Generation of Stomach Tissue from Embryonic Stem Cells

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Generation of Stomach Tissue from Embryonic Stem Cells

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Abstract

Embryonic stem cells (ES cells) have the ability to differentiate into all three germ layers, including gastrointestinal tract organs. Due to advances in developmental biology research, the pathway of gut cell differentiation is well understood. The development of three-dimensional (3D) intestinal organoids with both epithelium and muscle layer, which imitates intestinal development, has improved our understanding of intestinal development and intestinal diseases. In contrast, differentiation of stomach lineage from ES cells has not been well characterized. Recently, human gastric organoids (hGOs) were successfully generated from ES cells. Notably, these organoids included only antrum cell types, and their functions have not been completely understood. The requirement of *in vivo* mesenchymal-epithelial signaling in the gastrointestinal tract is a major challenge for achieving *in vitro* stomach lineage specification from ES cells. Mesenchymal BarH-like homeobox 1 (Barx1), which is strongly expressed in the mesenchymal region of whole stomach primordium, is indispensable for proper stomach development from the gut endoderm *in vivo*. Barx1 null mouse embryos show intestinalization in their stomachs by elongation of intestinal Caudal type homeobox 2 (Cdx2) expression, while ectopic mesenchymal *Barx1* expression induces gastrulation in the intestinal epithelium by induction of gastric SRY (Sex determining region Y)-box 2 (Sox2). For this reason, I hypothesized that induction of mesenchymal Barx1 may differentiate gut endoderm to stomach lineage *in vitro* by recapturing *in vivo* stomach-intestine specification.

In this thesis, I describe protocols for induction of mesenchymal Barx1 and differentiation of stomach lineages from ES cells. This thesis includes five chapters. Chapter 1 provides important background information for this study. Chapter 2 presents the detailed determination of appropriate culture conditions for mesenchymal Barx1 induction in differentiated ES stem cells. By combining several growth factors, I specifically differentiated ES cells into a stomach primordium composed of $Sox2^+$ foregut endoderm and $Barx1^+$ mesenchyme. I used an embryoid body (EB)-based differentiation method that allows ES cells to form gastrointestinal tissue-like structures (gut-like structures), and screened appropriate culture conditions for induction of mesenchymal Barx1 expression in gut-like structures. In addition, I found that Sonic hedgehog (SHH) induction and Wingless-type MMTV integration site family (Wnt) inhibition, which are observed in stomach/intestine specification *in vivo*, were effective for the induction of mesenchymal Barx1 in gut-like structures differentiated from ES cells. Furthermore, gut-

like structures cultured under these conditions gave rise to spheroids that resembled early stomach primordium-like structures. By analyzing these spheroids in detail, I found that these structures comprised of $Sox2^+$/Epithelial cell adhesion molecule $(EpCAM)^+$ epithelium and $Barx1^+$ mesenchymal structure, and differentiated into $Sox2^+$ anterior stomach region and Pancreatic and duodenal homeobox 1 $(Pdx1)^+$ posterior stomach region, suggesting that they had been specified into stomach primordium from ES cells *in vitro*.

Chapter 3 describes methods used to differentiate stomach primordium-like spheroids to a more matured stomach tissue state *in vitro* and the functions of the derived stomach tissue. I used a Matrigel-based 3D culture method for differentiation of stomach primordium-like structures to a more mature state. After supplementation of 3D culture with medium containing specific growth factors, the stomach spheroids grew and resembled stomach tissue. The innermost area was comprised of $EpCAM^+$ epithelium with ATPase H^+/K^+ exchanging beta polypeptide $(Atp4b)^+$ parietal cells, Pgc $(Pepsinogen)^+$ chief cells, and $Muc5ac^+$ pit cells. Importantly, I detected Pgc secretion by enzyme-linked immunosorbent assays and acid secretion following histamine stimulation, indicating that stomach tissue cells derived from ES cells had some of the functional features of the adult stomach.

