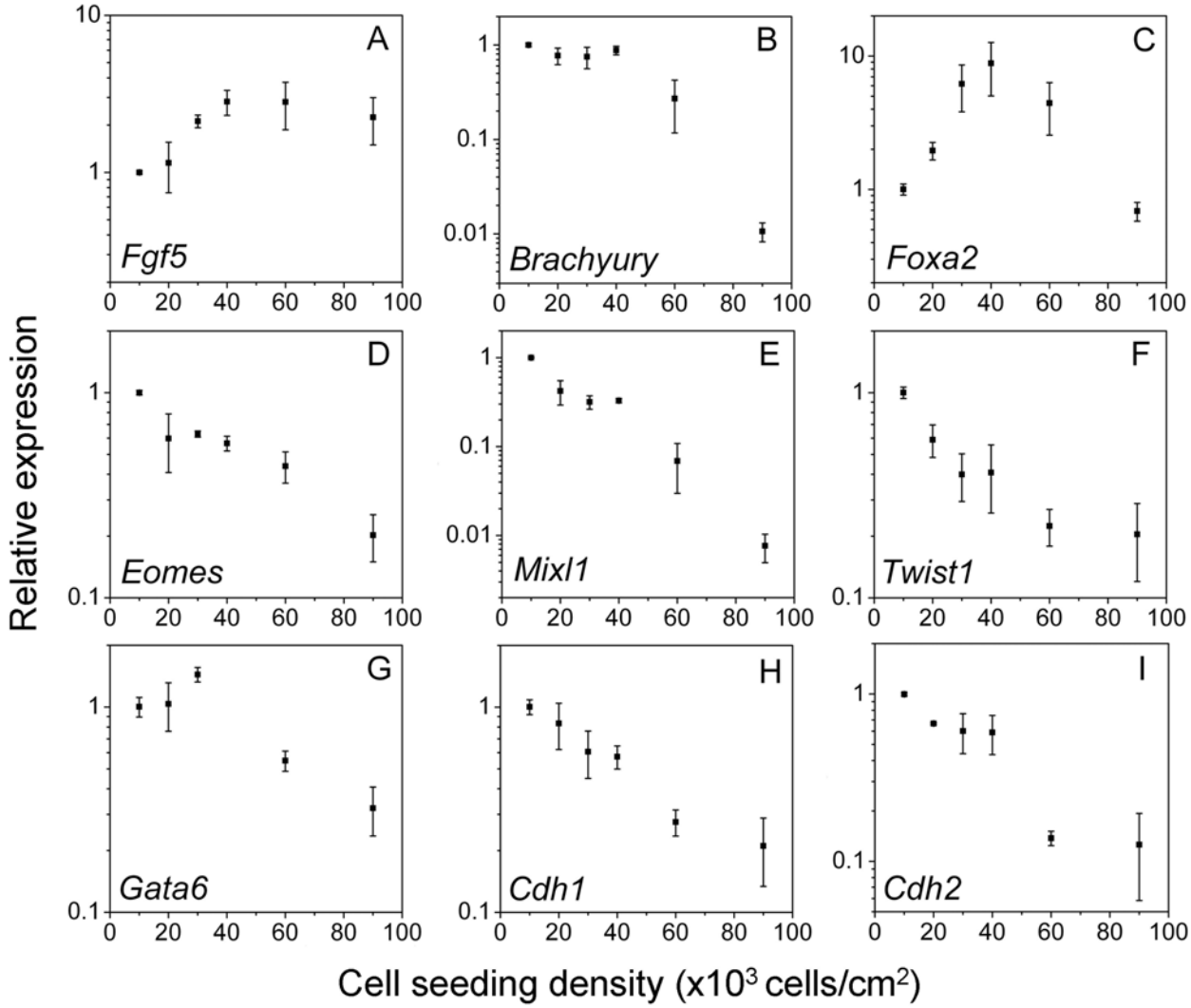

Figure 5. The effect of cell density on expression of putative markers of primitive ectoderm (*Fgf5* **[A]**) the primitive streak and mesendoderm precursors (*Brachyury* **[B]**, *Foxa2* **[C]**, *Eomes* **[D]**, *Mixl1* **[E]**,), anterior mesendoderm (*Twist1* **[F]),** primitive endoderm (*Gata6* **[G]**) and cadherins 1 and 2 (E- and N-cadherin; *Cdh1* **[H]** and *Cdh2* **[I]**) in cultures of ESCs 6 days after plating. Expression of all genes was normalised to the housekeeper, *Gapdh*, and results are expressed as relative increase compared to expression in cells 6 days after plating at a density of 10 000 cells/cm^2.

Expression of markers of primitive streak at day 6

Because of the substrate-dependent differences in expression of *Brachyury* and *Foxa2*, we next investigated whether there were relationships between the expression of other genes expressed in the primitive streak and in progenitors of the mesoendoderm. Both *Mixl1* and *Eomes* are expressed in the primitive streak during gastrulation (the latter gene is also expressed in the trophectoderm and extraembryonic ectoderm; Hart *et al*., 2002; Pearce and Evans, 1999; Ciruna and Rossant, 2001; Tam *et al*., 2007; Arnold *et al*., 2008) and have putative roles in mesendoderm differentiation and patterning. Expression of *Brachyury*, *Foxa2*, *Mixl1* and *Eomes* were positively correlated with substrate stiffness when analysed by Pearson's correlation ($p < 0.001$; Fig. 4). *Twist1*, which in Drosophila is necessary for gastrulation but which in mammals is expressed in anterior and lateral tissues and not in the primitive streak (Fuchtbauer, 1995), showed no significant correlation with substrate stiffness ($p = 0.36$). *Gata6*, a marker of primitive endoderm, and *Sox1*, a neuroepithelial marker, where both negatively correlated with substrate stiffness ($p < 0.001$). *Cdh1* (E-cadherin), a gene which is expressed in primitive ectoderm but which is downregulated in cells migrating though the primitive steak, was negatively correlated with substrate stiffness ($p < 0.001$; Fig. 4), while in contrast *Cdh2* (N-cadherin), which is expressed in the nervous system and in mesoderm cells during gastrulation (Winklbauer *et al*., 1992; Yang *et al*., 2008), was positively correlated with substrate stiffness ($p = 0.003$).

The effect of cell density on gene expression

We next investigated whether the increased cell density (associated with increased growth on stiffer substrates) could be the cause of the relationships between gene expression and substrate stiffness. We seeded ESCs at various cell densities and measured gene expression at day 6. *Brachyury*, *Mixl1*, *Eomes, Twist1, Cdh1* and *Cdh2* were

