Modeling of human neurulation using bioengineered pluripotent stem cell culture

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ABSTRACT

Leveraging the developmental potential and self-organizing property of human pluripotent stem (hPS) cells, researchers have developed tractable models of peri- and post-implantation development of the human embryo. Owing to their compatibility to live imaging, genome editing, mechanical perturbation and measurement, and screening applications, these models offer promising quantitative experimental platforms to advance human embryology and reproductive and regenerative medicine. Herein, we provide a review of recent progress in using hPS cells to generate models of early human neural development or neurulation, including neural induction of the ectoderm and regional patterning of the neural tube. These models, even in their nascent developmental stages, have already revealed intricate cell-cell signaling and mechanoregulation mechanisms likely involved in tissue patterning during early neural development. We also discuss future opportunities in modeling early neural development by incorporating bioengineering tools to control precisely neural tissue morphology and architecture, morphogen dynamics, intracellular signaling events, and cell-cell interactions to further the development of this emerging field and expand its applications.

1. Introduction

Neurulation is a key embryonic developmental process that gives rise to the formation of the neural tube (NT), the precursor structure that eventually develop into the central nervous system (CNS). Neurulation occurs soon after the gastrulation of the embryo, during which the epiblast resolves into a trilaminar germ disc structure containing the ectoderm, mesoderm and endoderm (Fig. 1). Neurulation is initiated by a neural induction process, during which the dorsal ectoderm is specified into a spatially patterned multicellular tissue containing the neural plate (NP) and the non-neural ectoderm (NNE) separated by the neural plate border (NPB) (Fig. 1). After neural induction, the NP folds towards the dorsal side of the embryo and fuses to form the tubular NT. The development of the NT continues with differentiation of distinct classes of neuronal progenitor cells located at defined positions within the NT along both the anterior-posterior (AP) and dorsal-ventral (DV) axes. Allocation of neuronal fates in the NT is directed by secreted inductive factors (*i.e.*, morphogens) emanated from surrounding tissues. These inductive signals are transduced through intracellular signaling events and genetic networks to activate distinct transcriptional factors that restrict the progressive development and specification of progenitor cells in the NT towards different neuronal subtypes [1].

Disruption of the NT development leads to the NT defects (NTDs), one of the most common birth defects [2]. Many neurodevelopmental disorders such as autism spectrum disorder and Down's syndromes are also connected with dysregulations of the early CNS development [3,4]. Understanding the molecular mechanisms and morphogenetic events underlying human neurulation is thus important for prevention and therapeutic development and treatment of NTDs and neurodevelopmental disorders. However, analyses of animal models have shown limitations in revealing some of the most fundamental aspects of development, genetics, pathology, and disease mechanisms that are unique to human CNS development. Furthermore, technical difficulty and ethical constraint in accessing neurulation-stage human embryonic tissues have significantly limited experimental investigations of early human CNS development and developments of human CNS disease models.

Pluripotent stem (PS) cells, including embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, provide a new paradigm for studying human development and disease modeling [5-9]. Significant progress has been achieved in directed differentiation of human PS (hPS) cells into specific neuronal subtypes by modulating dynamic chemical signals based on developmental principles [10]. Importantly, self-assembled three-dimensional (3D) aggregate cultures of hPS cells have been successfully developed to generate brain organoids with cell types and cytoarchitectures that resemble curtain aspects of embryonic brain [6,11,12]. Brain organoids have emerged as novel model systems to investigate human brain development and disorders at the molecular, cellular, structural and functional levels.

Recently, we and others have leveraged the developmental potential and self-organization property of hPS cells in conjunction with 2D and 3D bioengineering tools to achieve the development of spatially patterned multicellular tissues that mimic certain aspects of early human neurulation process, including neural induction and DV patterning of the NT. These models are important alternatives to animal models to study the self-organizing principles

involved in autonomous patterning during human neurulation. In this review, we discuss these hPS cell-based models and further provide perspectives on future efforts in incorporating advanced bioengineering tools to generate controllable experimental systems with hPS cells to advance knowledge of human neurulation and use such systems for disease modelling and high-throughput screens.