Chapter 4 illustrates the application of the differentiated stomach tissue to generate an *in vitro* stomach disease model. I established an ES cell line that overexpressed Transforming growth factor alpha (TGFα), which is associated with Ménétrier disease, under Tetracycline (Tc)-Off regulatory control and differentiated the ES cells into stomach tissue cells. After induction of TGFα, the inner and outer cells of the stomach tissue were characterized by a hypertrophic epithelium, which was structurally similar to that observed in Ménétrier disease-model mice. Moreover, the population of $Atp4b^+$ parietal cells decreased with TGFα overexpression. Thus, TGFα overexpression in stomach tissue derived from ES cells led to Ménétrier disease-like characteristics, such as overgrowth of epithelium and gastric achlorhydria. Taken together, these data showed that *in vitro* stomach tissue differentiated from ES cells could mimic *in vivo* early stomach development, and could be applied to develop *in vitro* stomach disease models.

Finally, Chapter 5 summarizes the conclusions of Chapters 2–4, highlights the significance of this study, and suggests possible applications of these data in understanding human developmental biology and developing clinical assays in the future.

Materials and Methods

Mice

ICR mice (E11.5, E13.5, neonatal, and 3 months old) were used for the studies described in this thesis. Samples were harvested from pregnant or adult mice, dissected in cold phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde in PBS (Wako, Osaka, Japan) overnight at 4°C or used directly for RNA isolation or functional assays. Samples for *in situ* hybridization were dehydrated in series of methanol-PBS after fixation, and stored in 100% methanol at −20°C.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan), or RNeasy mini kits (Qiagen, Limburg, Netherlands). RT reaction for synthesizing cDNA was carried out using 1 μg total RNA as template using Prime Script II First Strand cDNA Synthesis Kit (TaKaRa Bio, Shiga, Japan). The following protocol was performed for RT-PCR reaction: denaturation at 98°C for 10 s, followed by 30 cycles of annealing at 58–68°C for 30 s and polymerization at 72°C for 30 s. For quantitative-PCR (qPCR) analysis, 200 ng total RNA was used as template for RT reaction using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). The following protocol was performed for qPCR reaction using Thunderbird qPCR Mix (Toyobo) in a Chromo4 Real-time Detector (Bio-Rad, Hercules, CA, USA): primary denaturation at 95°C for 40 s, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/polymerization at 72°C for 30 s. PCR primers used are listed in Table 1.

ES cell culture

The E14 mouse ES cell line (ATCC, Manassas, VA, USA; passage 60–65) was cultured on mitomycin C- (Kyowa Hakko Kirin Co. Ltd., Tokyo, Japan) induced mouse embryonic fibroblasts (MMC-MEFs) in ES medium (DMEM high-glucose [Wako] containing 15% fetal bovine serum [FBS; Nichirei Bioscience, Tokyo, Japan], 0.1 mM nonessential amino acids [NEAAs; Sigma-Aldrich, St. Louis, MO, USA], 0.1 M β-mercaptoethanol [Sigma-Aldrich], 100 U/mL penicillin/streptomycin [Wako], and 1000 U/mL human recombinant LIF [Wako]) at 37°C in an atmosphere containing 5% CO_2. The medium was changed every alternate day, and ES cells were passaged every 4 days.

Differentiation of ES cells to stomach primordium-like spheroids

For EB formation, trypsinized ES cells were harvested and seeded on gelatin-coated dishes for 45 min to remove contaminating MMC-MEF. ES cell suspensions were then transferred to low-attachment 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA; 500 cells/well) in ES medium in the absence of LIF. After culturing for 6 days without medium changes, EBs were harvested and placed on gelatin-coated culture dishes and differentiated in KSR Medium (DMEM high-glucose [Wako] containing 15% knockout serum replacement [KSR; Life Technologies, Carlsbad, CA, USA], 0.1 mM NEAAs [Sigma-Aldrich], 0.1 M β-mercaptoethanol [Sigma-Aldrich], and 100 U/mL penicillin/streptomycin [Wako]) supplemented with 500 ng/mL recombinant human DKK1 and SHH (R&D Systems, Minneapolis, MN, USA). The medium was exchanged every 3 days. Attachment culture was continued until day 19, and stomach primordium-like spheroids were gradually formed in culture.