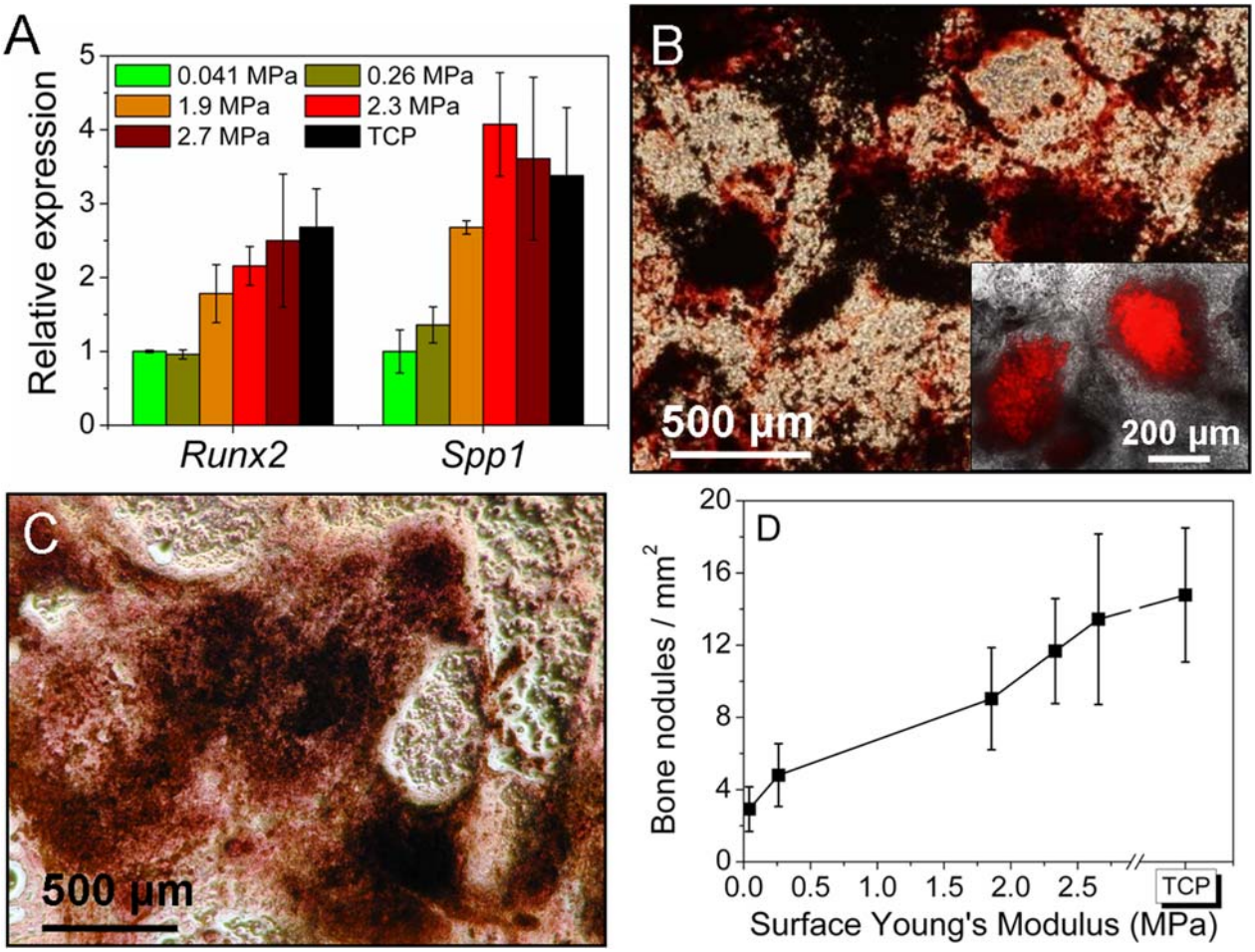

Figure 6. Osteogenic differentiation of ESCs grown on PDMS substrates and TCP for 28 days in the presence of osteogenic supplements. **(A)** Both *Runx2* and *Spp1* (osteopontin gene) are upregulated on stiffer compared to softer substrates in cultures of ESCs 16 days after plating. Expression of genes was normalised to *Gapdh* and is expressed relative to expression on 0.041 MPa PDMS. **(B)** Mineralised areas sequester alizarin red S and fluoresce at wavelengths of >590 nm under excitation at 510-560 nm (inset) or **(C)** sequester Ag^{2+} when stained using Von Kossa's method. **(D)** 28 days after plating, ESC cultures grown in osteogenic supplements were fixed, stained with alizarin red S, and mineralised areas were manually counted as discrete fluorescing areas. The number of mineralised areas increased in relation to substrate stiffness.

all negatively regulated by increasing cell density. *Foxa2* and *Gata6* showed a biphasic pattern of gene expression, with the greatest expression levels in cultures seeded at 40 000 cells/cm^2 while *Fgf5* expression was upregulated at higher cell densities (Fig. 5A-G).

