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Patterning pluripotency in embryonic stem cells

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Abstract

Developmental gradients of morphogens and the formation of boundaries guide the choices between self-renewal and differentiation in stem cells. Still, surprisingly little is known about gene expression signatures of differentiating stem cells at the boundaries between regions. We thus combined inducible gene expression with a microfluidic technology to pattern gene expression in murine embryonic stem cells. Regional depletion of the *Nanog* transcriptional regulator was achieved through the exposure of cells to microfluidic gradients of morphogens. In this way, we established *pluripotency-differentiation boundaries* between *Nanog* expressing cells (*pluripotency zone*) and *Nanog* suppressed cells (*early differentiation zone*) within the same cell population, with a gradient of *Nanog* expression across the individual cell colonies, to serve as a mimic of the developmental process. Using this system, we identified strong interactions between *Nanog* and its target genes by constructing a network with *Nanog* as the root and the measured levels of gene expression in each region. Gene expression patterns at the pluripotency-differentiation boundaries recreated *in vitro* were similar to those in the developing blastocyst. This approach to the study of cellular commitment at the boundaries between gene expression domains, a phenomenon critical for understanding of early development, has potential to benefit fundamental research of stem cells and their application in regenerative medicine.

Keywords

Pluripotency; Differentiation; Tissue regeneration; Technology; Stem cell-microenvironment interactions; Experimental models; Embryonic stem cells

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Introduction

Cell fate choices in murine embryonic stem cells (mESC) are regulated by orchestration of LIF-gp130-Stat3, BMP-TGF- β -Smad and MAPK-ERK signals that control a network of transcription factors such as Oct4, Sox2, *Nanog* and Tcf3 [1-8]. During embryonic development, the spatial distribution of these signals guides the arrangement of cells into organized structures that subsequently evolve into tissues and organs [9]. The precise molecular changes accompanying cell responses at the boundaries between regions are still not fully elucidated, due at least in part to the limited capability to analyse cells at tissue interfaces. *In vitro* recapitulation of gene expression domains and boundaries between differentiating cells that are normally established *in vivo* would markedly advance our understanding of gene regulation in early development.

Geometrically defined multicellular patterns have been established by laser ablation and printing of proteins [10-13]. In our previous study, the differentiation of adult human mesenchymal stem cells was modulated in a microfluidic platform by doxycycline (Dox) induction of bone morphogenic protein (BMP-2), to generate an interface between osteogenic and fibrotic tissue [14]. To date, direct patterning of developmentally important gene expression at the transcriptional level has not been attempted.

To gain new insights into the processes at the pluripotency-differentiation boundaries, we investigated if the pluripotent states of mESC can be induced in a topologically specified manner by patterning the expression of *Nanog*, a key factor in safeguarding the pluripotent state [15, 16]. To this end, we used microfluidic gradients of morphogens in conjunction with an inducible lentivirus-based genetic complementation system we previously established [17, 18]. In this lentiviral system, a constitutively active short hairpin RNA (shRNA) depletes endogenous *Nanog* mRNA, and normal levels of *Nanog* can be restored in a Dox-dependent manner from a shRNA “immune” mRNA. In this way, in the absence of doxycycline (Dox), both the rescue mRNA and the GFP are not expressed, whereas, in the presence of Dox the exogenous shRNA-immune mRNA is expressed as well as the GFP reporter.

Different *Nanog* levels in the individual mESCs define a *Nanog-high* population that is resistant to differentiation signals and a *Nanog-low* population that, while pluripotent, is sensitive to differentiation-inducing signals [19]. We reasoned that patterning *Nanog* expression in a laminar microfluidic system could modulate cell self-renewal and early differentiation commitment in a spatially defined manner (Fig. 1).

Materials and Methods

Embryonic Stem Cell Culture and Differentiation Assays

A murine ESC line with controllable *Nanog* expression (NanogR) was constructed and characterized as in our previous studies [16, 17]. Briefly, NanogR cells contain two expression cassettes, a constitutively active shRNA designed to deplete the Nanog gene product and an inducible shRNA-‘immune’ expression system that rescues its depletion in order to evaluate and confirm the target-specific effect of the shRNA. This engineered lentivirus based-rescue vector contains a human H1 promoter- driven shRNA targeting the 3' untranslated region (UTR) of Nanog and a TRE promoter driving and exogenous shRNA-immune rescue mRNA and GFP, which is translated via an IRES element. Since the shRNA targets the 3' untranslated region of Nanog, the unmodified coding sequence of Nanog under the TRE promoter is suitable for rescuing the shRNA-mediated effect. This system requires an ESC line, such as the Aniv15 line expressing the TetOn rt TA transactivator [20].

