DIFFERENTIAL ENVIRONMENTAL SPATIAL PATTERNING (δESP) RECREATES PROXIMAL-DISTAL AXIAL PATTRENS IN EMBRYONIC STEM CELL COLONIES

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ABSTRACT

Understanding embryonic development has significant implications in stem cell research and applications as well as drug testing. Developmental biology is still largely studied using animal models because of their inherent complexity, but are expensive and difficult to manipulate. Here, we develop a two-step, multi-component micropatterning technique (termed Differential Environmental Spatial Patterning, δESP) technique as an *in vitro* means to generate developmentally relevant differentiation patterns. We demonstrated that δESP could emulate proximal-distal differentiation patterns in an early developing embryo in embryonic stem cell colonies. δESP potentially paves the way for more biomimicry *in vitro* models as animal surrogates.

INTRODUCTION

The understanding of embryonic development has significant implications in stem cell research and applications as well as drug testing, and the ultimate goal of developmental biology research is to decipher how organized cell differentiation emerges in a structurally heterogeneous environment, comprising of different localized pockets of cells and extracellular matrices [1]. Existing *in vitro* embryonic models, such as monolayer embryonic stem cell (ESC) cultures or embryoid bodies, are typically restricted to the control of environmental composition around the cells but cannot control the *spatial organization* of that environment. Animal models, meanwhile, recapitulate the complex spatial organization of the microenvironment, but are time-consuming, expensive and difficult to experimentally manipulate. Our aim here is to maintain the experimental accessibility of ESC culture while incorporating spatial information to enable studies on how environmental organization affects ESC differentiation patterns.

To this end, we developed a two-step, multi-component micropatterning technique (termed Differential Environmental Spatial Patterning, δESP) [2], to organize representative microenvironments from an embryo around an ESC colony (Figure. 1A). Specifically, we wished to determine whether we could emulate, in a dish, proximal-distal (PD) patterning of the epiblast (*i.e.*, the initial step of germ layer formation by pluripotent cells within an embryo), which is modulated by two juxtaposed extraembryonic tissues.

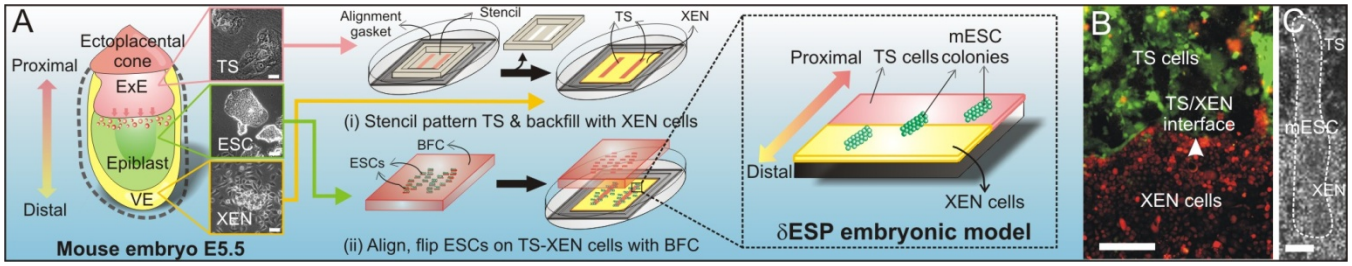

Figure 1. *Recapitulating proximal-distal (PD) epiblast patterning with δESP. (A) Concept for translating embryonic and extraembryonic tissues organization in an E5.5 mouse embryo to δESP model: (i) Trophoblast stem (TS) cell and extraembryonic endoderm (XEN) cell lines, derived from extraembryonic ectoderm (ExE) and visceral endoderm (VE) respectively, were stencil-patterned to generate the designated PD orientation. (ii) Mouse embryonic stem cell (ESC) colonies were then patterned on the TS-XEN interface with the Bio Flip Chip (BFC). (B) Stencil-patterned GFP-labeled TS cells and Cell Tracker Orange-labeled XEN cells formed a defined interface. (C) Phase images of mESC colonies on TS-XEN interface. Scale bars in (A) = 50 mm; scale bars in (B-C) = 200 μm.*