Osteogenic differentiation on PDMS substrates

We finally investigated whether substrate stiffness affected the terminal differentiation of ESCs by culturing them in the presence of supplements known to induce osteogenic differentiation (Gentleman *et al.*, 2009; Buttery *et al.*, 2001). At day 11, we found significant upregulation of both *Runx2* and *Spp1* (osteopontin) on stiff compared to soft substrates with a significant positive correlation between gene expression and substrate stiffness ($p < 0.001$; Fig. 6A). On all substrates, ESCs formed mineralised deposits containing Ca^{2+} salts that sequestered either alizarin red S, or Ag^{2+} using von Kossa's method, suggesting the formation of mineralised bone-like tissue (Fig. 6B and 6C). The amount of alizarin red S staining was positively correlated with the stiffness of the substrate on which the cells were plated ($p < 0.001$; Fig. 6D).

Discussion

We have demonstrated here that substrate stiffness affects cell spreading, growth rate, gene expression and osteogenic differentiation of ESCs. While cell attachment was unaffected by the stiffness of the growth substrate, cell spreading and cell proliferation were increased as a function of substrate stiffness. Similarly, several genes expressed in the primitive streak during gastrulation, and implicated in early mesendoderm differentiation, were upregulated in cell cultures as substrate stiffness increased from 41 kPa to 2.7 MPa. This effect was not dependent on cell density, and suggests that increasing stiffness of the substrate promotes mesendoderm differentiation. Finally,

we demonstrated that osteogenic differentiation of ESCs was enhanced as substrate stiffness increased illustrating the importance of mechanical environment in both early and terminal ESC differentiation.

We chose PDMS as a substrate because it is easy to prepare, transparent, non-toxic, and flexible, and does not either dissolve or swell in cell culture medium. We were able to fabricate reproducibly substrates ranging in stiffness from 41 kPa to 2.7 MPa (in a similar range to those previously reported (Tzvetkova-Chevolleau *et al*., 2008; Goffin *et al*., 2006; Cheng *et al*., 2009; Brown *et al*., 2005)). At high crosslinker concentrations (23% [w/w]) we noted a decrease in substrate stiffness, which can be attributed to the presence of unbound, mobile crosslinker molecules (Lee *et al*., 2004). Surface elastic moduli reported for adult tissue range from 17 Pa for fat to > 1 GPa for bone (Levental *et al*., 2007; Hengsberger *et al*., 2002; Mankani *et al*., 2006), although most tissues have elastic moduli less than 0.2 MPa. Here we used AFM to determine the surface Young's moduli of liver and calvarial bone and found these to be 0.018 MPa and 0.150 MPa respectively. The modulus we measured for calvarial bone was several orders of magnitude lower than found in another recent study (Balooch *et al*., 2005), although this may be explained by differences in sample preparation: our AFM measurements were conducted on the surfaces of the fresh calvarium in wet conditions, whereas Balooch *et al*. conducted experiments on epoxy-embedded bone cross-sections in dry conditions. There is a paucity of data available for embryonic tissues, but *E* has been measured at around 1 kPa in the blastula wall of sea urchin (*Strongylocentrotus purpuratus*) embryos (von Dassow and Davidson, 2007). We suspect that the elastic moduli of substrates fabricated in the current study are higher than those that exist in the early embryo. However, attempts to fabricate substrates with lower elastic moduli by using crosslinker concentrations of < 1% were unsuccessful – substrates were difficult to handle and never solidified. Future studies may use polyacrylamide (PA) gels, which range in *E* from < 1kPa-100 kPa (Engler *et al*., 2006; Khatiwala *et al*., 2007; Pelham, Jr. and Wang, 1997), to probe the effect of softer substrates on ESC differentiation.