The NanogR cells were maintained on primary mouse irradiated embryonic fibroblasts (MEFs) in DMEM media supplemented with 15% FBS (Hyclone), 100 mM MEM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 1 mM L-glutamine (Invitrogen), 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate and 10^3 units/ml of LIF (Chemicon). For experimental assays, NanogR cells were cultured on 0.1% gelatin-coated tissue culture plates without MEFs. To induce differentiation doxycycline (Dox, $1 \mu\text{g ml}^{-1}$) was withdrawn, and cells were maintained in LIF containing, LIF free, or LIF free plus Retinoic Acid ($5 \mu\text{M}$) media. Cell cultures were maintained at $37^\circ\text{C}/5\% \text{ CO}_2$.

Microfluidic system

The flow system consisted of a flow chamber, a syringe pump and a tubing loop. The flow chamber (Fig. 3) consisted of a 1 mm thick borosilicate glass plate attached to the bottom of a groove within a Plexiglas block, overlaid with another glass slide with pre-seeded cells. The two plates were held together with screws through a Plexiglas lid, and separated by a uniform thickness rectangular silastic spacer (McMaster), creating a flow chamber that was 5.2 cm long, 1.2 cm wide, and $154 \mu\text{m}$ high. Fluid enters and exits the chamber from two ports connected to cylindrical reservoirs at the inlet and outlet of the Plexiglas block (Fig. 3). Plastic tubing (soft high-temperature silicone rubber tubing, 1/16" ID, 1/8" OD, McMaster) connects the entrance port to a digital syringe pump and the exit port to waste collection bottles.

Prior to the experiment, the flow chamber and tubing were assembled, connected to a syringe pump, and primed with proper culture medium. The flow chamber and tubing were sterilized by autoclaving. The sterile plastic slide was coated with 0.1% gelatine for 4 hours, and air-dried. NanogR cells were seeded onto the slide at a density of 5,000 cells/ cm^2 in LIF and Dox (500 ng/ml) supplemented culture medium and cultured overnight in a humidified $37^\circ\text{C}/5\% \text{ CO}_2$ incubator. The system maintained two parallel laminar microfluidic streams, each at a flow rate of $4 \mu\text{l/min}$ (shear stress at the cell culture surface = 2.8 mPa, Reynolds number $\text{Re}=0.022$). To generate patterned pluripotent zones, NanogR cells were exposed to laminar flow for 3 days.

In dye separation experiments, cells were subjected to 30-60 min of medium flow supplemented with $2 \mu\text{M}$ Calcein-AM, and then to 10-30 min of flow of complete medium.

Alkaline Phosphatase Staining

Alkaline phosphatase (AP) activity was measured using a commercial detection kit (Sigma-Aldrich). The cells were fixed in 4% paraformaldehyde in $1\times$ PBS for 2 minutes. After a wash with TBST (20mM Tris-HCL, pH7.4, 0.15M NaCl, 0.05% Tween-20), cells were immersed into alkaline-dye mixture consisting of fast red violet, Naphthol AS-BI phosphate solution and water in 2:1:1 ratio, for 20 min in the dark (or 7 minutes when staining experimental samples for flow cytometry), and were again washed in TBST. AP staining images were captured by microscopy (Olympus IX81, Center Valley PA).

qRT-PCR

For gene expression analyses, total RNA was TRIzol®-extracted (Invitrogen) and column-purified with the RNeasy kit (Qiagen). Total RNA ($1 \mu\text{g}$) was reverse transcribed using the high capacity reverse transcription kit (Applied Biosystems). All quantitative PCR analyses were performed using the Fast SYBR® Green Master Mix (Applied Biosystems) following the manufacturer's protocol on a LightCycler480 Real-Time PCR System (Roche). All expression levels were normalized to β -actin. Gene-specific primers used for amplification are listed in Supplementary Table 1.

