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研究課題名（和文）iPSから継代培養と凍結可能な原始腸内胚葉細胞の誘導及び機能細胞への分化

研究課題名（英文）Multiple Endodermal Organoid Generation from Robustly Amplified Human Posterior Gut Progenitors

研究代表者

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研究成果の概要（和文）：近年、ｉＰＳ／ＥＳ細胞などの多能性幹細胞より誘導した組織・臓器を利用して、新たな医薬品を開発するための創薬スクリーニングや、失われた臓器の機能を補う再生医療を実現化することが注目されている。多能性幹細胞を分化させた後、原始腸内胚葉細胞（Ｐｒｉｍｉｔｉｖｅ　Ｇｕｔ　Ｅｎｄｏｄｅｒｍ　Ｃｅｌｌｓ：　ＰＧＥＣｓ）」の誘導を確立した。ＰＧＥＣｓは細胞増幅したのちに、機能細胞への分化誘導にも成功した。肝細胞、膵細胞及び腸細胞への分化が可能であり、ＣＸＣＲ４などの癌の悪性度に関係するマーカーを発現せず、フィーダー細胞を用いることなく調製することができるので、臨床応用が容易であるという優位性を持つ。

研究成果の概要（英文）：Early human developmental progenitors naturally possess robust amplification potential to ensure organ growth; thereby, are considered as a promising source for therapy due to minimal risks for tumor or ectopic tissue formation. Here, we first demonstrated the reproducible generation of human CDX2+ posterior gut endoderm cells (PGECs) from multiple induced pluripotent stem cell (iPSC) clones. We were able to amplify storable PGECs over a number of passages up to 1021 cells, showed much more stable differentiation propensity into endodermal lineage cells. Furthermore, human PGECs were capable of producing hepatic and intestinal tissues. PGEC-liver organoid transplantation showed therapeutic potential in treating lethal liver failure. Thus, the use of PGECs may be not only a promising alternative therapeutic source of pluripotency for self-organizing endodermal organoids but also a unique approach to study the developmental biology and disease model of the human gut.

研究分野：　肝臓再生

キーワード：　幹細胞　原始腸内胚葉細胞　分化　肝臓　移植

1. 研究開始当初の背景

Early human developmental progenitors naturally possess robust amplification potential to ensure organ growth; thereby, are considered as a promising source for therapy due to minimal risks for tumor or ectopic tissue formation. One remarkable example is posterior gut endoderm progenitors, which eventually develop majority of meter-long gastrointestinal tracts in humans.

Posterior gut specification occurs at a caudal part of the primitive gut endoderm on embryonic day (E) 8.5 in mice and day 20 in human stem cell culture1, and this process primarily contributes to the formation of the small and large intestines in adults2. Relative to the anterior domain of the endoderm, posterior gut progenitors elongate, forming a significantly longer segment of the gut through extensive proliferation and migration3. It is likely that PGEC re-arrangement and proliferation are required to achieve the expansion of the gut endoderm. One current major unmet challenge involves the recapitulation of the differentiation process of PGECs in a dish from pluripotent cells.

2. 研究の目的

The current study sought to differentiate and amplify CDX2+ human posterior gut progenitors from human induced pluripotent stem cells (iPSCs) by translating these molecular knowledge of modeling early endoderm organogenesis. We also investigated the self-organizing potential of these cells to develop into organoids of multiple endoderm derivatives with the aim of applying these cells for future therapeutic use

3. 研究の方法

We used a specified step-wise differentiation protocol in serum-free medium (SFD) that has previously been shown 29 to induce definitive endoderm (DE) (Figure S1A) cell fate in human iPSCs. This protocol mimics embryogenesis by initial treatment with Activin A and Wnt3a for 2 days, followed by BMP4, FGF2, VEGF and Activin A treatment for 4 days to induce DE. The generated DE cells were subsequently re-plated onto Matrigel-coated culture dishes at a 1:1 ratio to specify posterior gut endoderm cell, and then differentiate into endoderm lineage cells.

4. 研究成果

1) iPSCクローンからの原始腸内胚葉細胞（PGEC）の樹立.

Immunostaining revealed that the endoderm cell marker FOXA2 was present in both DE cells and PGECs, whereas CDX2 was specifically expressed in PGECs (Figure 1A). qPCR analysis further revealed low expression of the DE cell-specific markers CXCR4 and

Cerbelus1 and high expression posterior gut marker CDX2 as well as similar expression level of endodermal marker FOXA2 and SOX17 in PGEC compared with DE (Figure 1B).