EXPERIMENTAL

Devices were fabricated by polydimethylsiloxane (PDMS) molding according to [2]. Cell lines used include mouse ESCs with GFP-Sox1 reporter (46C, provided by Austin Smith), mouse trophoblast stem (TS) cells and extraembryonic endoderm (XEN) cells (provided by Janet Rossant), which were maintained according to [2-4]. The first step of δESP uses stencil patterning to pattern cells derived from the two juxtaposed extraembryonic tissues (*i.e.,* TS cells [3] and XEN cells [4]) for de-

fining the PD orientation (Figure. 1Ai, 1B). Next, mouse ESCs were filled into the micro-wells of the Bio Flip Chip (BFC) [2], aligned to the TS-XEN cell interface using a PDMS gasket, and flipped onto the receiving substrate containing the extraembryonic cells (Figure. 1Aii, 1C). Wnt signaling during PD axis formation was disrupted by addition of 50 ng/ml of recombinant mouse WNT3a (1324-WN, R&D Systems) or 200 ng/ml of recombinant mouse DKK-1 (5897-DK, R&D Systems). Expression of markers for different cell fates were determined after five days of culture by fluorescence imaging and quantified by image processing.

RESULTS AND DISCUSSION

After five days of differentiation, we examined the localization of proximal epiblast markers which included Brachyury (T), Wnt3a, Cripto1 and Fgf8. We found that the proximal epiblast markers were polarized in mESC colonies patterned at the TS-XEN cell interface (Figure. 2A-D), while control colonies on either TS or XEN cells alone exhibited symmetric expression patterns (Figure. 2E). This suggests that the cells are indeed creating a PD pattern *in vitro*. To test the functionality of this δESP embryonic model, we examined whether the polarized expression patterns were sensitive to disruption of Wnt signaling gradient, which is known to regulate PD axis formation *in vivo* [1]. In the presence of exogenous Wnt agonist (WNT3a), Wnt3a and T expression were more pronounced (Figure. 3A), while in the presence of exogenous Wnt antagonist (DKK-1), their expression was attenuated (Figure. 3B), as expected. Interestingly, PD patterning of Wnt3a and T in the mESC colonies was abolished in both cases since the PD intensity ratios of Wnt3a (Fig. 3C) and T (Figure. 3D) in colonies on TS/XEN cells were not significantly different from control colonies.

CONCLUSION

In conclusion, we successfully used δESP to demonstrate that creating a synthetically organized version of the early embryo is sufficient to initiate patterned differentiation, establishing for the first time *in vitro* proximal-distal pattern in differentiating stem cell colonies, which is the first step of gastrulation.

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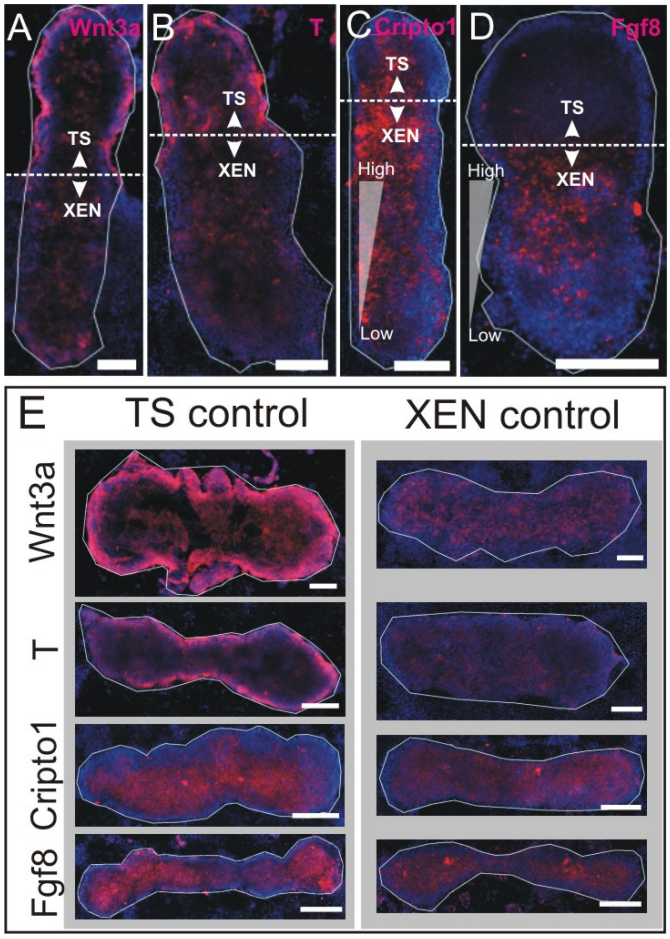