2. Neural induction

Neural induction, as an evolutionarily conserved developmental event, initiates the neurulation process by patterning the dorsal ectoderm into spatially organized NP, NPB, and NNE regions (Fig. 1). Accompanying progressive cell fate specifications during neural induction, morphogenetic events occur as the ectoderm becomes thickened in the dorsal region to form pseudostratified, columnar neuroepithelial (NE) cells in the prospective NP domain [13] (Fig. 1). Classic studies of animal models have revealed the importance of integrated signaling networks involving BMP, FGF and WNT signaling to regulate neural induction [14-17]. Graded BMP signaling has been identified to guide ectoderm patterning, with high BMP signaling promoting NNE differentiation, low BMP activity required for NP formation, and intermediate BMP promoting NPB development [18,19]. A balance between FGF and BMP signaling is also important for establishing and maintaining the NPB region [20]. Recent studies further show a role of WNT signaling in refining the boundary between NP and NNE by regulating the ectoderm's response to BMP and FGF signaling [21,22].

Recently, we and others have utilized bioengineering tools such as microcontact printing to generate two-dimensional (2D) colonies of hPS cells with defined shapes and sizes. In microcontact printing, adhesive extracellular matrix (ECM) proteins are printed onto 2D culture surfaces using a stamp containing regular micropatterns and inked with ECM proteins. After seeding, hPS cells are confined within adhesive micropatterns on 2D culture surfaces. Pre-patterned geometrical confinement induces emergent patterning of hPS cells, mimicking neuroectoderm regionalization during early neurulation. In the neural induction model developed from our group [23], patterned circular hPS cell colonies are cultured under a neural induction condition with dual Smad inhibition and a transient activation of WNT signaling. While hPS cells differentiate, they gradually self-organize into a radial symmetrical pattern of NE and NPB cells, with NE cells localized at the colony center and NPB cells accumulating at the colony border (Fig. 2A). Interestingly, differentiating hPS cells in circular colonies exhibit graded BMP activity along the colony radial axis, with low and high BMP activities at the colony center and border, respectively. By measuring cell shape and contractile force using micropost array force sensors, it is revealed that while hPS cells respond to neural induction signals, they self-organize and display graded mechanical properties, including cell shape and contractile force, along the colony radial axis [23]. By using a customized cell stretching device to stretch only the central region of cell colonies, NE differentiation is inhibited but NPB differentiation is promoted at the colony center. These observations suggest that self-organization of cell shape and cytoskeletal contractility may feedback to regulate graded BMP activity and thus instruct NE / NPB patterning. This work provides evidence of tissue

mechanics-guided neuroectoderm patterning and establishes a tractable model to study signaling crosstalk involving both biophysical and biochemical determinants in neuroectoderm patterning.

The neural induction model established by us does not contain the sensory placode and NNE, two additional ectodermal lineages. Brivanlou, Warmflash and colleagues have recently reported two micropatterned, hPS cell-based neurulation models [24,25]. Although slightly different, both models feature an early Nodal inhibition using SB431542 (TGF-β inhibitor) to induce ectoderm differentiation followed by exogenous BMP4 stimulation to induce radial patterning of all four ectodermal lineages, corresponding to the mediolateral aspects of ectoderm development. Notably, the neuruloid reported by Brivanlou and colleagues features a spherical lumenal NE cyst at the colony center surrounded by a NC domain, placode cells forming a concentric ring structure within the NC domain, and all these three domains covered by a single layer of NNE cells (Fig. 2B). Importantly, the neuruloid was further utilized to model developmental aspects of the Huntington's disease (HD) using disease-relevant cell lines. HD neuruloids show a reduction in NE lumen size, an increase of the central NE domain, and loss of collagen layer at the basal side of the central NE domain. Single-cell sequencing data suggest the involvements of WNT/PCP pathway and cytoskeleton as mechanisms underlying the morphogenetic defects observed in HD neuruloids. The study of HD neuruloids points to an important application to understand the link between early developmental defects and late onset neurodegenerative diseases, an area remaining largely unexplored.

Besides demonstrating radial patterning of NE, NC, placodal, NNE cells in 2D circular colonies, Warmflash and colleagues utilized their model to examine the roles of classic developmental signaling events (Fig. 2C); their observations support a two-step model of NC induction: an early activation of WNT and FGF signaling for NPB specification followed by BMP and WNT activation for NC maturation [26,27]. Their data further suggest low BMP and high WNT signaling conductive for NC development and high BMP and low WNT for placode specification.

