様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）Directing Human Induced Pluripotent Stem Cell-Derived Embryonic-like Organoid with 3D Biomimetic Cryogel Mechanical Microenvironment for Neural Induction.

研究課題名（英文）Directing Human Induced Pluripotent Stem Cell-Derived Embryonic-like Organoid with 3D Biomimetic Cryogel Mechanical Microenvironment for Neural Induction.

研究代表者

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交付決定額（研究期間全体）：（直接経費）　　2,200,000円

研究成果の概要（和文）：この研究では、特定のモルフォゲンを使用せずに、iPScs を拘束し、胚の特徴と神経運命を表示する 3D 成長期の分化を促進する単純なアガロースベースのクライオゲルが示されました. 特に、コア多能性タンパク質 Sox2+ 細胞のプライム状態多能性への移行は、安定な胚盤葉上層の状態にある Otx2 によって特徴付けられます。その後、Otx2 と T-Bra の間の相互排他的なパターンの促進に貢献します。したがって、これらの発見は、胚の状況における神経中胚葉運命の促進におけるiPScs/物質の相互作用についての新たな洞察を提供する可能性がある。

研究成果の学術的意義や社会的意義
The projects provide a simple approach that highlights cell/material interface platform. This platform can generate embryonic organoid derived from iPSCs. Further, useful to scale up to an organoid-on-chip model for drug testing to prevent fetal toxicity and miscarriage.

研究成果の概要（英文）：This project introduces the application of a simple agarose-based cryogel that can trap iPSCs and confine them. Thus, compacted cells sense mechanical constraints and promote differentiation in a 3D growth phase without the use of specific morphogens. The aggregates showed features of embryonic development, which display Anterior/Posterior (A-P) axis development and the presence of three germ layers along the axial convergence. The detailed cell specification reveals the capacity to self-organize into a rudimentary neural fate along the A-P axis. Notably, the transition of core pluripotency protein Sox2+ cells to prime state pluripotency is marked by Otx2 which is in the state of the stable epiblast. Later, it contributes to promoting mutually exclusive patterns between Otx2 and T-Bra. Thus, these findings can provide new insight into iPScs/material interaction in promoting neuromesodermal fate in an embryonic context.

研究分野：Embryonic organoid

キーワード：Cryogel　Mechanical Constraint　iPSCs　Axial convergence　Germ layers　Neural fate　Neuromesodermal

様　式　Ｃ－１９、Ｆ－１９－１（共通）

１．研究開始当初の背景

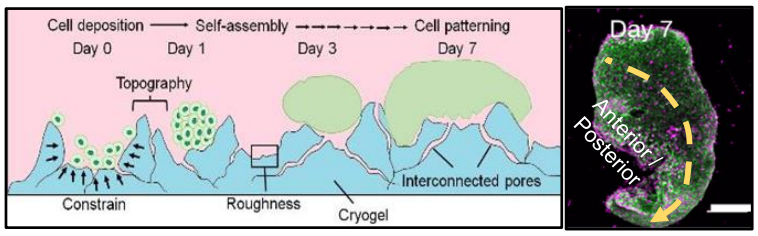

Figure 1: (A) Schematic showing embryonic growth (B) Growth of organoid with embryonic features at day 7

