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PAPER

Effects of shear stress on germ lineage specification of embryonic stem cells†

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Mechanobiology to date has focused on differentiated cells or progenitors, yet the effects of mechanical forces on early differentiation of pluripotent stem cells are still largely unknown. To study the effects of cellular deformation, we utilize a fluid flow bioreactor to apply steady laminar shear stress to mouse embryonic stem cells (ESCs) cultured on a two dimensional surface. Shear stress was found to affect pluripotency, as well as germ specification to the mesodermal, endodermal, and ectodermal lineages, as indicated by gene expression of OCT4, T-BRACHY, AFP, and NES, respectively. The ectodermal and mesodermal response to shear stress was dependent on stress magnitude (ranging from 1.5 to 15 dynes cm^{-2}). Furthermore, increasing the duration from one to four days resulted in a sustained increase in T-BRACHY and a marked suppression of AFP. These changes in differentiation occurred concurrently with the activation of Wnt and estrogen pathways, as determined by PCR arrays for signalling molecules. Together these studies show that the mechanical microenvironment may be an important regulator during early differentiation events, including gastrulation. This insight furthers understanding of normal and pathological events during development, as well as facilitates strategies for scale up production of stem cells for clinical therapies.

Introduction

The advancement of stem cell-based therapies is driving a need to better understand mechanisms of differentiation to generate particular phenotypes *in vitro*. Since the discovery of embryonic stem cells (ESCs) in the early 1980s,[1] very few techniques have been able to produce ESC-derived cell populations suitable for therapeutic use. Most efforts have examined differentiation towards a specific terminal phenotype; however, a shift in focus towards study of early lineage specification may prove useful for more efficiently generating downstream terminal phenotypes. In such cases, the processes that govern early embryonic development become especially insightful.[2]

Developmental processes are tightly regulated by complex and spatially disparate sequences of signals present in the chemical and physical microenvironment. While the earliest studies in embryology centered on the physical aspects of development, the advent of more modern technologies changed the focus to more biochemical- and biomolecular-based approaches.[3] As a result, the specific effect of mechanical cues during development is still poorly understood. Mechanical cues,

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Insight, innovation, integration

Mechanical forces have been shown to affect various stages of development, including initiation of asymmetry and cardiovascular organogenesis. Although cell sheet rearrangements during gastrulation induce mechanical deformations coinciding with germ layer specification, the relation between the two is unclear. Here, pluripotent stem cells and a custom engineered bioreactor were used to isolate the effects of mechanical cell deformation on pluripotency and differentiation towards the germ lineages *in vitro*. We found that shear stress affected early differentiation patterns in a magnitude and duration dependent manner. Thus, spatiotemporal changes in the mechanical microenvironment are important factors during initial cell specification. These results provide insight into development and can also be exploited to improve directed differentiation of stem cells for regenerative medicine applications.

or deformations caused by physical forces, can be transduced into biological signals which regulate cell behaviour and function. Some recent studies have shown that mechanical forces regulate several developmental stages, including establishment of left–right asymmetry[4] and organogenesis.[5] Yet the role of the physical microenvironment during germ lineage specification, among the earliest of the differentiation processes, has not been thoroughly investigated. This stage of gastrulation is of particular interest as the differentiation of the cells is concomitant with extensive changes in spatial re-arrangement and morphology, resulting in a host of physical changes including shear stress-induced membrane deformation.[6]

During early developmental processes, large-scale cell movements and reorganization of cell sheets (*e.g.* formation of the primitive streak, invagination, as well as convergence and extension) complicate the tracking necessary to correlate cell deformations to specific differentiation events. Although the complex nature of the microenvironment during embryonic development makes it difficult to study the relation of cell deformation to lineage commitment *in vivo*, it may be possible to identify the connection between mechanical deformation and early lineage commitment using stem cells *in vitro*. Pluripotent ESCs are derived from the inner cell mass of the blastula and can become cells in all three germ lineages. When differentiated in suspension, ESCs form cell aggregates, or embryoid bodies (EBs), that recapitulate spatial patterns of gastrulation.[7] In this 3D configuration, however, complex spatial arrangements and poor transmission of force to individual cells limit the ability to correlate mechanical cues to differentiation events. Adherent cultures, which allow for the application of well-defined physical forces, have been used extensively with differentiated cells to study the effects of cyclic stretch[8] or fluid flow[9] on biological processes.[10] Thus, adherent culture of ESCs provides the opportunity to study the effect of cellular deformation on differentiation.

The objective of this set of studies was to use an *in vitro* model system to determine the effects of cellular deformation on germ lineage specification on pluripotent cells. Our study used a parallel plate bioreactor system to apply fluid shear stress during the early differentiation of mouse embryonic stem cells in adherent culture. We observed that shear stress promoted differentiation towards specific germ lineages in a magnitude- and duration-dependent manner, activating multiple signalling pathways. These results elucidate the effect of mechanical forces on early stem cell differentiation, which not only provides insight into the mechanisms that govern germ layer development *in vivo*, but also allows for rational design in the directed differentiation of stem cells for clinical therapies.

Methods

Expansion of embryonic stem cells

Mouse D3 embryonic stem cells (ESCs) and embryonic fibroblasts (MEFs) were purchased from ATCC (Manassas, VA) and cultured as previously described.[11] Briefly, ESCs were expanded on mitotically-inactivated MEFs and then stored in liquid nitrogen. Just prior to use in experiments, ESCs were thawed and cultured on gelatin-coated tissue culture plastic without further subculture. Culture medium consisted of Dulbecco's Modification of Eagles Medium supplemented with 15% ES-qualified foetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1000 U ml^{-1} Leukaemia Inhibitory Factor (LIF: ESGRO® from Millipore, Billerica, MA), and antibiotics.