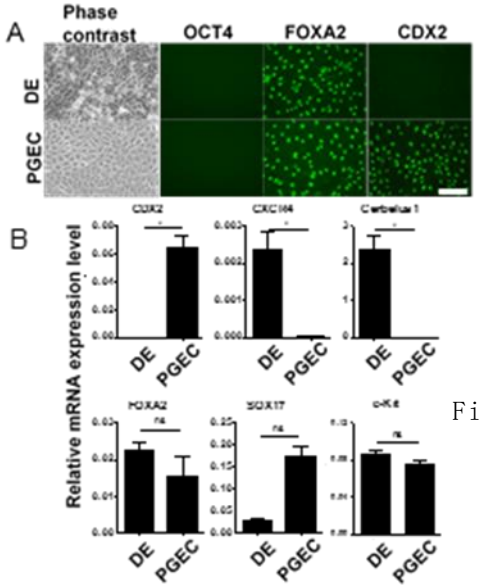
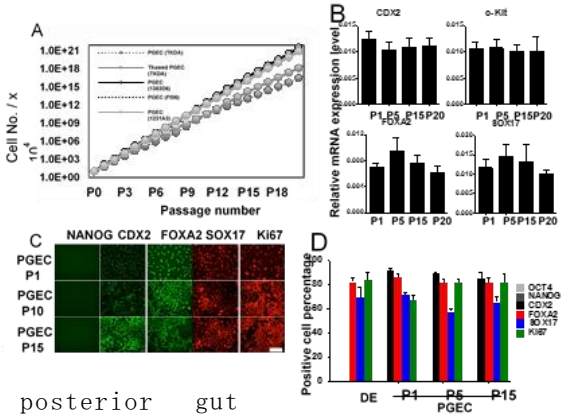


Fig1. Directed differentiation into CDX2+ posterior gut endoderm progenitor cells

2) 増幅したPGECの特性解析

iPSC clone-derived PGECs were maintained (for approximately 1015-, 1019-, 1019- and 1020-fold expansion for up to 70 days of TKDA3-4, FFI06, 1231A3 and 1383D6 iPSC clone-derived PGECs, with doubling times of 33.09 hours, 21.65 hours, 23.13 hours and 22.65 hours, respectively) for more than 20 passages and demonstrated epithelial-like morphology and proliferation (Figure 2A). Over a number of passages, serial qRT-PCR (Figure 2B) and immunostaining analyses (Figure 2C) showed that the posterior gut phenotype was maintained with stable CDX2, SOX17, FOXA2 and c-Kit expression, and the cell proliferative capability was maintained with active Ki67 expression (Figure 2).

Fig2. Extensive propagation of CDX2+ posterior gut endoderm progenitor cells



3) PGEC由来腸オルガノイドの作成

Following a step-wise, directed differentiation approach that mimics signaling events occurring during normal intestine development in vivo, we were able to generate intestinal organoids. Remarkably, Chir99021- and FGF4-treated PGECs underwent morphogenesis that was similar to embryonic hindgut formation.

After 3 days of Chir99021+FGF4 treatment, flat cell sheets condensed into epithelial tubes, many of which budded off and formed floating hindgut spheroids (Figure 3A). In vitro

hindgut-tube morphogenesis into spheroids was never observed without Chir99021+FGF4 treatment. These results support a previously described mechanism for gut development, in which synergy between Wnt signaling and FGF signaling is required for specification of the hindgut lineage.