Despite the relatively high elastic moduli of substrates in our experiments we were able to distinguish substrate-dependent biological effects. Both proliferation and cell spreading were significantly greater with increasing stiffness of the substrate, in agreement with several other studies (Rowlands *et al*., 2008; Khatiwala *et al*., 2006; Folkman and Moscona, 1978; Peyton *et al*., 2006; Yeung *et al*., 2005). We also measured an upregulation of genes expressed in the primitive streak and nascent mesendoderm – *Foxa2*, *Brachyury*, *Mixl1*, *Cdh2*, and *Eomes* – and a subsequent stimulation of osteogenic differentiation (a tissue derived in large part from the mesoderm) with increasing stiffness of substrates. We consider it unlikely that these effects are due to the chemical composition of the substrates as ICP analysis of cell culture medium revealed that the elemental Si (present in all components of PDMS) content remained below 2ppm on all substrates, including tissue culture plastic – a value similar to the trace amount found in blood plasma (Bercowy *et al*., 1994). Instead, our results suggest either a direct effect of the mechanical properties of the substrates on cell differentiation, or an indirect effect related to the increased cell proliferation and cell density measured on stiffer substrates; for example by paracrine growth-factor signalling, nutrient depletion, or direct cell-cell contact, rather than by substrate stiffness *per se* (Dietrich *et al*., 2002). But in direct contradiction to the latter hypothesis we found that *Brachyury*, *Mixl1*, *Cdh2* and *Eomes* were all down-regulated by increasing cell density in control experiments (Fig. 5). We therefore consider it likely that substrate stiffness directly stimulates the growth and differentiation of mesendoderm cells.

The mechanism underlying these observations may be related to the increased cell spreading that we observed with increasing substrate stiffness. Burdsal *et al*. demonstrated that cells derived from epiblast tissue (dissected from murine embryos) could be induced to flatten in culture and to differentiate to cells with the characteristics of mesoderm by incubating them with function-perturbing antibodies against E-cadherin (which inhibit cell-cell adhesion and stimulate cell-ECM adhesion) (Burdsal *et al*., 1993). Indeed, it is established that cells that undergo gastrulation lose their tightly packed epithelial morphology, and assume a stellate, migratory mesenchymal morphology (Baum *et al*., 2008; Tam *et al*., 1993). This epithelial to mesenchymal transition (EMT) allows these cells to actively adhere to and migrate through the ECM-rich space between the epiblast and the visceral endoderm, eventually forming the endodermal and mesodermal tissues of the adult organism. The importance of this process is revealed in embryos which lack gastrulation-related genes – *Brachyury*, *Eomes* and *Mixl1* mutants all have defects in mesendoderm patterning with an associated accumulation of cells at the primitive streak during gastrulation (Arnold *et al*., 2008; Tam *et al*., 2007; Wilson *et al*., 1995). This defect suggests that the accumulated cells are unable to migrate properly and pattern the mesendoderm, a finding that is also supported by *in vitro* observations of the impaired migration of mesodermal cells from *Brachyury* mutants (Hashimoto *et al*., 1987) (but not in *Eomes* mutants (Arnold *et al*., 2008)). These results suggest that one function of these genes may be to regulate the expression of cell attachment molecules involved in cell migration and cell-ECM attachment during gastrulation (Smith, 1997; Wilson *et al*., 1995). Thus in our experiments, stiffer substrates may support the growth and differentiation of more adhesive cells expressing these genes that arise in cultures of differentiating ESCs by providing an environment that more closely mimics the environment migrating mesendoderm cells experience in the early embryo. We did not directly test whether or not increased contractility in uncommitted cells on stiffer substrates stimulates differentiation directly. Future studies may seek to address this by examining the effect of inhibitors of cellular contractility on differentiation, or by immunostaining cells plated at low density on substrates of differing stiffnesses (Engler *et al*., 2006).

We also noted in our experiments that *Twist1* expression was unaffected by substrate stiffness. Unlike in *Drosophila* where its homologue has been shown to be

mechanosensitive, *Twist1* is not expressed in the primitive streak or in the regions surrounding it in the mouse but instead is expressed subsequently by cells that have migrated to the anterior part of the embryo (Fuchtbauer, 1995). Thus it is possible that the time-point we investigated (day 6) was too early to detect substrate-dependent changes in the expression of this gene. We also noted that the expression of *Foxa2* and *Brachyury* – genes that we found to be influenced by substrate stiffness – were expressed at much higher levels in EBs (where there is no substrate) than on either PDMS substrates or TCP. This is likely to be due to the three-dimensional nature of EBs, where cells are permitted to undergo similar morphological arrangements to those in the early embryo, a situation which is prevented in 2D cell culture and which in 3D culture is known to accentuate early cell differentiation events in ESCs (Levenberg *et al*., 2003; Levenberg *et al*., 2005)