Immunofluorescence Staining

Cells cultured at different Dox concentrations and at the respective days 0, 1, 3 and 5 were rinsed twice with 1× PBS, fixed with 4% paraformaldehyde in 1× PBS for 20 min at room temperature, quenched with 0.2 M glycine in 1× PBS for 10 min, and permeabilized with 1× PBS containing 0.5% Triton X-100 for 30 min. Nonspecific binding sites were blocked by incubation with 1× PBS containing 0.1% BSA for 30min at room temperature. Cells were then incubated with specific primary antibodies: 1:100 anti-mouse Oct4 (Santa Cruz, cat no. sc-8628), or 1:150 anti-mouse *Nanog* (Cosmo Bio, cat no. REC-RCAB0002P-F), diluted in 1× PBS containing 0.2% Triton X-100 and 0.1% of BSA for overnight incubation at 4 °C. After that, cells were incubated with one of the following secondary antibodies: Alexa Fluor® 546 donkey anti-goat (Life Technologies, cat no. A-11056) (1:1000 dilution) and Alexa Fluor® 488 donkey anti-rabbit IgG (Life Technologies, cat no. A-21206) (1:1000 dilution). After washing with 1× PBS containing 0.2% Triton X-100 for 3 times, the samples were incubated for 5 min with 1:1000 DAPI in water, followed by three washes with 0.2% Triton X-100 in 1× PBS. We confirmed the complete loss of GFP three days after fixation (Fig. S9), that was necessary to avoid GFP signal contamination in the *Nanog* immunofluorescence staining. Therefore, the staining was completed and fluorescence images were captured three days after fixation with a Nikon Eclipse TE 2000-U or Olympus B×41 fluorescent microscope.

Mathematical Model

The chamber geometry permits the wall shear stress to be directly calculated as a function of the laminar flow rate and fluid viscosity:

$$\text{Shear Stress: } \tau_w = \frac{6\mu Q}{wh^2} \quad (1)$$

$$\text{Reynolds number: } R_e = \frac{2Q}{\frac{\mu}{\rho}(w+h)} \quad (2)$$

where μ is the medium viscosity; ρ is the medium density; Q is flow rate; w and h are the width and height of the chamber. The hydrodynamic development length L in a channel of height h can be estimated as follows [21]:

$$\frac{L}{h} = 0.3125 + 0.011R_e \quad (3)$$

The concentration and velocity profiles were modelled using the finite element method (COMSOL Multiphysics v3.2, Burlington, MA). The concentration gradient driven by the syringe pump was based on the 3D incompressible flow Navier–Stokes equations:

$$\rho \left(\frac{du}{dt} + (u \nabla u) \right) = -\nabla p + \mu \nabla^2 u \quad (4)$$

$$\nabla u = 0 \quad (5)$$

and the convection-diffusion transport equation

$$\frac{dc}{dt} + (u \nabla) C = D \nabla^2 C \quad (6)$$

where ρ , u , and p are the density, flow velocity and pressure of medium and C and D are the concentration and diffusivity of the molecules in the medium. At each time step, the flow velocity was first obtained independently of the concentration, which was then calculated based on fluid flow field. For the convection–diffusion equation, a constant concentration was set at the channel inlet, and no-flux conditions were imposed at the channel walls. The boundary conditions were as follows: (i) specified flow velocity at the inlet, (ii) zero normal stress at the outlet and (iii) no-slip conditions on the channel walls.

Image Analysis

For quantifying the intensity of *Nanog* and Oct4 expression in the fluidic chamber after the exposure to different differentiation media (Fig. 3), we divided the flow chamber image (12 mm in width) into 24 equal segments (each 0.5 mm wide) and quantified the staining intensity for each segment $I(i)$. Normalized intensity values were generated as a ratio of $I(i)$ over the smallest intensity value in the series. $N(i) = I(i)/\min(I(i))$. In this way, we determined how uniform was the distribution of the staining intensity across the chamber, and compared different rounds of experiments.

Sequential images were acquired using a phase-inverted microscope (Olympus IX81), stored, assembled, and analyzed digitally by Photoshop CS4 program (Adobe). For quantitative analysis of immunofluorescent staining, the assembled colour images were converted to black-white images. Integrated staining intensities were quantified using Matlab R2007 software (Matlab, Natick, MA) by normalized integration of segmented images (0.5 mm), with intensity moving across the y-axis (the width) of the chamber.

