Journal of Biomechanics 48 (2015) 1777–1781

Contents lists available at ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
www.JBiomech.com

Embryonic stem cells growing in 3-dimensions shift from reliance on the substrate to each other for mechanical support

Ailing Teo [a], Mayasari Lim [a], Daphne Weihs [b,*]

[a] Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637459, Singapore
[b] Faculty of Biomedical Engineering, Technion-Israel Institute of Technology, Haifa 3200003, Israel

ARTICLE INFO

Article history:
Accepted 6 May 2015

Keywords:
Cell biomechanics
Traction force microscopy
Mouse embryonic stem cells

ABSTRACT

Embryonic stem cells (ESCs) grow into three-dimensional (3D) spheroid structures en-route to tissue growth. *In vitro* spheroids can be controllably induced on a two-dimensional (2D) substrate with high viability. Here we use a method for inducing pluripotent embryoid body (EB) formation on flat polyacrylamide gels while simultaneously evaluating the dynamic changes in the mechano-biology of the growing 3D spheroids. During colony growth in 3D, pluripotency is conserved while the spheroid–substrate interactions change significantly. We correlate colony-size, cell-applied traction-forces, and expressions of cell-surface molecules indicating cell–cell and cell–substrate interactions, while verifying pluripotency. We show that as the colony size increases with time, the stresses applied by the spheroid to the gel decrease in the 3D growing EBs; control cells growing in 2D-monolayers maintain unvarying forces. Concurrently, focal-adhesion mediated cell–substrate interactions give way to E-cadherin cell–cell connections, while pluripotency. The mechano-biological changes occurring in the growing embryoid body are required for stabilization of the growing pluripotent 3D-structure, and can affect its potential uses including differentiation. This could enable development of more effective expansion, differentiation, and separation approaches for clinical purposes.

1. Introduction

Formation of embryoid bodies marks an important step in ESC development by enabling initial formation of tissue-like structures that may later be differentiated (Doetschman et al., 1985). Formation of 3D spheroids can be induced on tunable 2D substrates, but can also spontaneously form in 3D hydrogels such as alginate, which support long-term maintenance of ESC pluripotency (Siti-Ismail et al., 2008) and are convenient for use in bioreactors (Teo et al., 2014). Biochemical events supporting embryoid body (EB) formation include cell–cell interactions facilitated by E-cadherin – an essential component for cytoskeletal organization during embryo development and regulate proliferation and differentiation of pluripotent stem cells (Li et al., 2012).

Conventional methods entail growing embryoid bodies in free suspension, however, formation of embryoid bodies on 2D substrates provides tunable physical properties such as hydrophobicity and matrix stiffness that could give better control in uniformity of embryoid body population (Harris et al., 1980) and influence (eventual) ESC differentiation (Zheng and Zhang, 2011). Mechanical loading of cells through the substrate has also been shown to affect cell differentiation and development (Shoham and Gefen, 2012; Shoham et al., 2015), where stiffening of the cells themselves is also related to their function and can indicate disease states such as cancer (Gal and Weihs, 2012; Guck et al., 2005), or response of different cell types to changes in their environments (Poh et al., 2010). Those studies not only supply evidence to the significance of mechano-biology in stem cell development, but also provide important tools for controlling stem cells *in vitro*. Differentiating stem cells are highly affected by their biomechanical environment, where changes in substrate stiffness decides the lineage fate of the cells (Engler et al., 2006). Hence, it is important to monitor dynamic biomechanics during critical stem cell development stages, such as induction of EBs on 2D substrates using mechano-biology tools such as atomic force microscopy (Kiss et al., 2011) and traction force microscopy (Butler et al., 2002).