Conclusions

In this study we conclude that increasing substrate stiffness from 0.041 MPa to 2.7 MPa promotes cell spreading, cell proliferation, mesendodermal gene expression and terminal osteogenic differentiation of ESCs. As well as illustrating that the mechanical environment is an important factor in cellular differentiation in the developing embryo, these results suggest that the growth substratum should be carefully considered in any attempts to grow and differentiate relevant cell populations *in vitro* for clinical applications.

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Supplementary Table 1

Gene name	GenBank Accession	Sequence	Amplicon length	Cycling conditions	Starting conc RNA (ng/μL)
Runx2	NM_009820	CCAACCGAGTCATTTAAGGCT GCTCACGTCGCTCATCTTG	207	95°C 10s 56°C 15s 72°C 15s	4
Eomes/Tbr2	NM_010136	GGCCCCTATGGCTCAAATTCC CCTGCCCTGTTTGGTGATG	62	95°C 10s 56°C 15s 72°C 15s	4
Spp1	NM_009263	AGCAAGAAACTCTTCCAAGCAA GTGAGATTCGTCAGATTCATCCG	134	95°C 10s 56°C 15s 72°C 15s	4
Nanog	NM_028016	TTGCTTACAAGGGTCTGCTACT ACTGGTAGAAGAATCAGGGCT	106	95°C 10s 56°C 15s 72°C 15s	4
Mixl1	NM_013729	ACGCAGTGCTTTCCAAACC CCCGCAAGTGGATGTCTGG	196	95°C 10s 56°C 15s 72°C 15s	4
Twist1	NM_011658	GGACAAGCTGAGCAAGATTCA CGGAGAAGGCGTAGCTGAG	146	95°C 10s 56°C 15s 72°C 15s	4
Brachyury	NM_009309	GCTTCAAGGAGCTAACTAACGAG CCAGCAAGAAAGAGTACATGGC	117	95°C 10s 56°C 15s 72°C 15s	4
Fgf5	NM_010203	TGTGTCTCAGGGGATTGTAGG AGCTGTTTTCTTGGAATCTCTCC	137	95°C 10s 56°C 15s 72°C 15s	4
Foxa2	NM_010446	CCCTACGCCAACATGAACTCG GTTCTGCCGGTAGAAAGGGA	222	95°C 10s 56°C 15s 72°C 15s	4
Sox1	NM_009233	AAGGAACACCCGGATTACAAGT GTTAGCCCAGCCGTTGACAT	231	95°C 10s 56°C 15s 72°C 15s	4
Gata6	NM_010258	TTGCTCCGGTAACAGCAGTG GTGGTCGCTTGTGTAGAAGGA	105	95°C 10s 56°C 15s 72°C 15s	4
Cdh1	NM_009864	CAGGTCTCCTCATGGCTTTGC CTTCCGAAAAGAAGGCTGTCC	175	95°C 10s 56°C 15s 72°C 15s	4
Cdh2	NM_007664	AGCGCAGTCTTACCGAAGG TCGCTGCTTTCATACTGAACTTT	101	95°C 10s 56°C 15s 72°C 15s	4
Gapdh	NM_008084	AGGTCGGTGTGAACGGATTTG TGTAGACCATGTAGTTGAGGTCA	123	95°C 10s 56°C 15s 72°C 15s	0.04