Maturation of stomach primordium-like spheroids to stomach tissue in 3D culture

After culturing for 19 days, stomach primordium-like spheroids were further cultured in growth factor-free KSR medium until day 20–22. 3D culture was performed by transferring these spheroids to 100 μL Matrigel (BD Bioscience, San Jose, CA, USA) supplemented with 100 ng/mL recombinant human FGF10, 100 ng/mL WNT3A, 100 ng/mL NOGGIN, 100 ng/mLFGF10, and 250 ng/mL RSPO1 (all from R&D Systems) in 12-well dishes. The spheroids in Matrigel were incubated at 37°C for 30 min to allow polymerization. After incubation, the spheroids were cultured in FGF-based medium (DMEM/F12 [Wako] containing 100 ng/mL recombinant human FGF10, 100 ng/mL WNT3a, 100 ng/mL NOGGIN, 250 ng/mL RSPO1, and 50 ng/mL EGF [Peprotech, Rocky Hill, NJ, USA]) supplemented with N2 (Wako)/B27 (Milteny Biotec, Noordrhien-Westfalen, Germany). The medium was changed every 4 days until day 42–60.

Immunofluorescence staining

For cultured cells, samples were fixed in 3.7% paraformaldehyde in PBS at room temperature for 30 min. Tissue samples or cultured spheroids were fixed in 4% paraformaldehyde in PBS overnight at 4°C, and were dehydrated in 10% sucrose/PBS for 2 h to overnight at 4°C. After dehydration, the samples were embedded in OCT compound

(Sakura Finetek, Tokyo, Japan), and frozen on dry ice-ethanol. The frozen sections were microtomed into 6–20 μm slices. Samples for immunofluorescence staining were washed in PBS twice, and permeabilized with PBST (PBS containing 0.5% Triton X-100) for 5 min at room temperature. The samples were then blocked with FBST (5% FBS/PBS containing 0.1% Triton X-100) for 60 min at room temperature, and reacted with primary antibodies in 1% FBST for 12 h to overnight at 4°C. After primary antibodies reaction, the samples were washed with 1% FBST at least 3 times, and were reacted with secondary antibodies in 1% FBST containing DAPI. After washing with PBST at least 5 times, the slide samples were embedded with Aqua-polymount (Polyscience, Warrington, PA, USA). The cell culture samples were directly used for analysis in PBS. Image analysis was performed using Olympus IX71 microscope equipped with a Photometrics CoolSNAP HQ2 CCD digital camera and MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). Antibodies used are listed in Table 2.

Generation of tetracycline-inducible ES cell lines

Tc-inducible TGFα overexpression ES cell lines were established using a published protocol (Masui *et al.*, 2005). Linearized pMWROSATcH DNA (60 μg) was transformed in 1×10^7 ES (E14 line) cells using electroporation (Eppendorf, Hamburg, Germany; 300 V and 500 μs). The electroporated ES cells were seeded on MMC-MEF and cultured in ES medium supplemented with 200 μg/mL hygromycin (Thermo Fisher Scientific) for 6 days to select transformed cells. ES cell colonies were harvested and propagated for transfection of plasmids. Human *TGFα* cDNA was cloned from human stomach samples (Takara Bio), and subcloned into pPthC vector between the XhoI-NotI restriction sites. Transfection of the pPthC vectors-carrying human *TGFα* into the knock-in ES cells were performed by Lipofectamine 2000 (Thermo Fisher Scientific). Five hours later, the medium was changed with ES medium supplemented with 1 μg/mL Tc for inhibition of transgene expression. After culturing for 2 days, the medium was changed with fresh ES cell medium supplemented with 1 μg/mL puromycin (Sigma-Aldrich). The knock-in ES cells were maintained in this condition for 6 days to obtain puromycin-resistant ES cell colonies. TGFα overexpression in several resistant colonies was evaluated using Venus fluorescence in the absence of Tc. Tc-inducible ES cell lines were cultured and maintained in ES medium supplemented with 1 μg/mL tetracycline and 1 μg/mL puromycin on MMC-treated Drug Resistant fibroblasts (DR4; ATTC). The medium was changed every day.