Dynamic Array Analysis

For qPCR analysis, 24 samples of 10 cells each from the NanogR rescue clone harvested from each of the three regions of interest: right (R), middle (boundary) (M) and left (L) were sorted directly into a mixture of CellsDirect 2× Reaction Mix (component of CellsDirect™ One-Step qRT PCR Kits, Invitrogen), 0.2× TaqMan Assay Mix (Applied Biosystems), and SuperScript™ III RT/Platinum® Taq Mix (Invitrogen). Reverse transcription (RT) and specific target amplification (STA) were performed sequentially. RT was performed for 15 min at 50°C. Samples were then incubated for 2 min at 95 °C to inactivate the RT enzyme and activate Taq polymerase. Thermal cycling conditions for STA were 18 cycles of 95 °C for 15 s and 60 °C for 4 min. Pre-amplified cDNA was diluted with TE buffer (1:5) for real-time PCR using BioMark™ 48-48 Dynamic Arrays (Fluidigm) [22]. The PCR profile included a 10 min, 95°C hot-start to activate the Taq polymerase, followed by 40 cycles of a two-step program: 15 seconds at 95°C (denaturation) and 60 seconds at 60°C (annealing and extension). Data were analyzed using BioMark™ Real-Time PCR Analysis Software v2.0 and previously established methods [22].

Data Processing

All real-time PCR values were normalized to the endogenous control by subtracting, for each sample of $n=10$ sorted cells, the value of the *GAPDH* expression level. Delta Ct values are represented in Fig. 6. Additionally, relative values represented in Fig. S8 were obtained using the perfusion medium R (LIF,Dox)/L(LIF,Dox) as reference sample.

Network

Nanog ChIP-seq targets were obtained from published data [23-28]. The network was built in the yEd Graph editor software http://www.yworks.com/en/products_yed_about.html.

Statistical Analysis

Experimental results were expressed as average $\pm$ standard deviation (SD) of the measured and calculated values. Data were analyzed by multiway ANOVA for multiple comparisons, followed by Tukey's post hoc analysis, with the confidence level set to 0.05 for all tests, using Statistic software. $P < 0.05$ was considered significant in all cases. For gene profiling, comparisons between experimental groups were performed using permutation analysis for differential expression [29].

Results

Regulation of *Nanog* expression and pluripotency by Dox, leukemia induction factor (LIF) and retinoic acid (RA)

The induction parameters of *Nanog* in the rescue clone NanogR were characterized for a range of Dox concentrations (0 – 1,000 ng/ml) in the presence of the differentiation factors LIF and RA, using LIF-containing medium, LIF-free medium, and LIF-free medium supplemented with RA (Fig 2; Figs. S1, S2, S3). Specifically, following Dox titration studies and harvesting the *Nanog* rescue clone at three different time points (Day1, Day3 and Day5), we performed three runs of differentiation: (1) Titration of Dox concentration in NanogR cells in ESC media with LIF (Figure S1); (2) Titration of Dox concentration in NanogR cells in ESC media without LIF (Figure S2); and (3) Titration of Dox concentration in NanogR cells in ESC media without LIF and with RA (Figure S3).

Figure 2 shows that 100 ng/mL of Dox during 3 days in media without LIF and with RA is the best condition for detecting if the pluripotency state is compromised and early cell fate commitment is occurring. Since our main focus was to study the patterns of gene expression at the boundaries where cells start their commitment to differentiation pathways, we selected day 3 as the most adequate time point for capturing the cell fate transitions in Fluidigm experiments.

Exposure of cells to > 100 ng/ml of Dox resulted in expression of exogenous shRNA immune *Nanog* mRNA, in spite to the constitutive suppression of the endogenous shRNA-targeted *Nanog* mRNA. Intermediate concentrations of Dox (100-1000 ng/ml) induced a graded expression of *Nanog*, resulting in varying degrees of mESC pluripotency states.

Exposure of cells to LIF at low *Nanog* levels did not induce cell differentiation, and the expression of *Oct4* was sustained even when *Nanog* expression was completely suppressed by the removal of Dox (Fig. 2; Fig. S1). Therefore, in the presence of LIF, down regulation of *Nanog* alone was insufficient to induce differentiation, suggesting the existence of compensatory mechanisms maintaining the pluripotent state.

In the absence of LIF (Fig. 2; Fig. S2), we observed Dox-concentration dependent decreases in alkaline phosphatase (AP) activity and *Oct4* expression, and increase in the expression of *Fgf5*, indicative of ectodermal differentiation [30].