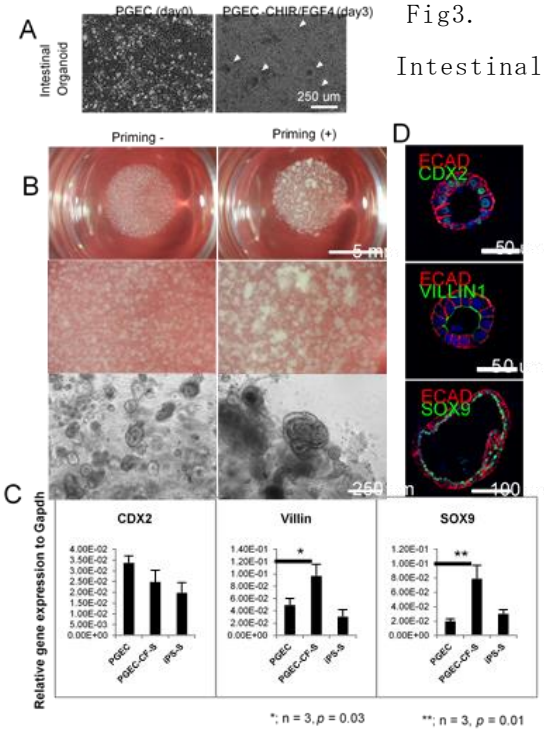


Fig3. Intestinal organoids from posterior gut endoderm cells

We next investigated whether the spheroids could develop and mature into intestinal organoids in vitro by using three-dimensional culture conditions that support the growth and renewal of adult intestinal epithelium. When the spheroids were embedded into this culture system, the spheroids developed into intestinal organoids (Figure 3B). After 7 days of culture, the epithelium of the spheroid matured and protruded into the lumen of the organoid (Figure 3B). However, PGECs without Chir99021+FGF4 pre-treatment failed to develop into intestinal organoids. These results were confirmed by immunofluorescence and qPCR analysis. After 7 days in 3D culture, enterocytes (VILLIN+) and intestinal epithelial cells (E-CAD+) were both readily detected (Figure 3C and 3D). The intestinal transcription factors CDX2 and SOX9 were broadly detected, suggesting that the PGEC-derived spheroids embedded onto the matrix retain an intestinal phenotype. Thus, directed differentiation of PGECs into intestinal organoids in vitro is highly efficient for generating three-dimensional intestinal organoids.

4) 劇症肝不全モデルに対するPGEC由来肝芽移植による治療効果

With our previous protocol for liver organoid generation, we cultured PGECs with endothelial (HUVEC) and mesenchymal (MSC) lineages (Figure 4A) to generate PGEC liver organoids (PGEC-LBs). After co-culture, human PGECs self-organized into macroscopically visible 3D cell clusters with an intrinsic organizing capacity up to 72 h after seeding (Figure 4B).

To examine whether this protocol was appropriate for PGECs, we first analyzed the human albumin secretion between different culture media of HCM and HCM/EGM. After culture for 8 days, significantly higher human albumin secretion was detected under HCM/EGM culture conditions (427 ± 34 ng/ml and 552 ± 85 ng/ml, respectively) (Figure 4C). qPCR analysis further revealed the significantly increased expression of early hepatic marker genes such as ALB, G6P and CYP3A7 by PGEC-LBs (Figure 4D). Another important indicator of hepatocyte function, ammonia removal, was also carried out and showed detoxifying ammonia capability in in vitro conditions (Figure 4E). Human serum albumin was tracked by ELISA under long-term culture of PGEC-LBs, and the results showed that human albumin was secreted into the culture media at approximately day 7, and up to 1,063 ± 127 ng/ml of human albumin was produced by day 28 of culture. Additionally, there was a significantly higher human albumin concentration in the PGEC-LB group compared with the PGEC only group (Figure 4F) ($P<0.0001$, n=5). To evaluate the functional maturation of human PGEC-LBs, we transplanted PGEC-LBs into an acute liver failure mouse model (Alb-TRECK/SCID) by kidney capsule transplantation. Notably, compared with the sham group, PGEC-LB transplantation significantly rescued mouse survival ($P=0.036$) (Figure 4G). These results suggest that PGEC-LBs with a 3D and vascularized structure achieved successful engraftment and maturation in vivo. The PGEC-LB-derived tissue exhibited hepatic cord-like structures (Figure 4H) at day 30, and immunohistochemistry showed positive detection of human nuclei, the vascular marker CD31, the bile duct marker CK19, and the mature hepatocyte marker human ALB, suggesting the maturation of human PGEC-LBs in vivo.

Fig 4. PGEC derived liver organoid transplant rescue liver failure

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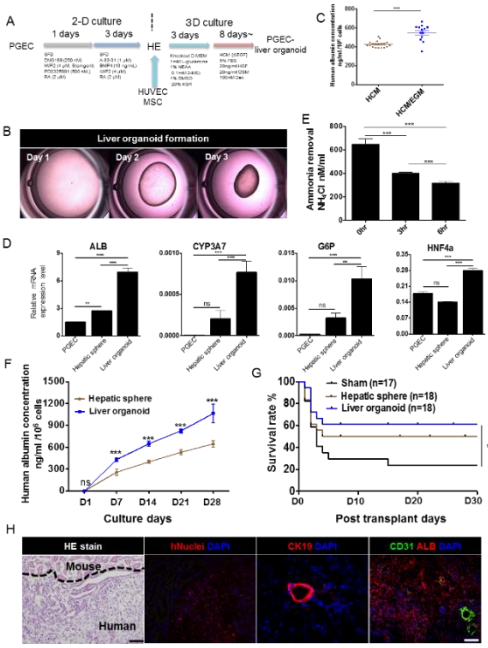

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