Here, we combine biomechanical and biochemical methods to show the mechanical changes occurring during development of a 3D spheroid/embryoid body of ESCs, providing insight into cell status and adherence. We studied the biomechanical development of 3D colonies of murine ESCs (mESCs), from a 2D plated colony, comparing the time-dependent colony size, cell-applied traction stresses, and various biochemical indicators, such as cell–substrate connections, cell–cell junctions, and maintained pluripotency of mESCs, at the initial stages of 3D colony development. We seeded

* Corresponding author.
E-mail address: daphnew@tx.technion.ac.il (D. Weihs).

http://dx.doi.org/10.1016/j.jbiomech.2015.05.009
0021-9290/

mESCs on a soft, elastic polyacrylamide (PAM) gel with sub-micron fluorescent particles embedded at its surface to facilitate traction force microscopy (TFM) measurements. We measured cell traction forces exerted by cells on PAM gels as the initially 2D mESC colonies expanded also vertically; TFM was measured on days 1–3 after the day of seeding ($t=0$), where beyond that time colonies merged on the surface and edges were indistinguishable. As a control, we have also evaluated the traction stresses applied by human hepatoma cells (HepG2) that grow and remain as a 2D monolayer; as those cells grow more slowly, HepG2 cells were monitored up to 5 days from day of seeding.

2. Materials and methods

2.1. ESC and HepG2 cell culture

Murine ESCs (E14TG2a cell line, ATCC, Manassas, VA, USA) were maintained on 0.1% porcine–gelatin coated (Sigma-Aldrich, St Louis, MA, USA) tissue culture flasks under high-glucose DMEM (Gibco, Lifetech, Carlsbad, CA, USA) with 10% fetal gold-standard bovine serum (FBS, GE Healthcare, Buckinghamshire, United Kingdom), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 2 mM L-glutamine (Gibco, Lifetech), 1x penicillin–streptomycin (GE Healthcare) and 1000 U/mL leukemic inhibitory factor (LIF, from Chemicon, Millipore, Billerica, MA, USA). LIF was present in the medium to prevent any spontaneous differentiation of mESCs, thus ensuring their pluripotency. Medium was changed daily and cells were passaged every 2–3 days when 70–80% confluence was reached.

Human hepatoma cells (HepG2 cell line, ATCC) was used as control cells to compare with mESCs. To prepare human hepatoma cells for seeding on polyacrylamide gels, the hepatoma cells were first cultured in high-glucose DMEM with 10% FBS and 1x penicillin–streptomycin. HepG2s were then routinely passaged every 4 days prior to seeding onto the polyacrylamide gels.

2.2. Gel preparation and cell seeding

The polyacrylamide (PAM) gel was prepared according to an established protocol (Kristal-Muscal et al., 2013; Raupach et al., 2007). A plastic frame (Gene Frame, 25 μl, 10×10 mm^2, Thermo Fisher Scientific, Waltham, MA) was adhered onto a circular, glass coverslip, 25 mm in diameter (Fisher Scientific, Hampton, NH), to confine the liquid gel precursors. The glass coverslip was pre-treated to enhance gel adherence: coated with hydroxyl groups using 0.1 M NaOH (Sigma, St. Louis, MO), and activated with 3-aminopropyltrimethoxysilan (Sigma, St. Louis, MO, USA). Blue-fluorescent, particles, 1 μm diameter (Life Technologies, Carlsbad, CA) were adhered to the glass coverslip by pipetting 50 μL of the stock solution (0.002%) and drying overnight; particles provide constant location markers to remove any stage drift-motions between experiment days. The glass surface was then fixed with 0.5% (v/v) glutaraldehyde (Thermo Fisher Scientific, Waltham, MA).

PAM gels, 4200 Pa stiffness, were generated on ice using 8% (v/v) acrylamide and 0.025% (v/v) BIS acrylamide as a crosslinker (Bio-rad, Hercules, CA) in ultrapure water. Red fluorescent particles, 200-nm diameter (Life Technologies, Carlsbad, CA) were added to the solution. Gelation was induced by adding 0.04% (w/v) APS as initiator and 0.2% (v/v) TEMED as a catalyst (Sigma, St. Louis, MO). The gel solution was placed on the activated glass and covered with a flexible, plastic coverslip; exposure to oxygen inhibits PAM gel polymerization. During polymerization and gelation, the glass-plastic sandwich as centrifuged for 30 min at 300 g, to bring the 200-nm particles to the gel surface (Pelham and Wang, 1997; Raupach et al., 2007). Further gelation was at room temperature in an upside down configuration to keep the tracker particles at the gel surface. After gelation the flexible plastic coverslip and frame were removed and the gel was kept in PBS for at least 24 h, to allow any residual polyacrylamide monomers to diffuse out.