Mammalian development and early organogenesis are highly regulated processes that depend on cell fate transitions. Cellular reorganization during embryogenesis is a physical process and depends on mechanical constraints imposed by the surrounding maternal tissue. Elucidating the mechanistic influences on embryonic morphogenetic events is crucial to understanding tissue homeostasis. It is impossible to decipher this biological behavior in vivo due to ethical boundaries and lack of access to embryos developing in the womb. Hence, there is a growing demand for 3D reconstructions of the developmental process *in vitro* using pluripotent stem cell assemblies. Under certain culture conditions, human pluripotent stem assemblies grown in suspension culture give rise to three-dimensional constructs with entities of early development called gastruloids or embryonic organoids. The growth features include symmetry breaking and convergent extension of the body plan along the anterior-posterior axis. Realistically, In vivo, embryonic morphogenesis is determined by the mechanistic effect of extra-embryonic tissue (EXT). However, 3D gastruloid protocols lack the growth under (EXT). Thus, developing an embryonic organoid culture protocol that includes a mechanical microenvironment is important from this point of view. To our understanding, we aim to fabricate a platform allowing cells to dynamically sense the physical properties (stiffness and topological cues) via the interaction of cell/material interface. This study shows that agarose cryogels generate large, interconnected macropores that appear as void-like microcavities on the surface. As a result, they act as cell-substrate interfaces that trap and constrain cells. Similarly, it mimics the compression of the tissue after transplantation in the uterus. Interestingly, softer cryogels (0.5% agarose) cryogels promote the differentiation of hiPSCs into germ layers and facilitate tissue morphogenesis towards anterior-posterior (A-P) axial polarity, indicating the formation of three germ layers along axial convergence. It is associated with the identity of the protoderm-forming tissue, which is regulated by the expression of the primary embryogenesis marker, Goosecoid (Gsc). Interestingly, these polar aggregates exhibit epithelial-mesenchymal transition (EMT) transition, which identifies the replacement of E-cadherin (E-cad) by N-cadherin (N-cad) and vimentin. Furthermore, the presence of N-cad appears to co-localize with cells expressing T-Brachyury (T-Bra) and Otx2. Overall, this finding suggests that the interaction between the neuroectoderm and mesoderm is reciprocal and coexistent. In conclusion, this study presents a simple platform for generating iPSC-derived neuralmesoderm cell fates in an embryonic context

２．研究の目的

Purpose: Using human induced pluripotent stem cells (hiPSCs)to develop synthetic embryonic organoid models which are considered as 'gastruloid'. This model shows commitment to replicating embryonic development in vitro, which can overcome ethical boundaries due to the inaccessibility of studying embryos implanted in utero.

Challenge: Despite growing insights into advances in gastruloid culture,
The growth of tissue dynamics concerning the outcomes of mechanical microenvironment is not well defined.

Objective: To understand the range of physical microenvironmental inputs to cell dynamics
and tissue morphogenesis. Introducing a new cryogel-based cell-substrate platform Provides topological constraints that act on cells without exogenous chemical

Stimulation.

３．研究の方法

Preparation of agarose-based cryogels:.
Agarose (SeaKem, Lonza) based cryogels were produced by freezing and thawing techniques. Hydrogels were prepared by first dissolving agarose in distilled water at the boiling point and casting 50 µl of the solution into 96-well plates (Ikki Cell Biology). After cooling the agarose solution to room temperature, 70 µl of sterile distilled water was added. These gels were frozen overnight at -80° C and then thawed for 1 hour in a wet CO2 incubator at 37° C. Three different concentrations were prepared as 0.5, 1 and 2% by weight/volume (w/v).

Organoid growth on cryogels:.
Monolayers of the hiPSC cell line TKDN-4M (Stem Cell Bank, Centre for Stem Cell Biology and Regenerative Medicine, University of Tokyo) were maintained in tissue culture dishes coated with vitronectin (Thermo Fischer Scientific, USA) in exchange for fully supplemented cell culture dishes. Essential 8 (E8) medium (Thermo Fischer Scientific, USA). Succession numbers up to less than 40 were used for experiments.

Immunocytochemistry:.
Aggregates collected from the culture platform were fixed in 4% paraformaldehyde (PFA) for 24 h at 4° C. They were permeabilised with 1% Triton-X-100 (Thermo Fischer Scientific) for 1 h, followed by three washes with PBS, and the aggregates were treated with gelatine blocking buffer containing 0.2% PBS-Tween20 (Nacalai Tesque) for 2 h. The incubation with primary antibody was implemented for 12 h, followed by three times PBS washing and finally incubating with secondary antibody.

Quantitative real-time PCR:.
Isolated mRNA was reverse transcribed to cDNA using the ReverTra Ace qPCR RT Master Mix (Toyobo, Japan) according to the manufacturer's instructions. Aggregates grown on cryogels and hydrogels were incubated to elucidate the expression profile of markers involved in aggregation development.

４．研究成果

This study has shown that 0.5% cryogel has a less rigid substrate and a rougher surface. Hence, tissue deformation is facilitated and the AP axis is established. On day 7, on average, about 65% of the aggregates in each experiment showed an elongated morphology. The exposed control gels did not show any shape change and acquired a spherical morphology. A similar patch-patterning trend was also observed in the control gels, with a decrease in the Otx2+ cell area in response to gel concentration. Furthermore, Oct4 reactivated regions undergo detachment and acquire Otx2 expression, which may correlate with the requirement for early neuroectoderm formation. As is evident, we found that Goosecoid (Gsc) expressing cells are found in the differentiated regions.