Initial differentiation of ESCs (pre-treatment)

ESCs were initially differentiated on collagen IV-coated glass slides in medium without LIF, as described previously.[11] Briefly, collagen type IV (BD Biosciences, Bedford, MA) at a concentration of 3.5 μg cm^{-2} was allowed to adsorb for one hour onto glass slides. ESCs were seeded at 10 000 cells cm^{-2} and incubated at 37 °C/5% CO_2 in 25 ml of differentiation medium, which consisted of Minimum Essential Alpha Medium supplemented with 10% foetal bovine serum, 0.1 mM beta-mercaptoethanol, and antibiotics. Under these conditions, slides were cultured for up to six days for comparison to embryoid body culture (ESCs placed in differentiation medium in non-tissue culture treated petri dishes) or for up to three days for fluid shear stress studies.

Application of fluid shear stress

ESCs seeded onto collagen IV-coated glass slides were subjected to fluid shear stress using a parallel plate bioreactor system, as described previously.[11] Fluid shear stress was applied using a parallel plate bioreactor system, which has been used previously by our group with ESCs[11] and by many others with endothelial cells,[9b,12] osteocytes,[13] and chondrocytes.[14] In this system, $\tau = 6Q\mu/(bh^2)$, such that the shear stress (τ) is dependent on the flow rate (Q), viscosity of the medium (μ), and the width and height of the channel (b and h, respectively). We used this system to apply steady laminar shear stress in the range of 1.5–15 dynes cm^{-2} for up to 4 days.

Real-time RT-PCR analysis for mRNA expression

At the end of culture, samples were individually treated with trypsin to generate cell solutions, which were then lysed, homogenized, and stored at −80 °C until further processing. RNA was isolated (Qiagen RNeasy kit) and quantified using a Nanodrop® spectrophotometer for each sample. Standard analysis of mRNA levels for each sample was done on cDNA converted from 1 μg RNA (Invitrogen Superscript® III First-strand synthesis) and analysed using SYBR® Green (Applied Biosystems, Carlsbad, CA) on a StepOnePlus™ PCR System. Primers were custom designed (Primer Express® Software v3.0) for octamer-binding protein 4 (OCT4), alpha-fetoprotein (AFP), T brachyury (T-BRACHY), nestin (NES), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward and reverse primers are listed in Table S1, ESI†). Gene expression levels were quantified using standard curves and are all reported as normalized to GAPDH expression.

Analysis of genes associated with signal transduction pathways was performed using a RT^2 Profiler™ PCR array (SA Biosciences, Frederick, MD). A minimum of 3 replicates per group were pooled and then converted to cDNA and analysed using the manufacturer's reagents and instructions. For each sample, 84 signal transduction genes were normalized to housekeeping genes (listed in Table S2, ESI†). Fold regulation between

experimental samples and matched static controls was used to identify genes affected by shear stress.

Flow cytometry analysis for protein expression

Following static or shear stress treatment, cells were removed from glass slides using StemPro® Accutase® (Invitrogen, Carlsbad, CA). Cell suspensions were fixed with 4% formaldehyde (Polysciences Inc., Warrington, PA) for 15 minutes at 4°C and stored in buffer solution consisting of PBS with 0.3% bovine serum albumin and 0.001% polyoxyethylenesorbitan monolaurate (Sigma-Aldrich, St. Louis, MO). Samples were permeabilized using 0.5% triton-X (Sigma-Aldrich) and blocked with 10% serum solution prior to immunostaining. Cells were assessed for expression of mesodermal protein using T-BRACHY primary (Abcam, Cambridge, MA) and Alexa Fluor 488 secondary (Invitrogen) antibodies at dilutions of 1 : 80 and 1 : 100, respectively. Relative protein expression was assessed using a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ). Histograms generated from the flow cytometer were used to determine a median fluorescence value for each sample, which was averaged across replicates to represent each group.

Statistical analysis

Data are presented as mean ± SEM. Samples were compared using either a *t*-test or one-way ANOVA, with *p*-values of <0.05 considered statistically significant.

Results

Embryonic stem cell differentiation

Embryonic stem cells (ESCs; Fig. 1A) were differentiated either in suspension as embryoid bodies (EBs) or in adherent culture on collagen type IV-coated glass slides. EBs were cultured for up to 12 days, while cells on slides were limited to only 6 days to avoid confounding results due to over-confluence. Cells differentiated in EBs were in cell clusters governed by cell–cell contact (Fig. 1B). ESCs differentiated in adherent culture, affected by both adjacent cells and the underlying protein substrate, had a range of morphologies from small tight cells to more cuboidal cells (Fig. 1C). Gene expression levels of cells from the different groups were quantified for a pluripotency marker (OCT4), as well as markers of differentiation. As is consistent with published reports,[15] OCT4 expression decreased to negligible levels in EBs within 8 days. After the longest culture duration on glass slides, however, OCT4 levels in cell populations were still at 66% of that in undifferentiated ESCs (Fig. 2A). Furthermore, direct comparison of OCT4 levels at day 4 showed that expression in EB samples was significantly lower ($p < 0.05$, *t*-test) than that of slide samples. Thus, both types of differentiation conditions resulted in a loss of pluripotent markers, with adherent culture either supporting decelerated kinetics or different dynamics of differentiation compared to EBs.