Recently, Toh and colleagues have reported another micropatterned ectoderm patterning model that further incorporates mesoendoderm lineages and displays morphogenetic events resembling NP folding (SSRN doi: 10.2139/ssrn.3231850). A short mesoendoderm induction followed by neuroectoderm induction allows hPS cells at the colony border to differentiate into mesoendoderm cells and cells at the colony center to differentiate into NE cells. Apical constriction, marked by enriched ACTIN and p-MYOSIN at apical surfaces, induces tissue folding at the colony border toward colony center. A critical role of mesoendoderm cells involving TGF-β signaling is identified for folding of micropatterned ectoderm tissues.

Current neural induction models are based on 2D cultures, where large-scale cellular organization and morphogenetic movement are limited. Although these neural induction models recapitulate certain features of cell fate patterning during early neurulation, 2D neural induction models are limited in reconstructing the in vivo 3D cellular structure and fall short in mimicking some key aspects of early neural development such as folding and fusion of the NP to form the NT. The model developed by Toh and colleagues points to a future direction where additional germ layer lineages (mesoendoderm cells) can be incorporated into the model to recapitulate

tissue-tissue interactions that might be needed for morphogenetic organization at the tissue scale during neurulation. Since neurulation in vivo occurs in a 3D environment, there are indeed efforts over the last few years in using 3D cultures of mouse and human PS cells to develop 3D models of NT development. These models have shown great promise in mimicking progressive, regional patterning of the NT. In the next section, we will discuss such 3D models of NT development.

3. Regional patterning of NT

After neural induction, the NP folds toward the dorsal side of the embryo and fuses to form the NT. Studies using model organisms have shown that after neural fold closure, progressive development of the NT leads to its regional patterning along the AP and DV axes, which is controlled by morphogens produced by surrounding tissues to establish signaling gradients [30-32]. Pioneering work by Tanaka, Lutolf and colleagues shows for the first time that mouse PS cells seeded in 3D cultures can be utilized to model DV patterning of the NT (Fig. 3A) [33,34]. When embedded in Matrigel or synthetic hydrogels under a neural induction condition, mouse PS cells self-organize and form spherical lumenal structures before differentiating into NE tissues. Such spherical lumenal NE tissues are termed NE cysts. These NE cysts maintain a default dorsal identity and are responsive to patterning signals, such as retinoid acid (RA) for posteriorization. Interestingly, global treatment of NE cysts with RA induces DV patterning, featuring sequential emergence of the ventral floor plate, P3 and pMN domains in discrete, adjacent regions and a dorsal territory progressively restricted to the opposite dorsal pole. $FOXA2^+$ floor plate emerges first in mouse PS-derived NE cysts before inducing the development of P3 and pMN domains in adjacent regions, consistent with FOXA2 as a RA target and FOXA2 and SHH transcriptionally activating each other [35,36]. DV patterned NT model has recently been further demonstrated with hPS cells, with similar observations supporting that RA treatment might first induce $FOXA2^+$ floor plate formation, and SHH secreted from floor plate cells in turn induces the specification of P3 and pMN progenitors in their adjacent domains [37].

Another recent work by Takahashi and colleagues reports 3D induction of dorsal, intermediate, and ventral NT-like tissues in a free-floating cell aggregate culture by controlling relative levels of exogenous BMP and SHH signals (Fig. 3B) [38]. Although full DV patterning has not been achieved, Takahashi and colleagues have shown either local dorsal or ventral patterning of NE cysts. Notably, localized roof plate-like structures surrounded by dorsal neural progenitor cells are observed in dorsalized NE cysts, and localized patterning of ventral cell fate with spatially aligned floor plate/P3/pMN structures are observed in ventral NE cysts. However, this work does not recapitulate correct apical-basal polarity of the NT, with the apical surface of the NE cyst facing outside external environments. Similar apical-basal polarity has also been reported in hPS-based organoids developed in free-floating cultures [39,40], suggesting culture environments as a contributing factor for apical-basal polarity establishment of multicellular epithelial tissues.