In situ hybridization

In situ hybridization was performed as previously reported (Noguchi *et al.*, 2012). Antisense RNA probes of *Barx1*, *EpCAM*, and *Sox2* were prepared from each pGEM-T easy vector by *in vitro* transcription with T7 or Sp6 RNA polymerase.

Global gene expression analysis

Total RNA was extracted using RNeasy Mini kit (Qiagen), and Cyanine 3-labeled cRNA probe was amplified using Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). Whole Mouse Genome Oligo Microarray (Agilent Technologies) was used for hybridization of the cRNA probes. After hybridization, microarrays were washed and scanned using microarray scanner (Agilent Technologies). Scanned data were processed using Feature Extraction software (Agilent Technologies) and Gene Spring Software (Agilent Technologies). Microarray data for E14 ES cells, pancreatic islets, and liver were imported from Gene Expression Omnibus (GEO) database (GSE396240, Kunisato *et al.*, 2010; GSE832110, Vivas *et al.*, 2011; and GSE1195282, Antherieu *et al.*, 2014). The data were normalized at 75 percentile, and probes were selected that have at least one "P" flag in all samples. More than 2-fold difference in expression between negative and positive controls was considered significant. Hierarchical clustering was conducted using the average linkage rule and Euclidean distances. Heat maps were prepared using GeneSpring software. Microarray data have been submitted to GEO database (GSE60031).

Functional assay in e-ST

Secreted Pgc was measured using a Pgc ELISA kit (USCN Life Science, Kohaku, China) according to the manufacturer's protocols. Samples of cultured spheroids were harvested from the medium of e-ST at 42 days culture after an additional 96 h incubation with fresh FGF medium. Adult tissue samples were harvested from tissues cultured in FGF medium for 96 h. Multiskan JX (Thermo Fisher Scientific) was used to measure absorbance of each sample.

pH value change was measured using Compact pH meter B-711 (Horiba, Kyoto, Japan) to evaluate acid secretion. Samples were incubated in fresh basic medium (128 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 30 mM D-glucose, pH 7.0)

supplemented with 500 μM histamine (Wako), and pH values were measured in each medium every hour for 3 h. pH values were normalized to the values of averaged 0 h samples.

Statistical analysis and cell count

Statistical significance in two-group comparisons was analyzed using unpaired *t*-tests. To quantify immunopositive cells and spheroids, samples were counted in at least three random microscope fields from three biologically independent experiments.

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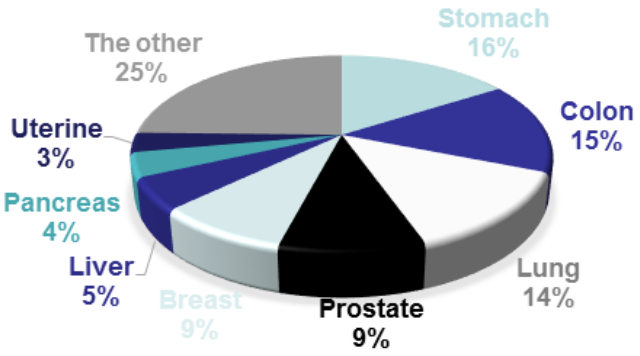
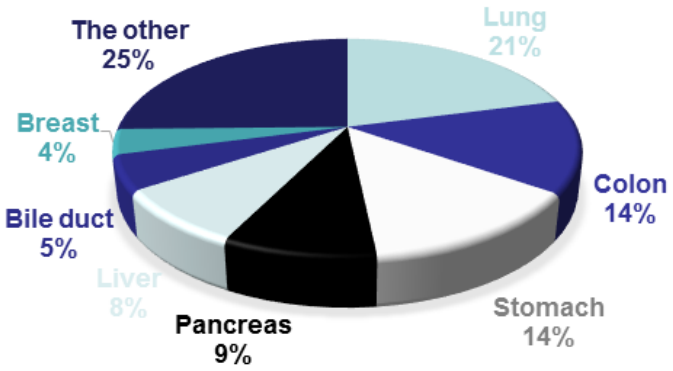
A



