The regulation of gene expression during onset of differentiation by nuclear mechanical heterogeneity

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ABSTRACT

Embryonic stem (ES) cells exhibit plasticity in nuclear organization as well as variability in gene expression. Although such physicochemical features are important in lineage commitment, mechanistic insights coupling nuclear plasticity and gene expression have not been elucidated. To probe this, we developed single cell micro-patterned assay to map nuclear deformation and its correlation with gene expression. We found an inherent heterogeneity in nuclear pliability of ES cells. Softer nuclei deformed to the underlying substrate geometry while the stiffer ones remained spherical. Stiffer nuclei were strongly correlated with decreased global histone (H3) acetylation and an increase in Lamin A/C expression. Interestingly, these cells also have higher nuclear accumulation of the transcription cofactor MRTF-A (myocardin-related transcription factor A) and an upregulation of its downstream target genes. Taken together, our results provide compelling evidence to show that the mechanical heterogeneity of stem cell nucleus can regulate transcriptional programs during onset of cellular differentiation.

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1. Introduction

Stem cells reside in 'niches' or 'microenvironments' which provide them with both physical stimuli and specific growth factor combinations leading to cell-fate decisions [1–3]. Stem cells indicate preferential lineage biases during differentiation by responding to mechanical features like the underlying substrate rigidity [4,5], geometric shape constraints [6,7] or variations in the cell spreading areas [6,8] corresponding to that of the physiological tissue elasticity [1]. These mechanical properties of cells are mostly defined by the cell membrane, cytoskeleton and the nucleus [9]. Previous studies show that the structural composition of both the nucleus [10–13] and the cytoskeleton are altered in these stem cells [1,14–16] and with onset of differentiation their nuclear stiffness increases [17,18]. The stem cell transcriptome also exhibits variable expression of key regulatory genes products [19–21] that is utilized for distinct differentiation potential [22,23] and possibly provides heterogeneous mechanical properties to stem cells. In addition, activation of MRTF-A (an actin dependant transcription cofactor) have been shown to upregulate its target genes involved in actin cytoskeleton pathway to stabilize nuclear morphology during cellular differentiation [24]. However, the coupling between the mechanical and transcriptional states of stem cells and its functional role during onset of stem cell differentiation has not been studied.

In this paper, we hypothesize that the nuclear mechanical properties may influence cytoplasmic- to-nuclear localization of transcription factors to modulate gene expression. In particular if Lamin A/C dependant nuclear mechanical pliability regulated actin related gene expression during early onset of mouse embryonic stem cell differentiation. To test this hypothesis, confocal fluorescence imaging experiments were carried out on stem cells cultured on fibronectin coated micro-patterns. Immuno-fluorescence staining with various differentiation markers, selected using whole genome microarray analysis, was then used to assess the coupling between nuclear mechanical heterogeneity and the spatial compartmentalization of transcriptional control.

2. Materials and method

2.1. Preparation of PDMS stamps and micro-contact printing

PDMS stamps were prepared from PDMS Elastomer (SYLGARD 184, DOW Corning) in a 10:1 ratio of silicone elastomer and a silicone elastomer curing agent. Both components are mixed to homogeneity and degassed in a desiccator for 10 min. The silicon wafers with desired micro-fabrications were cleaned of small particles using isopropanol and by sonicating at 20 °C for 20 min. The 10:1 ratio mixture of elastomer was poured onto the wafer and again degassed in the desiccators for at least 20 min to remove any trapped air bubbles. Later the elastomer mixture on the silicon wafer was cured at 80 °C for 2 h before it solidified into stamps were peeled off from the silicon wafer. Micro-patterned PDMS stamps were oxidized and

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sterilized under high power in Plasma Cleaner (Model PDC-002, Harrick Scientific Corp) for 5 min 10 μl of fibronectin solution (with unlabelled and labelled fibronectin with Cy3/Cy5 to a final concentration of 100 μg/ml) in 1XPBS was poured onto the surface of each PDMS stamp in dark and sterile conditions for 10 min and was allowed to dry. The extra fluid was removed using capillary action of a fine tissue. The stamp was examined till semi-dried under the light microscope, and then inverted onto the surface of non-treated hydrophobic plastic bottom dishes (ibidi). The stamped dishes were then inspected under fluorescent microscope to observe the micro-features of the stamp as it was marked using fluorescent tagged fibronectin. The stamped dishes were treated with Pluronic F-127 (2 mg/ml) for 30 min, to allow the cells to attach to only fibronectin islands.