Evaluation of differentiation to the three germ lineages (Fig. 2B–D) was assessed using T-BRACHY (mesoderm), AFP (endoderm), and NES (ectoderm), as has been done by others.[15a,16] T-BRACHY was transiently expressed in cells differentiated in EBs, reaching a peak expression level at day 4

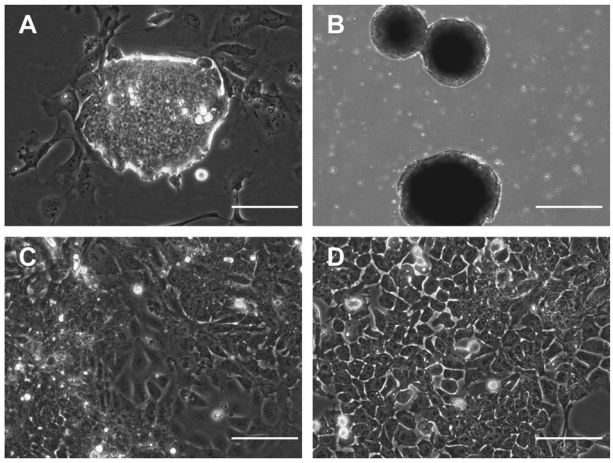

Fig. 1 Phase images of undifferentiated cells. As expected, undifferentiated ESCs grew in tight colonies (A). After 4 total days of differentiation, cells in EBs formed cell clusters (B) and cells in static adherent culture were heterogeneous with some tightly packed cells and other cuboidal cells (C). Cells statically cultured for two days and then exposed to 2 days of shear stress at 5.0 dynes cm^{-2} were more consistent as cuboidal in morphology (D). Scale bars represent 100 (A, C, D) or 500 μm (B).

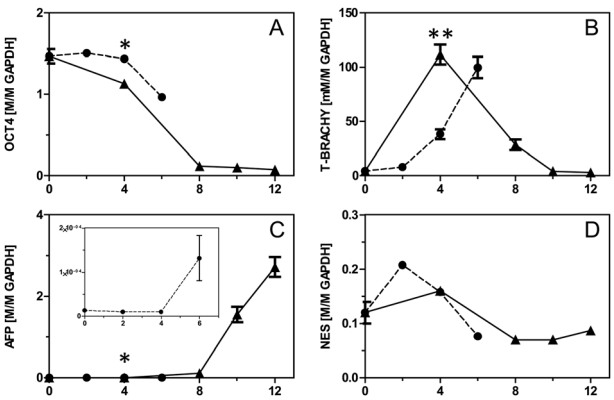

Fig. 2 Baseline ESC differentiation under adherent and suspension culture conditions. Gene expression was analysed for ESCs cultured under static conditions on collagen type IV-coated glass slides (circles, dashed lines) and in embryoid bodies (triangles, solid lines). Samples were analysed for pluripotency (A: OCT4) and differentiation to the mesodermal (B: T-BRACHY), endodermal (C: AFP), and ectodermal (D: NES) lineages. Inset graph in C presents AFP expression for slide samples on a more focused scale. Data are averages (mean ± SEM; $n = 3$–4) of gene expression normalized to GAPDH expression. Asterisks indicate a significant (* for $p < 0.05$, ** for $p < 0.01$) difference between groups at day 4.

and by day 10 returning to levels similar to undifferentiated ESCs (Fig. 2B). While cells differentiated on slides had a significantly ($p < 0.01$, *t*-test) lower expression of T-BRACHY after 4 days of culture, levels at day 6 were comparable to the peak expression level observed in EBs. AFP expression increased significantly with time in both EB ($p < 0.001$; Fig. 2C) and slide culture ($p < 0.02$; insert in Fig. 2C), with marked increases occurring at day 8 and 4, respectively. Relative expression levels, however, were strikingly higher in EBs compared to slides, as exemplified at day 4 with a >250-fold higher expression in EB samples. Conversely, NES levels were not different between

EB and slide samples at day 4 ($p = 0.85$) and did not vary notably across culture conditions or time (Fig. 2D). Overall, culture of cells on collagen type IV-coated glass slides supports differentiation within days and was deemed an appropriate model in which to subsequently study the effects of shear stress on germ lineage specification.

Shear stress magnitude

Effects of shear stress and its dependency on stress magnitude were assessed on samples initially allowed to differentiate for a pre-treatment duration of two days and then either continued to be cultured under static conditions (static control) or exposed to shear stress of 1.5, 5.0 or 15 dynes cm^{-2} (shear samples; Fig. 1D) for an additional 2 days. Gene expression (M gene/M GAPDH) of samples of each experiment was normalized to the average value of the static controls for that trial (Fig. 3). Consistently small variations for all the different static groups, which were all cultured identically, indicate the reproducible nature of this model system. Furthermore, the small variances in the samples exposed to fluid flow validate the use of gene expression analysis to understand the general effects of shear stress on non-homogenous pluripotent stem cell-derived populations. In terms of pluripotency, shear samples at all tested magnitudes had similar levels of OCT4 expression compared to static controls (Fig. 3A). The absence of a detected significant change in OCT4 expression after exposure to shear stress is likely due to sustained expression after 4 days of differentiation, as is seen even in embryoid bodies (Fig. 2A).

Differentiation to the three germ lineages, however, was distinctly regulated by applied shear stress magnitude. No changes in endodermal differentiation (AFP) were significant in response to shear stress (Fig. 3C). Conversely, both mesodermal and ectodermal differentiation were significantly ($p < 0.05$ for both) affected by shear stress and in a magnitude-dependent manner. T-BRACHY expression in samples exposed to 1.5 dynes cm^{-2} decreased significantly ($p < 0.01$) to 43% of levels in static controls (Fig. 3B). Stress at higher magnitudes of 5.0 and 15 dynes cm^{-2} did not result in the same decrease in T-BRACHY expression but with a possible trend towards upregulation of expression. The observed increased variability and loss of statistical power to establish significance between static controls and shear samples may indicate that such trends are a result of an upregulation in only a subpopulation. Statistically significant ($p < 0.05$) increases, however, were observed in ectodermal differentiation when the sample as a whole was evaluated in response to shear stress at >5.0 dynes cm^{-2} (Fig. 3D). In additional control studies, these observations of germ lineage specification were not fully recapitulated when conditioned medium from sheared samples was applied to static cultures of ESCs. Based on these results, shear stress at a range of magnitudes (1.5–15 dynes cm^{-2}) induced modest changes in germ lineage specification, but prompted investigation into additional treatment parameters such as duration.