Origin	Year	Incidence
Stomach	2011	132033
Colon	2011	124921
Lung	2011	111858
Prostate	2011	78728
Breast	2011	72472
Liver	2011	43840
Pancreas	2011	33095
Uterine	2011	26741
The other	2011	203744
Total	2011	827432

B



Origin	Year	Mortality
Lung	2014	73396
Colon	2014	48485
Stomach	2014	47903
Pancreas	2014	31716
Liver	2014	29543
Bile duct	2014	18117
Breast	2014	13240
The other	2014	88932
Total	2014	399817

Figure 1 Statistics relating to gastric cancer in Japan. (A) Cancer incidence in 2011; (B) cancer mortality in 2014. Statistical data are derived from Cancer Registry and Statistics, Cancer Information Service, National Cancer Center, Japan.

Accomplishments

[International Journals]

① **Takaaki K. Noguchi**, Hisako Ishimine, Yoshiro Nakajima, Kanako Watanabe-Susaki, Naoki Shigeta, Norio Yamakawa, Pi-Chao Wang, Makoto Asashima, Akira Kurisaki. Novel cell surface genes expressed in the stomach primordium during gastrointestinal morphogenesis of mouse embryos. *Gene Expr. Patterns*, volume 12, 154–163, March-April 2012

② Megumi Kowno, Kanako Watanabe-Susaki, Hisako Ishimine, Shinji Komazaki, Kei Enomoto, Yasuhiro Seki, Ying Ying Wang, Yohei Ishigaki, Naoto Ninomiya, **Takaaki K. Noguchi**, Yuko Kokubu, Keigoh Ohnishi, Yoshiro Nakajima, Kaoru Kato, Atsushi Intoh, Hitomi Takada, Norio Yamakawa, Pi-Chao Wang, Makoto Asashima, Akira Kurisaki. Prohibitin 2 Regulates the Proliferation and Lineage-Specific Differentiation of Mouse Embryonic Stem Cells in Mitochondria. *PLoS ONE*, volume 9, e81552, April 2014

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⑥ **Takaaki K. Noguchi**. Generating self-organizing stomach from mouse embryonic stem cells. *Pluripotent Stem Cells* (in press)

[Japanese Journals]

① **野口隆明**、栗崎晃、マウス ES 細胞を用いた胃組織の作製、*ライフサイエンス新着論文レビュー*、DOI: 10.7875/first.author.2015.102

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(Experimental Medicine)、vol. 34 (1); 74–77, 2016 年

[International Conferences]

① **Takaaki K. Noguchi**, Pi-Chao Wang, Akira Kurisaki. Novel cell surface genes expressed in the stomach primordium during gastrointestinal morphogenesis of mouse embryos. YABEC2011. Incheon, Korea. October 2011

② **Takaaki K. Noguchi**, Pi-Chao Wang, Akira Kurisaki. Directed differentiation of stomach tissue from murine embryonic stem cells. CiRA International Symposium. Poster Presentation 048. Kyoto, Japan. March 2013

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① **野口隆明**、栗崎晃, 王碧昭, 浅島誠、マウス胃形成期に胃組織特異的に発現する Adra2a 遺伝子、平成 22 年度つくば化学工学バイオ部会 *つくば学生研究交流会*、つくば,日本、2011 年 3 月

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④ 伊藤泰斗、**野口隆明**、関根麻莉, 二宮直登, 高田仁美, 栗崎晃、ヒト iPS 細胞から分化させた肺前駆細胞における細胞表面マーカーの解析、平成 26 年度産総研・産技連合同発表会、2015 年 2 月

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