To allow cell adherence gel surfaces were made bio-compatible. The surface of the gel was cross-linked with Sulfo-SANPAH (Thermo Fisher Scientific, Waltham, MA), conjugated with collagen (Rat tail type 1, Sigma, St. Louis, MO, USA), and kept at 4 °C for 16 h (Butler et al., 2002; Raupach et al., 2007). Following that, mESCs or HepG2 cells were seeded onto the PAM gel at concentrations of 2×10^5 cells/cm^2 or 1×10^5 cells/cm^2, respectively, in their original growth media that was changed daily. mESCs and HepG2 were cultured for 3 and 5 days on the gels, respectively, until colonies merged and were no longer distinguishable.

2.3. Traction force microscopy

An inverted Axiovert 200 M fluorescence microscope and AxioCam MRC camera (Zeiss, Oberkochen, Germany) were used to collect images of the cells and fluorescent particles embedded in the PAM gel and on the glass. Random colonies on the gel were images that were large enough to fill a field-of-view were observed already on day 1. The applied stress fields were determined through displacements of the fluorescent, sub-micron particles embedded within the PAM gel (Dembo and Wang, 1999; Krishnan et al., 2011). We used MATLAB 2011b based codes kindly provided by Ramaswami Krishnan from Harvard for the analysis of traction in a region within a monolayer. Briefly, when lateral, traction force is applied by cells to the gel, the gel is deformed, and the particles at the gel surface move by an equivalent strain. We measure the resulting gel-deformation by comparing images with and without cells, producing a displacement map of the beads; after cell removal the gels returns to an undeformed state. The displacement map is used to evaluate the cell-applied stresses through the known stiffness of the gel, its Young's modulus.

2.4. Quantitative polymerase chain reaction (PCR)

For analyzing gene expression levels involved in cell adhesion activities, mESCs were cultured on collagen-conjugated, 25 mm × 25 mm PAM gel sheets in 6-well plates. Those cells were then extracted on each day for quantitative PCR gene analysis. Total RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany) and quantified with a UV spectrophotometer (Nanodrop 2000c, Thermo-Fisher Scientific, Waltham, MA). The RNA (1 μg) was used to synthesize the first strand of complementary DNA, using M-MLV reverse transcriptase, dNTP mix, RNasin and oligo-dT primers and nuclease-free water (reagents from Promega, Madison, WI, USA) all used according to manufacturer's instructions. PCR amplification and measurement was then carried out with CFX Connect™ Real-time PCR Detection machine (Bio-rad, Hercules, CA, USA) and SensiFAST™ SYBR Fluorescein Kit (Bioline, UK). The amplification consisted of an initial denaturation step at 95 °C for 2 min and 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 30 s. Gene expressions were compared using the $2^{-\Delta\Delta CT}$ method by normalization to the geometric means of four endogenous control genes (see Table 1 for primers list), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), TATA box binding protein (TBP), succinate dehydrogenase complex subunit A (SDHA), and phosphoglycerate kinase 1 (PGK1), and then normalized to day 1 samples.

2.5. Statistical analysis

Statistical reliability of root-mean-square (RMS) traction stress of the mESC and HepG2 cultures and gene expression fold-changes were assessed with *P*-values determined by two-sided student *t*-test with unequal variances. The *P*-values for

Table 1
Primer sequences used for quantitative RT-PCR.

Target gene	Forward sequence	Reverse sequence
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
TBP	GAAGAACAATCCAGACTAGCAGCA	CCTTATAGGGAACTTCACATCACAG
SDHA	GCTCCTGCCTCTGTGGTTGA	AGCAACACCGATGAGCCTG
PGK1	CTGACTTTGGACAAGCTGGACG	GCAGCCTTGATCCTTTGGTTG
PTK2	GACCTGGTTATCCTAGCCCGAGA	ATGCCTGACCTTGGTAAATGCTG
CDH1	ACTGTGAAGGGACGGTCAAC	GGAGCAGCAGGATCAGAATC
Nanog	CCTGATTCTTCTACCAGTCCCA	GGCCTGAGAGAACACAGTCC
Oct-4	TGTGGACCTCAGGTTGGACT	CTTCTGCAGGGCTTTCATGT