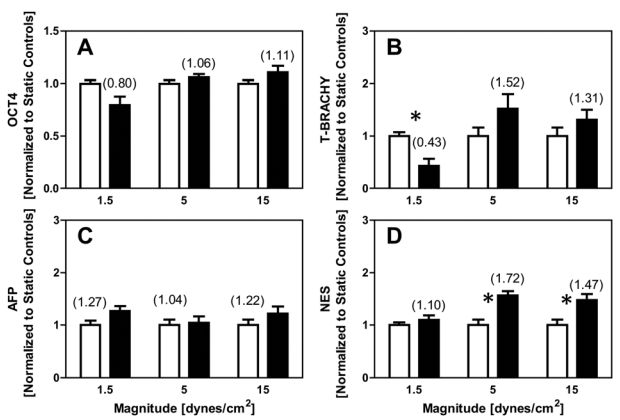

Fig. 3 Fluid shear stress at multiple magnitudes. Cells were initially allowed to differentiate for a pre-treatment duration of two days to promote adhesion to collagen type IV-coated slides. Experimental samples were then exposed to shear stress of 1.5, 5.0, or 15 dynes cm^{-2} (■) and compared with trial-matched static controls (□). Fold changes are indicated in parentheses and were calculated by normalizing the expression level of each sample to the average of its matched controls. Genes assessed were OCT4 (A), T-BRACHY (B), AFP (C), and NES (D). Data presented are averages (mean ± SEM; n = total of 9–12 replicates from 3 or 4 independent trials) and asterisks indicate a significant ($p < 0.05$) difference between static and shear stress groups.

Treatment duration

The well-defined but modest effect of shear stress on markers of differentiation motivated studies to determine the effect of sustained applied fluid flow. As above, the cells were initially allowed to differentiate for two days after which samples were exposed to shear stress ($\tau = 5.0$ dynes cm^{-2}) for 1, 2, or 4 days and compared to time-matched static controls (Fig. 4). Sample analysis indicated that shear stress duration had a significant effect ($p < 0.001$ in all cases) on gene expression for the pluripotency and germ lineage markers. OCT4 expression was largely unchanged between the shear samples and the static controls, though a significant ($p < 0.01$) difference was detected after 4 days of shear stress (Fig. 4A). In terms of differentiation to the mesodermal and ectodermal lineages, shear stress promoted differentiation with contrasting kinetics. T-BRACHY expression was not affected by shear stress after 1 day, but was increasingly upregulated with up to 4 days of applied shear (Fig. 4B). Inversely, the initial upregulation in NES expression after 1 day of applied shear stress was lost after 4 days (Fig. 4D). Observed differentiation towards the endodermal lineage between day 4 and 6 of culture under static conditions, on the other hand, was completely inhibited in the presence of shear stress (Fig. 4C). To distinguish the effects of extended application of shear stress from the application of shear stress at later time points, additional studies were performed that consistently applied only one day of shear stress but after varying durations of pre-treatment (Fig. S1, ESI†). Those studies did not recapitulate the effects on differentiation observed with extended application of shear stress. Thus taken together, these results indicate that persistent shear stress may bias differentiation of pluripotent cells from one germ lineage to another.

Signalling pathways analysis

Assessment of signalling pathways was performed to explore the upstream changes and potential mechanisms in shear stress-induced germ lineage specification. PCR arrays were

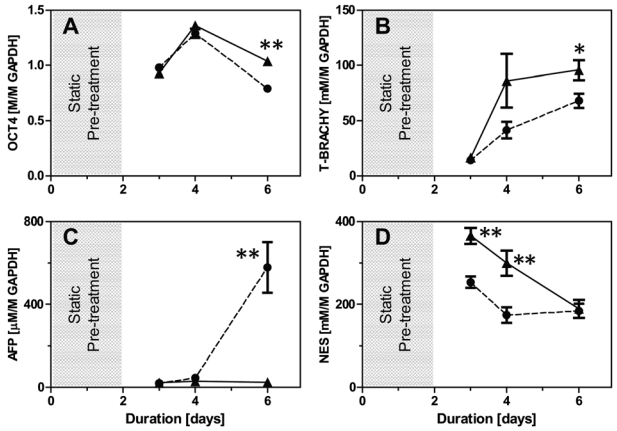

Fig. 4 Effect of shear stress duration. Cells were initially allowed to differentiate for a pre-treatment duration of two days to promote adhesion to collagen type IV-coated slides (grey shading). Experimental samples were then exposed to shear stress of 5.0 dynes cm^{-2} for a duration of 1, 2, or 4 days (▲, —). Control samples were matched in duration but cultured under static conditions (●, - - -). Gene expression (normalized to GAPDH) was assessed for OCT4 (A), T-BRACHY (B), AFP (C), and NES (D). Data presented are averages (mean ± SEM; n = total of 9–12 replicates from 3 or 4 independent trials) where asterisks indicate a significant ($*p < 0.05$, $**p < 0.01$) difference between experimental and control groups.

used to analyse fold regulation of gene expression on selected experimental conditions described above. Scatter plots of shear samples *versus* static controls displayed the overall number of genes that were affected by various shear stress conditions (Fig. 5A–D). Genes up- or down-regulated by greater than twofold were identified and listed on a color-coded plot (Fig. 5E and F).