In the presence of RA without LIF, a stronger differentiation inducer, we observed robust differentiation, evidenced by the loss of AP activity and *Oct4* expression (Fig. 2; Fig. S3). Concomitant expression of the endoderm marker, *Gata4*, and a lack of expression of *Fgf5* suggest RA-mediated endodermal differentiation and suppression of ectodermal differentiation.

Computational modelling of concentration and velocity profiles

The flow field and the concentration profiles of Dox, LIF and RA were analysed in detail with the aid of mathematical modelling and COMSOL Multiphysics® software (Fig. 3). The flow rates of 4 $\mu\text{l}/\text{min}$ in each channel correspond to the Reynolds number $\text{Re} = 0.022$ and the hydrodynamic length of 0.048 mm at which a fully developed flow is established (corresponding to $\sim 0.1\%$ of the total length of 52 mm). Thus, the flow of medium over the cells could be considered as fully developed, with negligible variation in the direction transverse to the flow. The wall shear stress acting at the cells was estimated to 2.8 mPa, a level well under the physiological values of shear stress associated with the interstitial flow of fluids in tissues [31] and the flow of blood [32].

We developed a simple fluidic model to study the profiles of flow and morphogen concentrations at steady state. Different concentration profiles of Dox, LIF, and RA were modelled, to show that the bi-laminar-fluidic system permits alignment of flow with different concentration profiles, and the creation of a concentration interface (Fig. 3B). The model predictions of the concentration profiles were verified in experiments with Calcein-AM (diffusion coefficient of Calcein-AM is $D_{\text{Calcein}} = 2.6 \times 10^{-6} \text{cm}^2/\text{s}$) that was used as a model molecule for flow studies at the inlet concentrations of 2 μM and 0, and the flow rates of 4 $\mu\text{l}/\text{min}$ per channel. The basic modelling approaches were those developed in our previous studies [14].

Patterning of embryonic pluripotency by microfluidic gradients of Dox, LIF and RA

In microfluidic flow experiments, we generated patterned zones of mESC pluripotency and differentiation, by applying gradients of Dox, LIF and RA across the flow chamber. The streams of culture medium with supplements selectively inducing cell pluripotency or differentiation were introduced at the right (R) and left (L) of the microfluidic chamber (Fig. 1B, Fig. 3), to establish the following four experimental groups:

Group 1: *Control (no gradient)*: R(LIF+Dox)/L(LIF+Dox)

Group 2: *Gradient of Dox*: R(LIF+Dox)/L(LIF)

Group 3: *Gradients of Dox and LIF*: R(LIF+Dox)/L(none)

Group 4: *Gradients of Dox and LIF and RA*: R(LIF+Dox)/L(RA)

Uniform concentrations of Dox and LIF in the flow chamber (control, Fig. 4 first column) elicited cellular responses consistent with those observed in the corresponding static cultures. *Nanog* and Oct4 expression levels and the staining for AP, *Nanog*, Oct4 and cell nuclei were spatially uniform across the chamber and without apparent effects of flow (Fig. 4, Fig. S4).

Cellular responses to a gradient of Dox only (Fig. 4A, second column; Fig. S5) or to the combination of Dox and LIF gradients (Fig. 4A, third column; Fig. S6) were comparable. In both cases, patterned *Nanog* expression was observed within the same population of cells, along the gradient of Dox or Dox/LIF, and with spatially uniform expression levels of Oct4. Cells exhibited, within a short time period patterned AP staining along the Dox or Dox/LIF concentration profiles. Most interestingly, high-magnification fluorescent images demonstrated patterned *Nanog* expression within the individual cell colonies (Fig. S7, third and fourth column). LIF gradient had no apparent effect on cellular responses (Fig. S5-S7). *Nanog* suppression, with or without LIF, affected AP activity but did not affect expression of Oct4. Suppression of *Nanog* and removal of LIF were thus not sufficient to induce significant mESC transition from pluripotency to differentiation.