2.2. Cell culture and pharmacological inhibitors treatments

Mouse embryonic cells like R1ES cells and H2B-EGFP expressing ES cells were maintained by culturing on gelatin (0.1%) coated dishes with reconstituted media to rule out any contamination with PMEFs while plating on the stamps. ES cell Knockout DMEM was supplemented with 15% Knockout Fetal Bovine Serum, 1 mM sodium pyruvate (Sigma), 0.1 mM nonessential amino acids, 2 mM L-Glutamine, 0.1 mM β-mercaptoethanol (Sigma), and 500 U/ml leukaemia inhibitory factor (LIF) (Merck Millipore) and penicillin−streptomycin. All cell culture reagents, unless otherwise indicated, are from Life Technologies. Cells were maintained at 37 °C in a 5% CO_2 incubator. For both live-cell imaging and fixed-cell experiments, cells were split and 65,000 cells were plated on the micro-contact printed ibidi dishes or fibronectin coated dishes, and washed off after 30 min to remove the remaining unattached cells. Various pharmacological inhibitors treatments were used like Cytochalasin D (Sigma) at 2 μM for 5 min at 37 °C for actin depolymerization, Blebbistatin from 5 to 50 μM for 20 min at 37 °C and then fixed for inhibiting myosin motor dependent actomyosin contractility.

2.3. Plasmids

Tractin-mRFP plasmid was obtained from Bershadsky lab. The pEGFP-Lamin A/C C1 plasmid was made by Protein Cloning and Expression Core facility at MBI, Singapore. For transfection of cells were plated on gelatin (0.1%) coated Nunc dishes for one day and then transfected with 500 ng of DNA using Lipofectamine 2000 and Opti-MEM. The cells were later split after 16−18 h and put on stamped ibidi dishes of fibronectin coated dishes for live-cell imaging.

2.4. Antibody staining

Immunostaining protocol has been described in the previous chapter. For immunostaining, the following antibodies were used MRTF-A (1:150 dilution, rabbit monoclonal, Santa Cruz), Lamin A/C (1:50 dilution mouse monoclonal, Abcam), Oct-4 (1:500 dilution rabbit polyclonal, Abcam), H3Ac (1:200 dilution rabbit polyclonal, Upstate) and phosphoMyosin Light Chain (1:100 dilution rabbit polyclonal, Cell Signalling) and secondary antibodies were used at 1:500 dilution (Invitrogen). F-Actin was stained using Rhodamine phalloidin (Sigma). DNA was stained using Hoechst 33342 (Sigma) at a concentration of 1 μg/ml.

2.5. Confocal imaging

Nikon A1R Confocal microscope and Images were taken using Carl Zeiss 710 Meta and was used in our experiments. Imaging and FRAP experiments on cells were carried out using CFI Plan ApochromatVC 100×, 1.4 N.A oil immersion objective (Nikon) and C-Apochromat 63×, 1.2 N.A water immersion objective (Zeiss). For comparison in protein expression the samples were labelled similarly and imaged with identical acquisition settings. EGFP-tagged proteins and FITC/Alexa-488 were excited with the 488 nm line of an Argon-ion laser (Melles Griot Multi Line Argon 65 mW) and the emission collected with a 525/50 band pass filter. Fluorophores like Cy3/Rhodamine and Alexa-647 were excited using 543 nm (Coherent Sapphire 561 nm 20 mW) and 633 nm (Coherent CUBE 644 nm 40 mW) laser line and collected using 630/75 band pass and 700/75 filters, respectively. 405 laser line (Coherent CUBE 405 nm 100 mW) was used to excite Hoechst 33342 and collected using 460/50 emission filters. Confocal images (512 × 512 pixels, 12 bit images, with optimal pinhole sizes) were acquired. Z-stacks of nuclei with a step size of 0.8 μm were acquired.