A total of 28 genes were regulated ≥ 2-fold by the application of two days of shear stress at either 5.0 or 15 dynes cm^{-2} (Fig. 5A and B), but only 12 were similarly regulated across both examined magnitudes (Fig. 5E). The comparably regulated genes are involved in estrogen, NF-κB, and Wnt pathways. In particular, Bcl2l1 and Nfkbia, which are associated with estrogen and NF-κB pathways, respectively, undergo a 5-fold upregulation in response to applied shear. Several genes involved in the Hedgehog pathway, on the other hand, were found to be differentially regulated by shear stress at different magnitudes. These results suggest that the presence of shear stress activates multiple pathways, but the response of select pathways may be stress magnitude dependent.

Application of extended durations of shear stress had a striking effect on the number of signalling genes affected (Fig. 5C and D). When cells were exposed to only one day of shear, only 5 genes were found to be shear stress responsive. With two and four days of applied shear, 23 and 20 genes were responsive, respectively (Fig. 5F). Furthermore, only 4 of the genes found to be responsive with the longer durations were consistently regulated for both two and four days of shear. The previous discussed studies looking at markers of differentiation indicated that extended application of shear stress altered specification towards the three germ lineages. This signalling analysis further establishes that altered specification with continued application of shear stress may be due to activation of distinct pathways.

Mesoderm differentiation by shear stress

Effects of treatment duration described above suggested that application of shear stress for multiple days may be useful to enhance mesodermal populations of cells. To further explore the effects of extended durations of shear stress, cells were cultured under static conditions for two days followed by four days of either continued static or shear stress (τ = 5.0 dynes cm^{-2}) treatment. Under these conditions, shear stress significantly ($p < 0.05$) upregulated the gene expression of mesodermal homeodomain MEOX1 (Fig. 6A) and paraxial mesoderm marker PDGFRα (Fig. 6B) by 2.4- and 1.7-fold (respectively) compared to static controls. Flow cytometry assessments of similar samples indicated that shear stress also increased ($p < 0.05$) the protein expression levels of T-BRACHY (Fig. 6C and D) by 2.0-fold. Together, these results indicate that four days of shear stress promotes gene expression of the mesodermal germ lineage markers (both T-BRACHY and MEOX1), and that these changes are not transient, as indicated by increased protein expression of T-BRACHY and gene expression of the downstream mesodermal marker PDGFRα.

Discussion

In these *in vitro* studies we used a parallel plate bioreactor system to apply fluid-based shear stress to embryonic stem cells (ESCs) cultured on collagen type IV-coated glass slides. Germ lineage specification, which normally occurs during gastrulation *in vivo*, was shown to be affected by exposure to shear stress, known to deform the cell. In particular, shear stress most significantly affected mesodermal and ectodermal differentiation, where extended durations of shear stress promoted mesoderm differentiation (Fig. 7). Application of shear stress also activated multiple signalling pathways, including Wnt, NF-κB, and estrogen. These pathways are consistent with some of the genes necessary for proper primitive streak formation and mesodermal separation.[17] Furthermore, the observed magnitude- and duration-dependent activation of shear-sensitive signalling may help orchestrate the extensive sequence of events that guide germ layer rearrangement *in vivo*. Overall, these studies imply that cellular deformation due to spatial and temporal mechanical dynamics may be an important regulator of early differentiation.

Forces experienced by cells can be imposed externally or generated internally. External cues originate in the cellular microenvironment, where deformation of surrounding tissue or adjacent cells causes stresses and strains (normalized forces and deformations, respectively) at the cell membrane and throughout the cytoskeleton. For example, the flow of blood across the cell lining in the vasculature leads to shear stresses that directly deform the endothelial cells.[18] Actin–myosin molecular motors instead generate forces within the cells, which are transmitted along cytoskeletal proteins.[19] The interfaces at which forces propagate between the external and internal cellular compartments are comprised of transmembrane protein complexes that govern cell–cell and cell–matrix binding.[20] Due to the strong link between the two types of forces, the study of mechanobiology must include both observational studies *in vivo* to provide complete context of tissue level responses, as well as