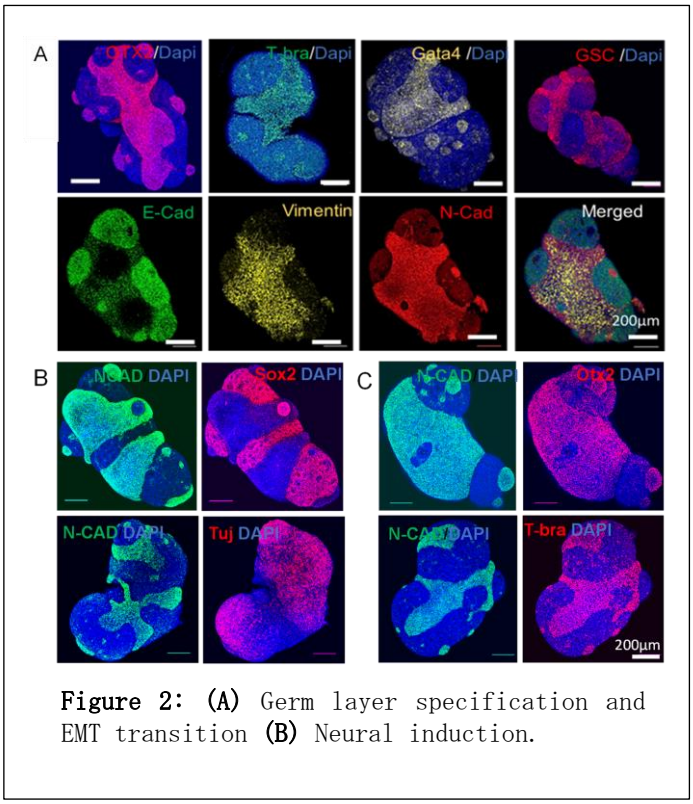

Figure 2: (A) Germ layer specification and EMT transition **(B)** Neural induction.

Gsc is expressed during the early stages of gastrulation. It is a specific marker of the embryonic organizer during formation of the human primitive streak. Next, we observed that cadherin switching acts on the loss of pluripotency by down-regulating Oct4 expression. E-cadherin (E-cad) replaces N-cadherin (N-cad), together with the upward regulation of the mesenchyme marker vimentin. Furthermore, vimentin expression persists in mesodermal induction and in mesodermal derivatives such as body segments. On the other hand, N-cad, also called neural cadherin, is essential for developmental events during neurogenesis. It is strongly expressed in the neuroectoderm. Interestingly, we found that N-cad-positive cells coincided with the Otx2 and the mesoderm marker T-bra. The association of T/Bra-positive and Otx2-positive forms a complementary pattern of expression through the elongation of the body axis. The next step is to determine whether N-cad may be induced by Sox2 and Tuj-1 in neuronal domain expansion. N-cad is responsible for the identity of migrating neurons but did not co-localize with Sox2 and Tuj-1. Our observations suggest that the expression of Sox2 and Tuj-1 started earlier than the maintenance of the neural progenitor cell state. To further assess cell fate specification, we evaluate the expression of Epha-1, a known marker of neuralmesoderm progenitors (NMPs). We found that Sox2 co-localizes with Epha-1 but not with T-Bra. This may indicate that Sox2+/Epha-1+ co-expression is involved in early differentiation event generation (MNP) fate. Importantly, we note that Sox2 interacts heterotypically with both T-bra and Otx2 at the same localization. This mutually exclusive pattern suggest involvement of Otx2 in the specification of heterogeneous NMP populations and reinforces the mixing of neural and mesodermal identities.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計1件（うち招待講演　0件／うち国際学会　1件）

１．発表者名 Raja Vadivelu, Rubina Khadim, Gandhi Torizal , Masaki Nishikawa , Masaya Hagiwara ,Yasuyuki Sakai
２．発表標題 Simple Cryogel-directed hiPSCs derived Gastruloid-like organoids with neural induction
３．学会等名 2023 RIKEN BDR-CuSTOM Joint Organoid Symposium（国際学会）
４．発表年 2023年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

I am writing a paper as outcome of the project and anticipating to submit soon.

６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関