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Generating Self-Organizing Stomach Tissue from Mouse Embryonic Stem Cells

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Abstract

Advances in developmental research have allowed for the differentiation of pluripotent stem cells into various somatic cells in vitro. Recently, it was revealed that the aggregation of pluripotent stem cells or their derivatives during differentiation in three-dimensional (3D) cultures with collagen gel could mimic the process of spontaneous organogenesis in vitro as mimicking proper development in vivo. These methods are thought to be useful for monitoring the progress of organ formation and disease physiology, as could be done in an experimental animal. Here, we introduce a recently established method for stomach lineage differentiation from pluripotent stem cells with Matrigel-based 3D culture leading to stomach development. This method induces embryonic stem cell (ES cell) aggregates to spontaneously self-organize into stomach tissue; therefore, it has potential for modeling stomach organogenesis and development in vitro. We further discuss the ability of these in vitro stomach tissues to serve as a new model for gastric disease.

Keywords: Stomach, Pluripotent stem cells, Differentiation, Self-organization, Disease modeling

1. Introduction

Embryonic stem cells (ES cells) are derived from the early blastocyst and are in a pluripotent state with the ability to differentiate into all three germ layers and each type of somatic cell in vitro [1, 2]. Recently, defined transcription factors were found to be able to convert adult somatic cells into an ES cell-like state, termed induced pluripotent stem cells (iPS cells), providing great opportunities for developing in vitro developmental and disease models and future regenerative medicine including cell transplantation [3, 4]. To obtain somatic cells derived from ES or

iPS cells in vitro, we have established directed differentiation methods for several somatic cell types following the in vivo developmental stages step-by-step. However, little is known about the differentiation of ES and iPS cells into stomach lineages in vitro.

The stomach consists of the gastric epithelium, which forms a functional gland structure for the production of digestive enzymes and gastric acid and is surrounded by a muscle layer derived from the mesoderm. Gastrointestinal organs, including the stomach, recruit splanchnic mesoderm to obtain each organ-specific cell type in their epithelium [5]. In developmental studies in chicken and mice, the early stomach mesenchyme regulates the adjacent stomach epithelium specification and differentiation into functional stomach cells from the embryonic gut endoderm [5]. In 2005, Kim *et al.* showed that mesenchymal Barx1 regulates stomach epithelium development by inhibition of Wnt signaling [6]. Disruption of Barx1 or blocking of Wnt inhibition causes intestinalization in the stomach. This phenomenon is rescued by ectopic Barx1 in the adjacent mesenchyme and by restored Wnt inhibition [6, 7]. For this reason, we hypothesized that stomach specification from ES cells in vitro might require not only the gut epithelium, but also Barx1+ mesenchyme.

In this chapter, we describe recent advances in our knowledge of stomach lineage specification with both gut epithelium and mesenchymal Barx1 from pluripotent stem cells [8]. We found that ES cell aggregates differentiate into endodermal and mesodermal lineages in basic medium and are specified into early stomach lineages by induction of SHH and inhibition of Wnt signaling. Of note, this culture condition induced the formation of self-organizing stomach primordium-like spheroids composed of Sox2+ anterior foregut endoderm and Barx1+ stomach mesenchyme. Furthermore, these stomach primordium-like spheroids could differentiate into a more mature state in 3D culture forming a gastric gland with functional secretion abilities, such as the ability to produce gastric acid and digestive enzymes. These results indicate that stomach tissue can be generated from ES cells using specific conditions in vitro and that these self-organized stomach tissues would possibly be useful for modeling development and disease.

2. Self-organization of stomach primordium-like spheroids from ES cell aggregates

Recently, we have established a method for directed differentiation of ES cells into stomach primordium-like spheroids comprised of both gut epithelium and stomach mesenchyme (**Figure 1** [8]). Previous reports indicated that the small number of ES cell aggregates in floating culture form a definitive endoderm at day six [9], and they differentiated into an intestinal gut-like structure with gut epithelium and stromal mesenchyme in serum containing basic medium [10]. With respect to the dual generation of the endodermal and mesodermal lineages, we postulated that these ES cell aggregates differentiate into both definitive endoderm and early mesoderm in floating culture at day six, and they then have the potential to develop into several gut lineages including the stomach depending on growth factor stimulation. Therefore, a modification in culture conditions after formation of ES cell aggregates at day six could direct

the development of stomach lineages, including mesenchymal Barx1, in gut-like structures from the intestinal fate. To date, we have screened culture conditions with several growth factors cocktails for the induction of stomach lineages in ES cell aggregates and found that SHH and DKK1 efficiently induce mesenchymal Barx1 with epithelial Sox2, but not intestinal Cdx2. This condition also resulted in Sox2+/Barx1+ stomach primordium-like spheroids, and they spontaneously grew bigger and differentiated into anterior to posterior embryonic stomachs as the culture progressed, which can be categorized as self-formation or self-organization [11].

Figure 1. Self-organization of stomach tissue from mouse embryonic stem cells. Illustrated summary of differentiation and formation of stomach tissue from ES cells. Modified with permission from Noguchi *et al.* 2015 [8].