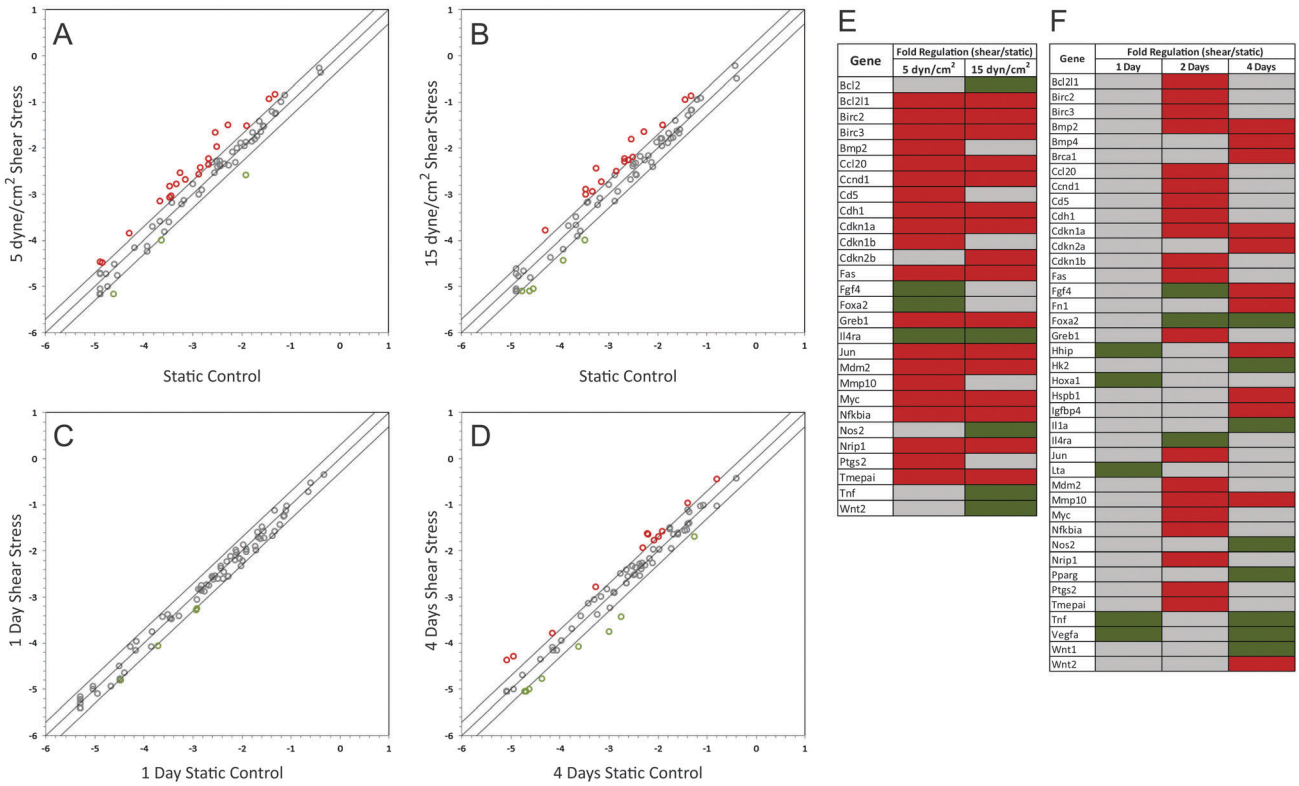

Fig. 5 Shear stress-activated signalling pathways. PCR-arrays of 84 genes were used to assess signal transduction pathways. Relative gene expression is displayed with scatter plots of shear *versus* static samples (A–D). Genes regulated by $\geq$ 2-fold were identified and are listed alphabetically (E and F) with upregulation (shear/static $\geq$ 2) indicated in red and downregulation (static/shear $\geq$ 2) indicated in green. For all samples, cells were pre-treated for two days as previously. Samples evaluated for stress magnitude were exposed to two days of shear stress at 5.0 or 15 dynes cm^{-2} (plotted in A and B, respectively, and summarized in E). Effects of extended duration of applied shear stress were evaluated in samples exposed to 5.0 dynes cm^{-2} of shear stress for one (C), two, or four (D) days (summarized in F). Data represented four samples.

mechanistic studies *in vitro* to understand the effects of force on cell behaviour.

External mechanical forces have long been associated with establishment of the body plan.[21] A recent resurgence of interest in the topic,[22] along with novel imaging techniques,[23] has helped create a detailed spatial and temporal mapping of key mechanical events during development. For example, one of the first instances of mass fluid movement is nodal flow, which helps establish left–right asymmetry.[4] Similarly, shear stress due to fluid flow has been identified as a critical component in normal development of cardiac,[5b,24] valvular,[25] vascular,[26] and hematopoietic[27] tissue. These studies have helped correlate *in situ* shear stresses to changes in phenotype, cellular arrangements, and tissue growth during the later stages of development.

Gastrulation during early development involves spatial rearrangements into layers that specify germ lineages. Major morphological changes include convergence and extension due to cell intercalation, involution caused by tissue inflection, and epiboly thinning as cell layers spread.[28] Computational models have shown that such mass movements of cell sheets can be accounted for by membrane deformations in individual cells.[29] Few studies, however, have directly investigated the effect of cell deformation on germ lineage specification. Farge found that compression of whole embryos in drosophila altered the spatial pattern of twist gene expression during germ band extension.[30] In mouse ESCs, it was recently shown that local deformation of cell membranes leads to downregulation of the pluripotency marker OCT3/4.[31] In the studies we presented here, it was further shown that shear stress applied to the cell membranes of mouse ESCs directly alters specification to the three germ lineages. Taken together, these initial few studies explicitly establish the importance of the mechanical microenvironment on early developmental patterning. General mechanistic understanding of mechanical regulation of germ lineage specification, however, requires additional *in vitro* and *in vivo* studies to build upon this nascent knowledge base.

Well-controlled studies investigating physical regulators of cell fate decisions can provide insights into mechanical events that pervade development. Advancing technologies[32] aid in better characterization of the mechanical milieu *in vivo*, but the highly dynamic spatial and temporal presentation of soluble factors, proteins, and cell–cell interactions together complicate mechanistic understanding of the physical cues on cell behaviour. Use of other organisms, such as flies, worms, and fish, provides simpler models that allow for techniques such as complete genetic analysis[33] and *in situ* imaging.[23b] Such assessments, however, are technically challenging in mammalian systems. Instead, the use of embryonic stem cells allows for direct quantification of differentiation effects due to isolated factors *in vitro* and studies with human cells that are not ethically permissible *in vivo*. Furthermore, while these studies focused primarily on