Clearly defined zones of pluripotency and differentiation were established when the cells were exposed to the same-direction gradients of Dox and LIF superimposed with an opposing gradient of RA (Fig. 4A, fourth column; Fig. S7). In this group only, both *Nanog* and Oct4 levels decreased, in parallel with the decrease in Dox and LIF, and with the increase in RA. Measured transitions in *Nanog* and Oct4 expression between the pluripotency and differentiation zones were consistent with the concentration profiles of Dox, LIF and RA (Fig. 4B-C, fourth column; Fig. S7). The AP staining pattern was similar to that in the Dox and Dox/LIF groups, except that AP activity in the pluripotency region was markedly lower (Fig. 4C, fourth column), presumably due to RA diffusion or direct interactions between the pluripotency and differentiation regions. Parallel staining with DAPI showed that the observed differences in *Nanog* and Oct4 levels were not caused by non-uniform distributions of cells. In sum, down regulation of *Nanog* alone in cultured cells could not induce definitive lineage commitment, a finding consistent with the previous reports [15]. Instead, a combination of *Nanog* suppression with proper gradients of LIF and RA was necessary for robust differentiation commitment.

Notably, high magnification images revealed patterning of the expression of *Nanog* and Oct4 within single cell colonies located at the boundary between the pluripotency and differentiation regions (Fig. 5). Perpendicular to the boundary, we observed gradual changes from aggregated cell populations, and clear boundaries with diffuse populations of flattened cells (Fig. 5D).

Gene expression signatures of differentiating stem cells at the pluripotency/differentiation boundaries

The R(LIF+Dox)/L(RA) group (Fig. 4, fourth column; Fig. S7) that showed clear patterns of pluripotency and differentiation was analysed in depth for molecular signatures in the region of pluripotency, at the boundary, and in the region of differentiation (Fig. 4 - right, boundary, and left, respectively). The cells were harvested from each region as shown in Fig. S8 to assess the expression of genes in cells at the interface between the two regions, following cell exposure to the gradients of morphogens.

A total of 21 genes from three groups: (i) pluripotency markers, (ii) chromatin modifiers and (iii) differentiation markers, were analyzed by microfluidic quantitative PCR (Fluidigm) [22] in cells from each region of interest. Cell exposure to microfluidic gradients resulted in significant downregulation of pluripotency mRNAs, including: *Tbx3*, *Klf4*, *Esrrb*, *Stat3*, *Lin28*, *Bmp4* and *Dppa4* along with downregulation of *Nanog* (Fig. 6A, Fig S8 B, C). In parallel, differentiation marker mRNAs such as: *Gata4*, *Gata6*, *Foxa2*, and *Olig1* were upregulated in the differentiation region of the flow chamber. This is consistent with the known roles of *Gata4* and *Gata6* as crucial regulators of primitive endoderm [33-35].

The switch towards differentiation in the cells within the -LIF-DOX+RA region was associated with the downregulation of important chromatin modifiers: *Wdr5*, *Smarcc1*, *Jarid2* and *Rai*, known to play important roles in maintaining the pluripotency. Our observations support the importance of these chromatin modifiers for pluripotency [2, 36-39] and suggest that their downregulation could promote expression of primitive endoderm markers.

Strong interactions between *Nanog* and its target genes were identified by constructing a network with *Nanog* as the root and the measured levels of gene expression in each region (Fig. 6B).

Withdrawal of Dox downregulated *Nanog* and suppressed the expression of *Nanog*-activated targets (*Nanog*, *Oct4*, *Sox2*, *Bmp4*, *Lin28*, *Stat3*, *Esrrb*, *Dppa4*, *Tbx3*, *Myc*, *Klf4*, *Rest*,

Wdr5, *Smarcc1*, *Jarid2* and *Rai*). *Nanog* was found to bind and repress *Foxa2*, preventing differentiation [3, 4]. Consistent with this previous finding, we observed gradual activation of *Foxa2* outside the pluripotency region.

Discussion

During morphogenesis, cells undergo massive changes in gene expression programs, with combinations of spatial and temporal gradients of morphogens coordinating tissue development. Our strategy was to pattern *in vitro* the expression of key master regulator genes and thereby mimic some of the *in vivo* mechanisms in which molecular signals orchestrate early development from multicellular patterns to increasingly complex structures. The mechanistic relationships and the transcriptional circuitry between developmental markers and cellular responses remain largely unknown. Transcriptional patterning *in vitro* could provide important insights, by spatiotemporal control of molecular signalling and by establishing boundaries between gene expression domains, such as those between the wing and the notum in *Drosophila melanogaster* [40], foregut and hindgut [41] and between somites [42–44] in vertebrate embryos.