The existing models of regionally patterned NT rely on autonomous cell fate patterning in a relatively homogenous culture environment, and thus the efficiency of generating patterned NE cysts remains relatively low [33]. When matrix composition, degradability, and stiffness are optimized using synthetic hydrogels, the shape of NE cysts became more uniform and 70% of NE cysts underwent proper DV patterning [34]. Regional patterning of the NT in vivo is controlled by morphogen signaling gradients. Thus, bioengineering techniques, such as microfluidics, which can provide precise spatiotemporal control of chemical gradients, are promising tools that can be incorporated in this direction to improve the efficiency of controlled modeling of NT development and patterning.

4. Outlook

Here we provide a concise review of current PS cell-based, neural development models mimicking neural induction and NT patterning. In conjunction with lineage and signaling reporter lines, these models will offer promising trackable systems to study pattern formation, morphogenesis, cell differentiation, and growth and how these developmental processes are regulated and coordinated during neural development. These models are also useful for elucidating intracellular signaling dynamics and gene regulatory networks and their cross-talk with mechanotransduction during embryonic development and for studying classic developmental biology questions, such as symmetry breaking, scaling and induction. In principle, these synthetic models can be integrated with multi-well plate formats to achieve highly parallelized assays compatible with existing automation workflows and screening infrastructure. By employing advanced bioengineering approaches to control dynamic cell-cell and cell-ECM interactions and soluble environments, new models of neural development, such as those simulating neural fold development and their fusion into the NT and AP / DV pattering of the NT, will likely emerge in the years ahead. Successful development of these new models will hinge on refined understanding of the self-organizing properties of neural development and the intricate interactions and interdependencies between soluble factors and insoluble biophysical signals and their roles in neural development. It is also important to recognize that the neurulation is a long-term process in which dynamic changes of cell-cell and cell-extracellular matrix interactions abound. How we can generate in vitro culture environments to mimic the dynamic nature and complexity of the in vivo neurogenic niche remains a significant challenge.

The existing models of neural development largely rely on autonomous self-organization of PS cells to form patterned multicellular structures de novo through spontaneous symmetry breaking. It can be envisioned that different bioengineering strategies can be incorporated into this emerging area to provide precise controls of different aspects of cell culture microenvironments. These bioengineering tools, which span different scales, from molecular to cellular to organ levels, allow researchers to generate dynamic culture environments, with the molecular, structural, hydrodynamic, and mechanical cues well controlled in conjunction with their spatial and temporal levels and combinations. Some of the techniques have already been successfully implemented in the examples discussed above, including microcontact printing and synthetic hydrogels. Others useful for controlling dynamic chemical signals and their gradients,

intracellular signaling, and cell-cell interactions, such as microfluidics, optogenetics, and bioengineered tissue co-culture systems, are expected to find important applications in this area. Specifically, development of microfluidic devices capable to precisely control cell colony size and position and generate dynamic morphogen gradients could lead to new controllable models of neural development such as AP / DV patterning of the NT. Bioengineered tissue co-culture systems, which could control the position of different cell types differentiated from hPS cells, would enable studies of tissue-tissue interactions during neural development [41]. For example, co-culture of NE and NNE cells or mesoderm cells could answer some key questions of neural crest induction. Recent development of optogenetic tools to control developmental signaling activities will enable controlled symmetry breaking, which could in turn induce the development of local signaling centers critical for progressive cell fate specification and tissue patterning (https://www.biorxiv.org/content/10.1101/665695v1). Another important area we expect new discoveries from these synthetic models is the study of feedback regulation by morphogenetic cues (such as geometric boundary, cell polarity and shape, and cytoskeletal contractility) on classic developmental signaling pathways to guide embryonic patterning and tissue morphogenesis during neural development.

All in all, bioengineered culture environment with tailored ECM composition, mechanical properties, and spatiotemporally controlled chemical signals and gradients would lead to more robust developments of patterned multicellular tissues mimicking the NT development. It should also be noted that the self-organizing properties of embryonic development have only become more appreciated recently, and hPS cells have intrinsic self-organizing abilities to develop into spatially patterned multicellular tissues without external spatial cues. How to leverage the intrinsic self-organizing properties of hPS cells while using bioengineered culture environments with spatiotemporally controlled mechanical and chemical signals and gradients to guide multicellular tissue development provides new opportunities for developing improved NT development models.