Figure. 2 *Immunofluorescence staining of proximal embryonic markers (in red) after 5 days of differentiation. δESP-patterned mESC colonies exhibited spatial polarization of embryonic proximal markers: (A) Wnt3a, (B) T, (C) Cripto1 and (D) Fgf8. (E) No marker polarization was observed in control colonies on TS or XEN cells alone. Cell nuclei were counterstained in blue. Scale bars = 200 μm.*

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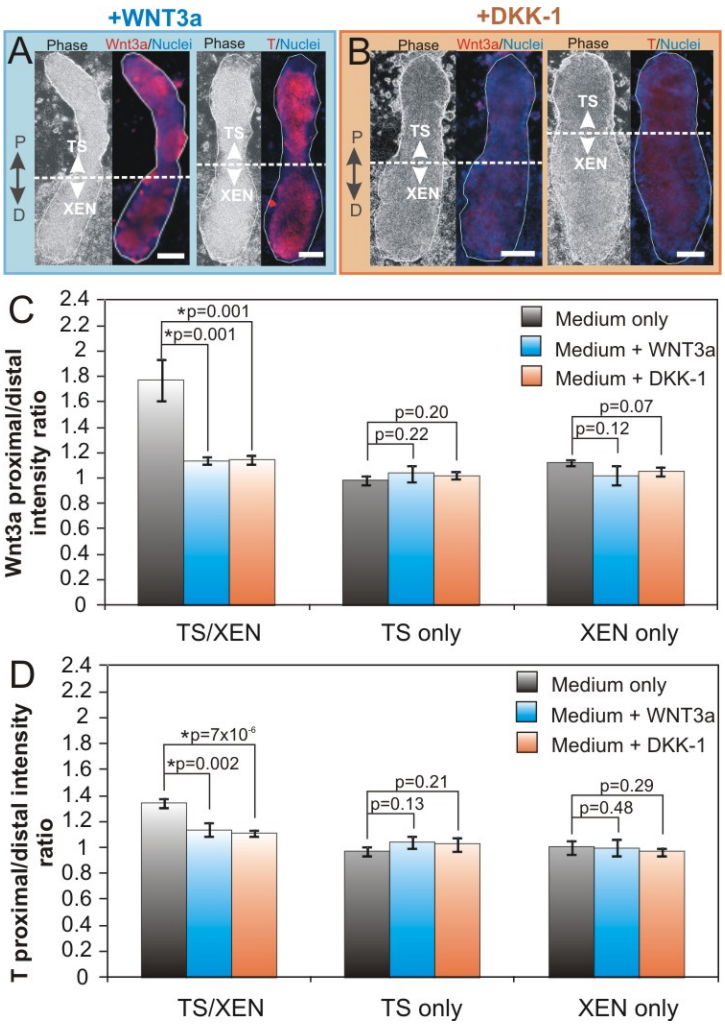

Figure. 3 PD patterning in *δESP embryonic model is modulated by Wnt-signaling. Disruption of polarized Wnt3a and T expressions by exogenous (A) WNT3a (50 ng/ml) or (B) DKK-1 (200 ng/ml). Quantitative assessment of (C) Wnt3a and (D) T PD asymmetries in co-culture medium or co-culture medium supplemented with WNT3a or DKK-1. Data are average of >10 colonies (on TS-XEN interface) or >3 colonies (on TS or XEN cells alone) from 2 independent experiments ± s.e.m. Pairwise comparisons (t-test) are indicated by connecting lines with the respective p-values. * indicates statistical significance (p<0.05). Scale bars = 200 μm.*

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