Using the microfluidic device (Fig. 1, Fig. 3) we patterned multicellular structures by applying localized gradients of soluble morphogens. The application of –LIF/–DOX/+RA gradients generated a boundary between the pluripotency state and the primitive endoderm state, with significant changes in gene expression of pluripotency markers, chromatin modifiers and early differentiation markers in the cells located at different positions down the morphogen gradients.

Our study established a direct link between the gradients of morphogens, gene expression profiles, and the lineage fate of differentiating stem cells. The results we collected suggest possible restrictions in the differentiation potential of stem cells to commit to specific lineage fates as a function of position along the gradient and the type of morphogen. Our findings also indicate that the cell exposure to gradients can provide patterning of cell differentiation cues. Finally, we identified the gene expression levels of *Nanog* targets that initiate dynamic processes leading to cell fate decisions at the boundaries.

In principle, pluripotent ES cells can be distinguished from differentiated cells by the specialized chromatin state required to epigenetically regulate the ES cell phenotype. Recent studies show that in addition to the factors specific to pluripotency, chromatin remodelling enzymes play major roles in regulating chromatin in ES cells and the capacity of ES cells to self-renew and differentiate. Here we show the efficient downregulation of some histone modifiers like *Wdr5*, *Rai1*, *Smarcc1* and *Jarid2* resulting in lineage commitment. This evidence supports the importance of chromatin dynamics for early lineage decisions, in line with the previously reported roles of histone modifiers in maintaining ES cells [36–38, 45].

Moreover, in this study we successfully show how the cells condition their gene expression programs in response to microenvironment signals. *Nanog* signalling circuitry is tightly regulated by feed-forward regulatory loops that maintain the pluripotent state [23–28]. The identification of the gene expression levels of *Nanog* targets at the boundaries enables quantitative insights into the dynamic processes involved in cell fate decisions.

An important result is that by application of the –LIF/–DOX/+RA gradient one can generate a boundary populated by the cells with low transcriptional state of *Nanog*, that are poised for differentiation [15]. In this case, the microenvironment alone determines the fate commitment. In the absence of differentiation factors, these *Nanog*-low cells can be maintained in their pluripotent state. In contrast, the presentation of strong differentiation

signals like RA would make these same cells commit and differentiate preferentially into visceral endoderm [46].

Conclusion

The present study combined inducible gene expression with a microfluidic technology for patterning gene expression in mESCs, in order to investigate effects of gradients of morphogen on cell fate decisions. We demonstrate that the boundaries between pluripotency and differentiation domains can be established within an initially uniform population of mESCs by directly patterning of *Nanog* expression by applying combinations of gradients of Dox, LIF and RA. This innovative approach employs a simple and highly controllable *in vitro* system to modulate cellular function at the transcriptional level and engineer discrete cellular domains mimicking those present in a developing blastocyst. Further studies of the regulatory circuitries involved in cell fate decisions during early development would provide a more comprehensive view of how cells orchestrate morphogenetic processes, and help develop a roadmap for directing stem cell fates in regenerative therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Patterning of pluripotency and differentiation in mESCs by spatial regulation of gene expression

(A) *In vivo* patterning of gene expression during embryonic development, where distinct pluripotency and differentiation domains are defined by the expression of *Nanog*. (B) Microfluidic model for patterning gene expression in an initially uniform population of mESCs. Distinct regions of mESC pluripotency and differentiation are established by patterning the expression of the *Nanog* transcriptional regulator through the exposure of the cells to gradients of chemicals or morphogens. (C) Tet-on system for the doxycycline (Dox) dependent induction of *Nanog*. In the presence of Dox, the exogenous *Nanog* gene is expressed to maintain pluripotency.

Figure 2. Regulation of gene expression in mESCs by exposure to different levels of Dox in the presence of LIF and RA

(A) Alkaline phosphatase (AP) staining of NanogR cells. (B) Immunofluorescent staining of NanogR cells (blue: DAPI; green: *Nanog*; red: Oct4). (C) Expression levels of *Nanog*, *Oct4*, *Sox2*, *Esrrb*, *Fgf5*, *Gata4*, *Gata6* and *Foxa2*, measured by RT-PCR. *: $p < 0.05$ compares the corresponding conditions between two analytic days at the same Dox concentration. +: $p < 0.05$ compares the corresponding condition with cultures in the absence of Dox and at the same analytic day. Data are shown for day 3 of cultivation in medium containing LIF (+LIF) as control, medium without LIF (-LIF) or without LIF and including retinoic acid (RA) (-LIF+RA). Scale bar: 100 μ m.