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Disclosures

The authors declare no conflicts of interest.

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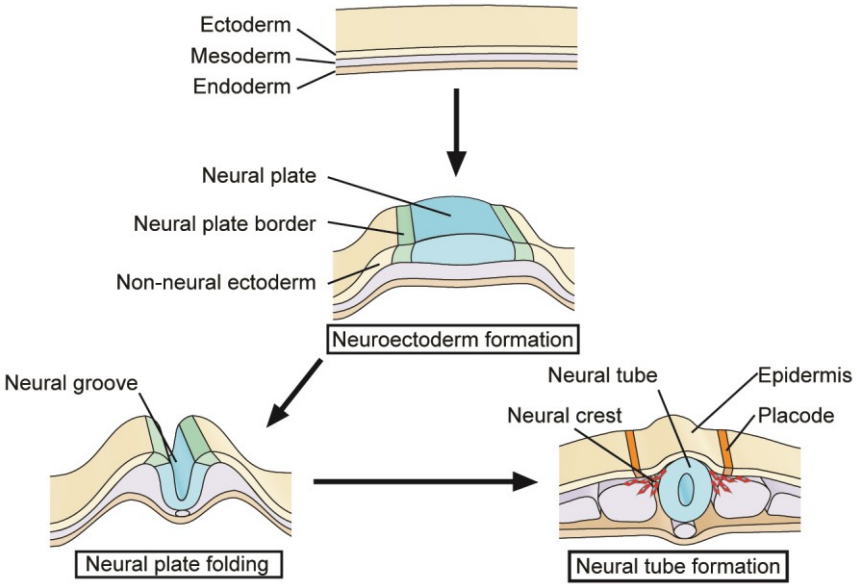
Figure legends

Figure 1. Schematics of early neural development. After gastrulation, the ectoderm is specified into a spatially patterned tissue, including neural plate, neural plate border and non-neural ectoderm. The neural plate then bends towards the dorsal side of the embryo to form a tubular structure, the neural tube. The neural plate border will continue to develop into migratory neural crest cells through epithelial to mesenchymal transition and delaminate from the neural tube.

Figure 2. hPS cell-based in vitro models of neural induction and ectoderm patterning. (A) A micropatterned colony with spatially organized $PAX6^+$ neuroepithelial cells and $PAX3^+$ neural plate border cells. Colony diameter, 400 μm. Scale bar, 100 μm. Adapted with permission from Reference 23. (B) Top and side views of the neuruloid model. Colony diameter, 500 μm. Scale bar, 50 μm. Adapted with permission from Reference 24. (C) Representative images of colonies stained for indicated markers showing radial organization of four ectoderm lineages. Colony diameter, 700 μm. Scale bar, 100 μm. Adapted with permission from Reference 25.

Figure 3. In vitro models of DV patterning of the NT. (A) DV-patterned neuroepithelial cysts derived from mouse ES cells cultured in Matrigel and synthetic PEG gels. Scale bar, 20 μm. Adapted with permission from Reference 33. (B) Human PS cell-derived dorsal and ventral spinal cord-like tissues. Scale bar, 200 μm. Adapted with permission from Reference 38.