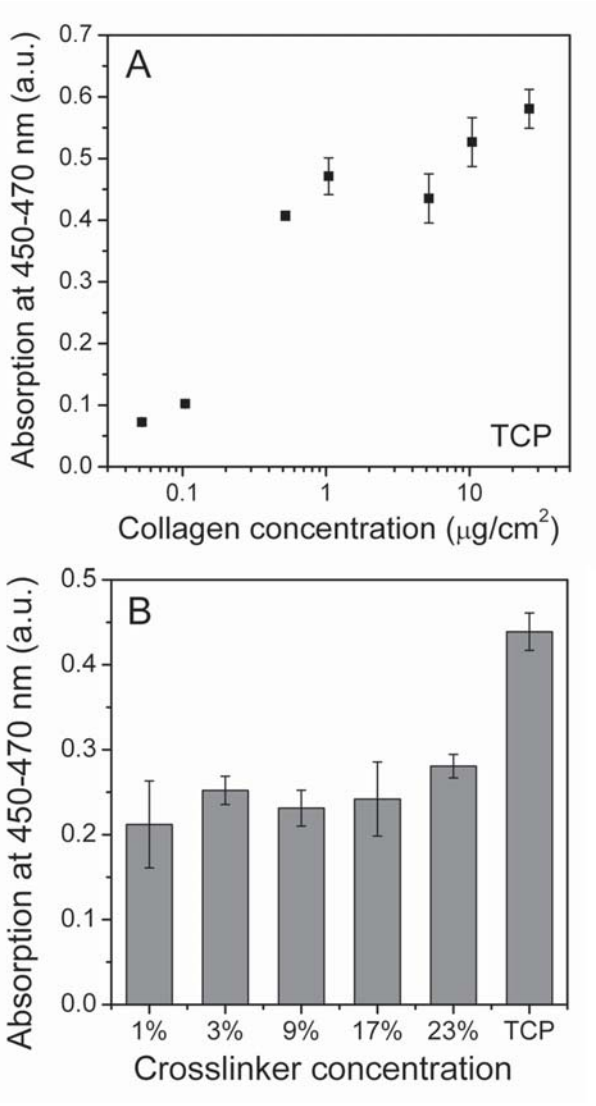
Supplementary methods

Collagen Quantification

Collagen attachment to PDMS surfaces was confirmed with an assay based on the binding of biotinylated fibronectin to Type I Collagen, as has been previously described (Gaudet *et al*., 2003). Briefly, fibronectin (Sigma) was biotinylated with an EZ-Link® Sulfo-NHS-Biotinylation Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. PDMS surfaces and TCP coated with Type I Collagen as described in the 'Materials Synthesis' section were rinsed 6 times with distilled water, treated for 30 minutes with a blocking buffer of 2% (w/v) BSA (Sigma) and 0.05% (v/v) Tween-20 (GE Healthcare formerly Amersham Biosciences) in PBS. Biotinylated fibronectin was then added to the surfaces and allowed to attach for 1 hour at 37 °C. Substrates were then rinsed three times with rinsing buffer consisting of 0.1% (v/v) Tween-20 in PBS. They were then incubated for an additional 30 minutes at 37 °C with 50 ng/mL horseradish peroxidise-streptavadin (Pierce Biotechnology) in blocking buffer. Substrates were rinsed 3 times and the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Pierce Biotechnology) was added and allowed to develop for 10 minutes at room temperature. The reaction was stopped with an equal volume of 2M H_2SO_4 and absorbance values were measured on a colorimetric plate reader at 450 nm.

ICP analysis of cell culture medium

Samples of cell culture medium were collected after 4 days from cell seeding and the presence of Si atoms from the substrate in the media was measured by inductively coupled plasma (ICP) spectroscopy (iCAP 6300, Thermo Fisher Scientific, Loughborough, UK).



Supplementary Figure 1

Reference

Gaudet C, Marganski WA, Kim S, Brown CT, Gunderia V, Dembo M, Wong JY (2003) Influence of type I collagen surface density on fibroblast spreading, motility, and contractility. *Biophys J* **85:** 3329-3335.

Discussion with Reviewers

J Hayes: Have the authors attempted to compare non-stimulated (i.e. without osteogenic supplements) embryonic stem cells to see if a stiffness specific differentiation response exists?