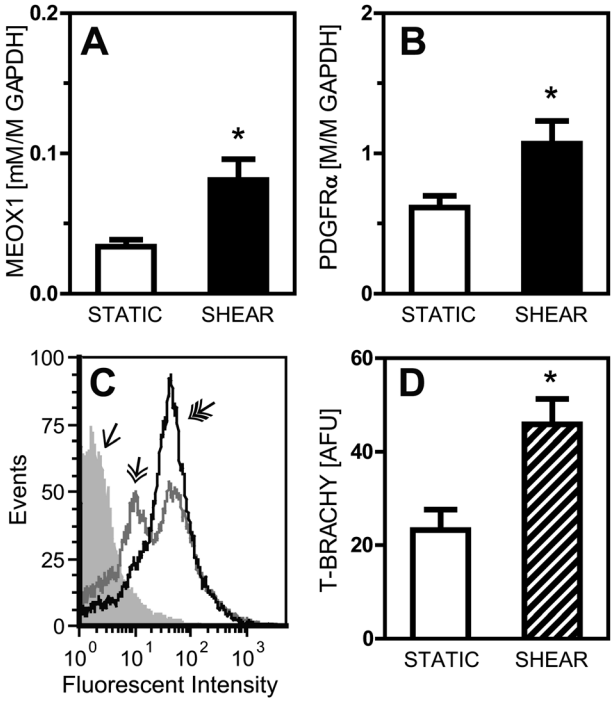

Fig. 6 Extended durations of shear stress and mesodermal differentiation. Cells were cultured under static conditions for two days followed by four days of either continued static culture (STATIC) or shear stress treatment (5.0 dynes cm^{-2}; SHEAR). Cells were assessed for gene expression of mesodermal markers MEOX1 (A) and PDGFRα (B) normalized to GAPDH. A representative histogram of T-BRACHY protein expression (C) is shown for an immunostaining 2° antibody-only control (filled; single arrowhead), static experimental control (double arrowhead) and shear stress experimental sample (triple arrowhead). Median fluorescence levels (D) in Arbitrary Fluorescent Units (AFU) was calculated from individual histograms and averaged for both static control and shear stress experimental groups. Data presented are averages (mean ± SEM; n = 10–11 for A, B and n = 4 for D) where asterisks indicate a significant ($*p < 0.05$) difference between STATIC and SHEAR groups.

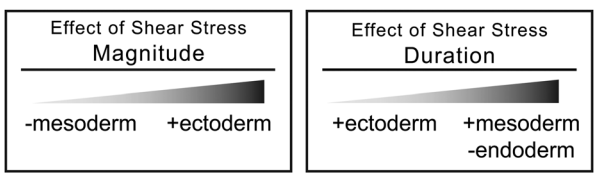

Fig. 7 Potential strategies for producing cell populations of phenotypes from the distinct germ lineages using fluid shear stress. This schematic diagram summarizes the response of pluripotent stem cell specification in response to varying magnitudes and durations of shear stress in adherent culture.

physical forces, the effects can be taken into context of additional biochemical cues present during development through supplementation with growth factors (*e.g.* VEGF) or altering the protein substrate.

Mechanical forces, such as shear stress, compression, and tension, have long been applied *in vitro* to differentiated cells, and more recently to pluripotent and multipotent stem cells. For example, a vast number of studies have investigated the effects of shear stress and cyclic tensile strain on the function of endothelial (reviewed in Chien, 2007[34]) and smooth muscle cells (reviewed in Zou *et al.*, 1998[35]), respectively. In recent years, similar *in vitro* systems have been used to study the effect of mechanical cues on differentiation of adult stem cells, such as bone-,[36] blood-,[37] or adipose-[38] derived cells. A less explored area is the effect of mechanical cues on embryonic stem cells. Some groups have explored the differentiation of ESCs in embryoid bodies using mass fluid flow in stir-based bioreactors,[16,39] which excel in maintaining a well-mixed system to replenish nutrients and remove waste products in close proximity to cells.[40] Primary focus on many of those studies has been to enhance the subpopulation of a target differentiated phenotype, but mechanistic understanding of differentiation is hindered by a spatially and temporally variable chemical and mechanical microenvironment.[41]

Application of a fluid shear stress to cell layers induces membrane deformation,[42] which in a cytoskeleton-dependent manner[43] can lead to persistent changes in cell stiffness[44] and shape.[9b,45] Previous studies have shown that ESC-derived cells sorted for FLK1+,[10a] CD31+/CD45−,[46] and CD41+[10b] have taken on more mature endothelial and hematopoietic phenotypes in response to applied shear stress. In those cases cells were significantly differentiated, either on adherent surfaces or in embryoid bodies, prior to the application of stress in order to examine the effects of mechanical manipulation on the later stages of phenotype commitment. Other studies also used shear stress at later stages of differentiation to evaluate ESC-derived cells for endothelial functionality, focusing on alignment to the direction of fluid flow and gene expression modulation.[46,47] Fewer studies, however, have investigated the effects of mechanical cues during early differentiation on unsorted ESC populations. It has been shown[11,48] that even earlier application of shear stress also results in endothelial differentiation. In other *in vitro* models, however, it was shown that mechanical inputs can direct earlier fate decisions. Cyclic tensile strain has been shown to promote self-renewal,[49] while passive substrate elasticity affects germ lineage specification.[50] Our study is unique in that it utilizes a well-defined *in vitro* system to explore the effect of applied cell deformation on germ lineage specification, one of the earliest processes in differentiation. These types of studies lend insight not only into complex developmental processes, but also into approaches that can leverage shear stress to direct early differentiation in pluripotent cells (Fig. 7).

We have shown that fluid shear stress may be an appropriate method for promoting mesoderm phenotypes (Fig. 6). Previous efforts targeting mesoderm differentiation have been typically mediated by the addition of growth factors, such as BMP4,[51] activin A,[51b] or using conditioned media.[52] EB-based studies in pluripotent mouse cells have shown that media supplementation by BMP4 can increase gene expression of T-BRACHY by 2.0- to 3.5-fold[51] after 1–4 days. However, these methods have been shown to concomitantly promote non-mesodermal lineages,[53] particularly endoderm.[54] In our adherent model system, four days of shear stress increased T-BRACHY gene expression by 1.4-fold and protein expression by 2.0-fold (Fig. 4 and 6, respectively) while downregulating the gene expression of the endoderm marker AFP by 96% (Fig. 4). This suggests that mechanical cues and soluble factors may

direct germ lineage specification through independent means. Thus, shear stress applied during early differentiation events may be a complementary approach to use of cytokines in generating downstream phenotypes for tissue engineering cell therapy applications.