Figure 3. Microfluidic chamber

(A) Flow chamber and its components (main chamber: $52 \text{ mm} \times 12 \text{ mm} \times 0.154 \text{ mm}$). (B) Modeling of velocity and concentration profiles. Using COMSOL Multiphysics®, the velocity and concentration profiles were defined for the flow rate of $4 \mu\text{l/min}$ per channel (with the Reynolds number of $\text{Re} = 0.022$ and the hydrodynamic development length of $48 \mu\text{m}$, corresponding to a fully developed laminar flow). The wall shear stress in the cell culture zone was 2.8 mPa for the fluidic chamber, well under the shear stress values of cells exposed to normal interstitial (31) or blood flow (32). Steady-state profiles of flow velocity and concentrations of Dox, LIF, and RA are shown for the input concentrations of $500 \mu\text{g/ml}$ Dox = $1.13 \times 10^{-6} \text{ M}$; $10^3 \text{ units LIF/ml}$ = $2.13 \times 10^{-10} \text{ M}$, and $5 \mu\text{M}$ RA. Diffusion coefficients: $D_{\text{Dox}} = 3.93 \times 10^{-6} \text{ cm}^2/\text{s}$, $D_{\text{LIF}} = 1.66 \times 10^{-6} \text{ cm}^2/\text{s}$, $D_{\text{RA}} = 2.3 \times 10^{-6} \text{ cm}^2/\text{s}$; flow viscosity ($= 10^{-3} \text{ Pa}\cdot\text{s}$); fluid density ($= 10^3 \text{ kg/m}^3$).

Figure 4. Patterning of mESC pluripotency by microfluidic gradients of Dox, LIF and RA
 (A) Experimental design: cultured NanogR cells were exposed to concentration gradients of Dox, LIF and RA by two microfluidic streams: Right and Left (R/L) containing (i) R(LIF +Dox)/L(LIF+Dox), (ii) R(LIF+Dox)/L(LIF), (iii) R(LIF+Dox)/L(none), and (iv) R(LIF +Dox)/L(RA). Concentration profiles calculated from mathematical models are shown for each of the four conditions. (B) Normalized intensities of Nanog/Oct4 immunostaining fluorescence across the flow chamber (n = 4-5). (C) Staining for AP, Nanog and Oct4 at the middle section of the flow chamber. Arrows represent the flow direction: from top to bottom in the respective panels (C). Scale bars: 1mm (C).

Figure 5. Patterning of mESC pluripotency within a single cell colony

Microfluidic gradients shown in Fig. 4 for the entire cell field were also established within the individual cell colonies. Panels (A-D) show the distribution of the cells (blue, cell nuclei stained by DAPI), Nanog (green) and Oct4 (red) under experimental condition: cultured NanogR cells were exposed to concentration gradients of Dox, LIF and RA by two microfluidic streams: (Ai) top(LIF+Dox)/bottom(LIF+Dox), (B) top(LIF+Dox)/bottom(LIF), (C) top(LIF+Dox)/bottom(none), and (D) top(LIF+Dox)/bottom(RA). Fourth column, normalized intensities of DAPI/Nanog/Oct4 immunostaining fluorescence across the interface (n = 3,4 selected zones from the same image). Arrows represent the flow direction: from left to right in the respective panels. Scale bars: 100 μm.

Figure 6. Spatial patterning of gene expression in mESCs by microfluidic gradients (A) Scatter dot plots of expression levels for 21 genes across the microfluidic chamber (right (R), middle (boundary) (M) and left (L)), measured using the BioMark HD System for multiplex quantitative PCR. The data are ΔCt to the reference gene *GAPDH*. Raw ΔCt data for all genes assayed is in Supplementary data. (B) Molecular signature of Nanog targets (pluripotency markers, chromatin modifiers and differentiation markers) in the right (R), middle (boundary) (M) and left (L) regions of the chamber. The colour of each node represents the average expression level for each gene at each location (R, M and L). The arrows represent protein-DNA interactions (Supplementary Methods). Data are shown for the group R(LIF+Dox)/L(RA) (Fig. 4, **fourth column**).