Authors: We have not attempted this experiment. On all substrates osteogenic supplements were added, therefore we would argue there is a stiffness-specific stimulation of osteogenesis. On stiff tissue culture plastic, we do not see bone nodule formation in the absence of osteogenic supplements. Therefore we would not expect to see bone nodule formation on PDMS substrates in the absence of supplements.

J Hayes: Did the authors observe any substrate stiffness changes in the expression of the housekeeping gene?

Authors: To answer this pertinent question we have re-analysed our data from 7 separate time course experiments. We averaged the fluorescence intensity values at which the SYBR green signal crossed a given threshold (the Ct value) for each substrate and for EBs. We found Ct values of 18.1 ± 0.8 for PDMS1, 18.3 ± 0.5 for PDMS3, 18.9 ± 0.8 for PDMS9, 18.3 ± 0.6 for PDMS17, 18.2 ± 0.7 for PDMS 23; 18.4 ± 0.6 for TCP; and 18.5 ± 0.6 for EBs. No group was statistically significant from any other ($p > 0.2$). We also analysed a possible time-dependence of Gapdh expression by

examining Gapdh expression at each time point. We found Ct values of 19.9 ± 1.3 at day 0 (ESCs), 18.3 ± 0.8 at day 4, 18.5 ± 0.9 at day 6, 18.3 ± 0.8 at day 8 and 19.1 ± 0.9 at day 10. The Ct value was significantly higher in undifferentiated ESCs (day 0) than at any other timepoint ($p< 0.05$) but there were no significant differences between other timepoints. Given the efficiency of the Gapdh primer pair we used (1.81), on average Gapdh expression is reduced by a factor of 2-3 in differentiated compared to undifferentiated ESCs. While equal masses of RNA were added to each PCR reaction tube, one should note that the mass of RNA in each reaction was measured by its absorbance, Values obtained using this technique may be affected by the presence of protein which could interfere with measurement –as undifferentiated ESCs contain less protein than differentiating ESCs this may account for some of the discrepancy.

J Hayes: The authors state that they suspect that the elastic moduli of the substrates used in their study are higher than those in the early embryo. Do they believe therefore, that the results presented are merely an outcome of an *in vitro* 'artefact'? Obviously the data are still of great interest. I am just wondering if the authors can assign specific biological relevance to their data, given that bone differentiation – according to the authors – appears to be influenced at much lower magnitudes of tissue stiffness.
Authors: It is difficult to answer this question concisely. The *in vitro* environment can never hope to replicate *in vivo* complexities, but it does allow us to reduce the number of variables we encounter in an experiment and to pin firmer conclusions to given observations. Strictly speaking, any *in vitro* experiment is artificial. We chose to take one variable and test whether it affects ESC differentiation. Of course, the embryo is not a static system and much more complex. But our results show that the substrates in our manuscript still do have significant effects on differentiation, for reasons we suggest in the discussion.

J Hayes: The author's show that many of the genes studied were expressed at much higher levels in the embyroid bodies and attribute this observation to differences between 2D and 3D culture, which of course is very plausible. I am wondering however if, by chance, the authors measured the Young's modulus of the EBs (if this is even possible?) and if so how did this compare to the liver and calvarium samples and did the measurements correlate to their observations?
Authors: Unfortunately we did not measure EB stiffness. It would be interesting in future studies to measure the stiffness of both EBs and early embryos. We believe this to be experimentally feasible.

G Reilly: Some of the authors recently published a very interesting paper in Nature Materials showing that the mineralisation achieved in ESC culture is not of the same structure and quality as that created by adult stem cells (MSCs) or fully differentiated osteoblasts. Do they think this also applies to the mineralisation seen in these cultures? Would they expect substrate stiffness to affect the quality/ structure of mineralised nodules
Authors: In answer to the first question – yes. These experiments were conducted in parallel with those reported in the Nature Materials paper. We think that the quality of the mineralised nodules formed from ESCs in this study is comparable to those in the Nature Materials paper. In answer to the second question – we don't know, as we haven't tested it. There is more mineralisation on harder substrates than soft ones but we don't know whether it is qualitatively different. This is very interesting and the subject of ongoing research in our group.