2.6. Microarray sample preparation and analysis

Gene expression changes upon induction with fibronectin were compared in a time course manner. Control cells were grown on gelatin coated dishes and the experimental cells were plated on fibronectin coated dishes for 4 h, 12 h and 24 h respectively. 10^6 cells were seeded on each petridish for the specified time duration. Non-adhered cells were removed after 1 h of plating and cultured in the above mentioned reconstituted ES cell media. After growing cells for the required time duration on the dishes, cells were harvested for extracting the total RNA using RNeasy Minikit (Qiagen) RNA isolation kit. Duplicate experiments were done for gelatin control and all time points. RNA concentration and purity was determined using Nanodrop® ND-1000 spectrophotometer (Wilmington, DE) and the integrity of RNA verified on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip

(Agilent Technologies, California). Equal amounts of RNA was labelled using Agilent dye Cyanine 3-CTP for single colour microarray and hybridized to Mus musculus Gene Expression Array 4 × 44 K. The slides were scanned using Agilent Microarray Scanner G2565BA, and data was extracted from images using Feature Extraction software v9.5.3 from Agilent. Mean expression of each gene was calculated by taking an average of all background corrected probes for the same gene. Microarray data analysis services were provided by Genomax technologies, Singapore. The microarray data was analysed using the Genespring 12.0 software.

2.7. Data analysis

Relative nuclear area fluctuation was obtained by subtracting the mean area from the time series. From relative area fluctuation, the autocorrelation analysis was carried out to determine the timescale of fluctuation. All the data analysis was carried out using MATLAB and graphs were plotted in Origin 8.0 (OriginLab Corporation). p value was obtained from Student's t-test. * in all graphs shows significance level below $p < 0.05$.

2.8. Functional analysis of genes

The functional analysis of upregulated genes was performed using DAVID. Based on Gene ontology annotations genes were organized and plotted.

3. Results

3.1. Whole transcriptome analysis during stem cell differentiation

ES cells were grown on fibronectin coated dishes for 4 h, 12 h and 24 h to study early temporal changes in the global genome expression patterns following onset of differentiation [25]. Fig. 1a shows changes in cell spreading and emergence of actin stress fibers during early onset of differentiation on these fibronectin coated dishes. Fig. 1b shows differential regulation of genes after 4 h of fibronectin signalling compared to control cells grown on gelatin. More than 200 genes were found to be upregulated (Fold change = 2 & $p < 0.05$) after 4 h of differentiation as compared to control undifferentiated cells whereas around 140 genes were downregulated. The number of differentially regulated genes changes as the differentiation proceeds for 12 h and 24 h (Fig. 1b).

Gene ontology analysis was carried out for the upregulated genes at different time points to get insight on their functional clustering. As expected, due to activation of integrin signalling by fibronectin, most of the upregulated genes were found to belong to the class of cytoskeletal genes including actin and intermediate filaments by 4 h of differentiation. The subset of the upregulated genes was also found to be part of contractile actomyosin stress fibers. Since onset of differentiation is coupled with cellular spreading, genes of focal adhesion family were also significantly upregulated. Interestingly, the biological process attributed to these genes at 4 h, included a diverse family of functional modules that converge to early onset of cell-fate commitment modules by 24 h (Fig. 1c) towards matrix attachment, suggesting a reduction in cellular functional heterogeneity upon activation of integrin signalling. Consistent with this, the upregulated genes included target genes of integrin and MRTF-A/SRF pathway (Fig. 1d) facilitating cell matrix attachment. To validate the target genes obtained from the microarray analysis, Real time PCR experiments were carried out for vinculin and zyxin known targets of MRTF-A SRF pathway (Supplementary Fig. S1). In the next section, we carry out time lapse single cell measurements to study the correlation between MRTF-A mediated gene expression, during early onset of differentiation, and cell-matrix attachment.

3.2. Expression of cell-matrix genes during early onset of differentiation

Stem cells cultured on fibronectin coated dishes were stained with MRTF-A and its target gene actin at different time points. Starting with a homogeneous population of Oct-4 positive cells