Figure 1


Ectoderm
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Neural plate
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Neural groove
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Neural tube
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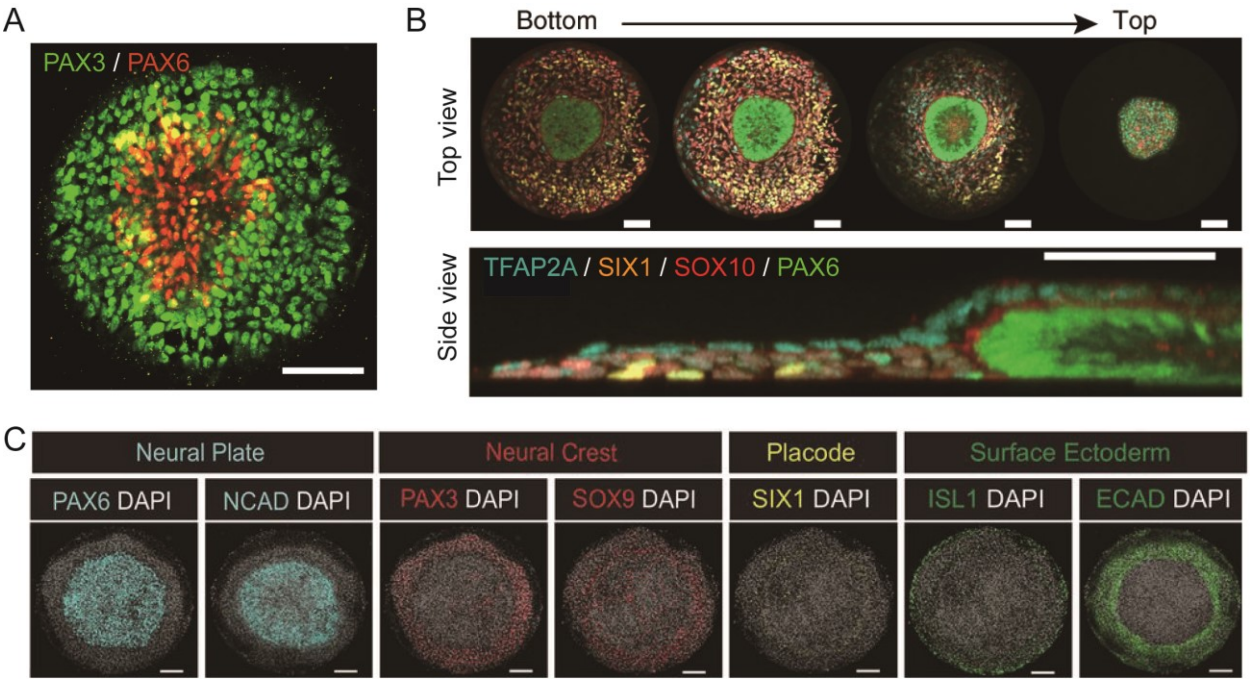
Neural crest
Placode
Neural tube formation

Figure 2


A
PAX3 / PAX6
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TFAP2A / SIX1 / SOX10 / PAX6
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Neural Plate
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PAX6 DAPI
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PAX3 DAPI
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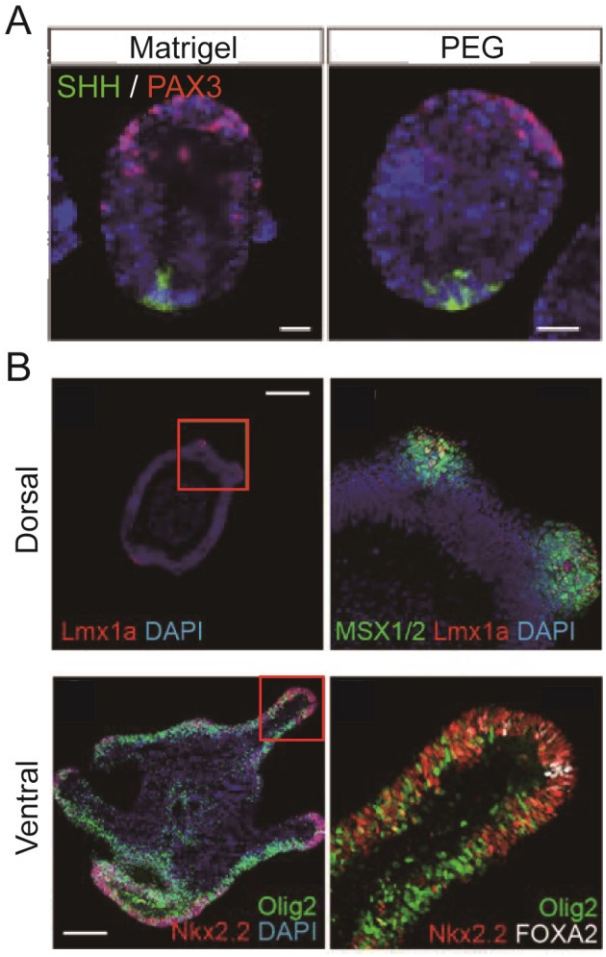
ECAD DAPI

Figure 3


A
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