Though there is evidence that cyclic strain and shear stress may activate discrete pathways in other cell types,[55] little is known of overall mechanotransduction pathways in ESCs. Initial studies have shown that the shear stress response of ESCs does involve epigenetic changes[56] and heparan sulphate proteoglycans.[57] Studies using differentiated phenotypes, however, have identified several pathways activated by shear stress. For example, shear stress applied to endothelial cells has been shown to activate FLK1,[58] PECAM1,[59] ion channels,[60] integrins,[61] and G-proteins.[62] These mechanosensors then activate multiple intracellular pathways, including focal adhesion kinase (FAK)[63] and nitric oxide (NO) release.[64] It is unclear, however, if unspecialized pluripotent stem cells (or early derivatives) have the same machinery as differentiated cells to sense and transduce externally applied mechanical forces. For example, studies in ESCs have shown that NO synthase elements are not activated during early differentiation[65] and that ESC-derived endothelial cells still produce markedly lower levels of NO compared to mature endothelial cells.[66] Conversely, enduring machinery of the source somatic phenotype may alter the differentiation response of induced pluripotent cells (iPSC) to applied mechanical forces. Thus, additional studies in stem cell mechanotransduction are necessary to identify target signalling pathways for directed differentiation.

The studies presented here, though limited to shear stress, are among the first to directly characterize the effect of mechanical force parameters on germ lineage specification, one of the earliest differentiation events. Differentiation of stem cells can be depicted by an adaptation of Conrad Waddington's illustration of the epigenetic landscape (Fig. 8). In that metaphor, Waddington compared a marble rolling down a slope to the differentiation of a cell, where the ultimate cell phenotype is represented by the valleys at the base of the slope where the marble eventually settles.[67] This classic metaphor of development can be expanded to integrate effects of mechanical forces: changes in the default path of the rolling ball (Fig. 8A) are influenced by the application of a force (Fig. 8B, arrow), its magnitude (Fig. 8C), the duration of application (Fig. 8D), and the stage of differentiation at which force is applied (Fig. 8E). As the mechanical milieu is only one of a host of modulating cues, its effects are mitigated by synergistic influences with other environmental factors (Fig. 8F, double arrow head), such as soluble gradients (*e.g.* VEGF), presented proteins, and adjacent cells. Thus, just as studies with chemical factors are considered with respect to dose, studies of mechanical signals must be interpreted in the context of the independent characterizing parameters.

It is important that these shear stress outcomes be followed by additional mechanobiology studies of pluripotent stem cells. Due to the practical limitations of our model system, shear stress was applied to ESCs that were differentiated for up to two days to promote cell–surface attachment. While assessment of the select germ lineage genes used in this study implied that major differentiation events had not yet occurred after two days of pre-treatment, confirmation through evaluation of additional germ markers is important. It is also important to ultimately evaluate effects of cell deformation using 2D and 3D model systems that better mimic *in vivo* development. For example, the current studies presented collagen type IV to the ESCs; future efforts may mimic the protein environment during early embryogenesis by presenting fibronectin[68] or laminin,[69] where functional matching of the matrix stiffness is likely important.[70] This model system can thus be expanded to multivariate studies to investigate the effects of other factors (*e.g.* soluble growth factors, extracellular matrix proteins, heterotypic cell interactions, *etc.*), either independently or synergistically, for a more comprehensive understanding of environmental regulators of germ specification. Furthermore, the mechanotransduction pathways responsible for converting the physical microenvironment into intracellular signals in stem cells need to be better characterized; not only do we need to identify force-related sensors, transducers, and effectors,[71] but determine whether mechanical effects can also activate known receptor–ligand pathways by inducing autocrine or paracrine soluble signals. Such fundamental knowledge of mechanical regulation of early differentiation processes can further understanding of normal and pathological developmental processes. These concepts can be leveraged to proficiently promote the initial fate decisions of differentiation that ultimately dictate terminal phenotypes. Improved efficiency using mechanical factors to generate large numbers of target phenotypes would markedly increase the clinical impact of stem cell-based therapies.

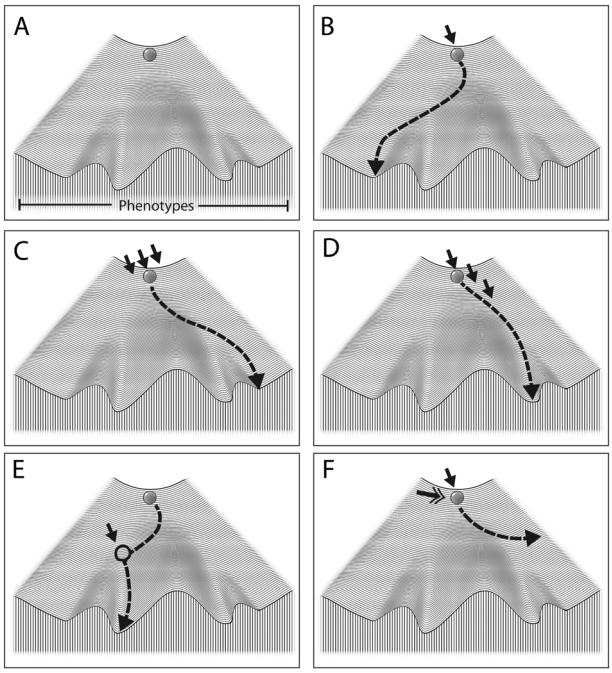

Fig. 8 Mechanical parameters depicted on Waddington's epigenetic landscape. A stem cell, represented as a ball at the top of a hill (A), can be influenced by forces (arrow; B) during cell fate decisions that lead to a specialized phenotype (valley). The path of differentiation is modulated by the shear stress magnitude (simultaneous arrows; C), the duration of application (sequential arrows; D), the stage of differentiation in which the force is applied (modified ball; E), and synergy with other environmental factors (double headed arrow; F).

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