3. Differentiation of ES cell-derived stomach primordium to stomach tissue in vitro

Self-organization was observed for several differentiation methods by using aggregate cultures of ES cells or their derivatives, such as for the optic cup [12], pituitary gland [13], and kidney [14]. These methods induce aggregates of pluripotent stem cells or their derivatives to form embryonic primordium-like structures, and frequently induce them to differentiate terminally and functionally. Our stomach-like spheroids, which are close to embryonic stomach primordium, also recapitulate further in vivo stomach maturation. Developmental studies indicated that mesenchymal Barx1 expression covers the anterior to posterior regions of the embryonic stomach [15, 16], and inside the stomach the epithelium further differentiates into Sox2+ forestomach/corpus region and Pdx1+ antrum region, forming gland structures in the corpus and antrum regions. As well as showing stomach development, our ES cell-derived stomach primordium surrounded by Barx1+ mesenchyme further differentiates into the whole region of stomach and builds mature stomach tissue in a long-term 3D

culture. In brief, ES cell-derived stomach primordium-like spheroids were cultured in growth factor-free medium and differentiated into a Sox2⁺ anterior stomach region and a Pdx1⁺ posterior region. Next, the spheroids were transferred to Matrigel-based 3D culture medium with mitogenic growth factors, as previously shown with gastric stem cell primary culture (Figure 1 [17]). These ES cell-derived stomach tissues, called e-ST, had similar gene expression profiles, morphology, and gastric cell zoning as a neonatal or adult stomach [8]. Of note, the ultrastructure of the gastric parietal cells and chief cells in the e-ST were very close to that of a neonatal or adult stomach, and the e-ST showed functional production of gastric acid in the presence of histamine and secretion of pepsinogen in the culture medium, which was not observed with previous stomach differentiation methods from pluripotent stem cells [18]. Accordingly, we suggest that the e-ST could be widely applicable as a new in vitro experimental model for mimicking and analyzing the in vivo physiology of stomach tissues, such as modeling gastric ulcers.

Our analysis, however, revealed that e-ST had abundant chief cells and low enteroendocrine cells, suggesting that they were mainly differentiated into the corpus region, rather than the antrum/pylorus. This differentiation tendency can be explained because our method included long-term SHH stimulation during stomach primordium formation [8]. During the early development of the stomach, the expression level of *Shh* is high in the endodermal forestomach, but low in the hindstomach [19], and *Shh* ^{-/-} embryos show a reduced size of the forestomach region [20]. Endodermal *Shh* expression is limited by FGF10, secreted from mesenchyme in the hindstomach, and maintains anterior-posterior balance during stomach development [21]. Consistent with this knowledge, our ES cell-derived stomach primordium might differentiate into mainly the forestomach region when cultured in SHH-containing medium during early-phase attachment culture, and then further differentiate into the corpus state during late-phase 3D culture. Thus, addition of recombinant FGF10 and reduction of SHH in medium at the early phase of e-ST culture could divert differentiation tendency of stomach primordium-like spheroids towards the hindstomach fate. These results indicated that our e-ST differentiation method could be useful for in vitro developmental studies, such as analyzing anterior-posterior patterning during stomach development.

4. Modeling diseases using e-ST

In terms of the possibility of modeling in vivo development using e-ST, as they self-organize into stomach tissue in vitro, their characteristics could be also explained as the recently defined “organoids” [22]. Organoids are 3D structures, in which each cell self-organizes into the proper tissue maintenance and differentiation state in vitro, similar to their in vivo tissue state, and are derived from either pluripotent stem cells, neonatal and adult stem cells, or their progenitors [22]. These organoids could recapitulate not only in vivo development and tissue homeostasis, but also disease states with gene manipulation by using gene-engineering tools including CRISPR/Cas9 [22].

As their definition and applications, we think that our e-ST can also be categorized as “organoids” and could mimic several diseases in vitro, as a Menetrier's disease model has

shown [8]. Briefly, we introduced a tetracycline-inducible transforming growth factor alpha (TGF- α) overexpression cassette into mouse ES cells and then differentiated these ES cells into e-ST. This model could mimic chronic TGF- α overexpression in the stomach, and as a result successfully modeled a Menetrier's disease state with hypertrophic mucosa [8, 23]. Of note, TGF- α -induced e-ST showed reduced gastric acid, indicating that this e-ST could also model disease physiology. These findings indicate that e-ST could model in vitro gene-modified gastric diseases, and gene editing tools such as CRISPER/Cas9 may be beneficial for modeling cancers stemming from gene mutations.

5. Current limitations of e-ST

The method presented herein for the generation of stomach tissue from ES cells, however, remains challenging for developmental and clinical applications in the future. First, it is unclear how ES cell aggregates form definitive endoderm and mesoderm layers in serum-containing medium. We do not know how the growth factors in serum induce ES cell aggregates to differentiate into endoderm and mesoderm fates, and how these factors might affect applications such as in vitro analysis of stomach development in e-ST. Furthermore, serum is not categorized as a good manufacturing practices (GMP)-complaint material, and thus, it is not suitable for clinical applications. To be fully acceptable for developmental models, we have to generate chemically defined conditions for the differentiation of ES cells aggregates into e-ST. In addition, defined conditions could also help us to establish more efficient ways to generate e-ST.

Second, it is not clear whether e-ST can actually be established from human ES cells to model human development and disease. Currently, aggregates of human ES cells or their derivatives could recapitulate self-organizing human optic cup [24], and human pituitary gland [25] in vitro, and the methods are deeply related to mouse ES cell differentiation methods. With respect to those reports, we think it could be possible to recapitulate self-organization during human stomach development from human ES cells, as well as mouse ES cells, with some modifications. By establishing human e-ST from human ES cells, functional features of human stomachs could be also modeled as recently published for human pituitary tissue [25]. To achieve the differentiation of human stomach tissue from human ES cells, as well as e-ST from mouse ES cells, we have to consider the differences between “naïve” mouse ES cells and “primed” human ES cells [26]. Recently, naïve-like human ES cells were established by several groups [27–29]. Naïve-like human ES cells possess similar characters to naïve mouse ES cells under specific conditions; therefore, conversion of human ES cells to a naïve-like state might have a potential to mimic the self-organization of the human stomach as naïve mouse ES cells did.

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