In vitro organogenesis of gut-like structures from mouse embryonic stem cells

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Running title:
Gut formation from ES cells

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ABSTRACT

Embryonic stem (ES) cells have pluripotency and give rise to many cell types and tissues including representatives of all three germ layers in the embryo. We previously reported that mouse ES cells formed contracting gut-like organs from embryoid bodies (EBs) (Stem Cells 20:41-49, 2002). These gut-like structures contracted spontaneously, and had large lumens surrounded by three layers, i.e., epithelium, lamina propria and muscularis. Ganglia were scattered along the periphery, and interstitial cells of Cajal (ICC) were distributed among the smooth muscle cells. In the present study, to determine whether they can be a model of gut organogenesis, we investigated the formation process of the gut-like structures in comparison with embryonic gut development. As a result, we found that the fundamental process of formation *in vitro* was similar to embryonic gut development *in vivo*. The result indicates that the gut-like structure is a useful tool not only for developmental study to determine the factors that induce gut organogenesis, but also for studies of enteric neuron and ICC development.

KEY WORDS

c-kit; development; enteric neuron; gut; interstitial cells of Cajal; spontaneous contraction

INTRODUCTION

Embryonic stem cells (ES cells) are pluripotent and able to give rise to all cell types found in the embryo and the adult animal including germ cells.[2,4,5,10-12,21] Recently we demonstrated that mouse ES cells differentiate and form gut-like structures that have morphological features similar to those of the mouse gastrointestinal (GI) tract. These structures showed spontaneous rhythmical contractions, and contained interstitial cells of Cajal (ICC) that were distributed specifically in the GI muscles and played the role of pacemakers.[6,17,19,20]

Gut organogenesis involves all three germ layers, i.e., the endoderm, mesoderm and neural crests originating from the ectoderm. It also shows regional tissue specificity from the oral to anal ends including esophagus, stomach, and small and large bowels. Because of these complexities, investigation of the gut organogenesis mechanisms is difficult, and little is known about the factors inducing gut formation.[3,9,13,14]

The information obtained from *in vitro* organogenesis of gut-like structures from mouse ES cells may be of value in understanding the mechanisms of gut development, provided the formation of gut-like structures *in vitro* occurs by the same mechanism as embryonic gut organogenesis *in vivo*. In this study we examined the fundamental morphology of gut-like structures derived from ES cells and the process of their formation. We focused on the development of ICC and the enteric nervous system, and compared the development of these cell types *in vitro* to that of embryonic gut organogenesis *in vivo.*

MATERIALS & METHODS

ES cell culture

Gut formation from ES cells

The G4-2 ES cells were derived from EB3 ES cells (a sub-line originated from E14tg2a) and carried the enhanced green fluorescent protein (EGFP) gene under the control of the CAG expression unit. They were maintained in ES cell medium (Dalbecco's modified Eagle's medium containing 10% fetal bovine serum) with 1,000 U/mL of leukemia inhibitory factor (LIF). They were then dissociated with 0.25% trypsin and cultured in hanging drops in the absence of LIF. Approximately a thousand of ES cells were incubated in 15 μL of ES cell medium for 6 days, and the resulting embryoid bodies (EBs) were plated onto dishes for outgrowth. The growth and differentiation of EBs were observed and recorded using a dissection microscope and an inverted light microscope.

Immunohistochemistry

Gut-like structures, and guts of mouse embryos (embryonic day (E) 10, 12, 17) and neonates were fixed with either Zamboni's solution for 30 min or ice-cold acetone for 2 min. They were processed for whole mount preparations or for making cryo-sections. Samples were stained with anti-PGP9.5 (1:8000, UltraClone), anti-α smooth muscle actin (1:800, Sigma), anti-p75 (1:400, Chemicon), anti-Id2(1:400, Santa Cruz), anti-c-Kit (ACK2; 1:400, kindly gift from Dr. Nishi), anti-nitric oxide synthase (nNOS; 1:2000, Eruo-Diagnostica), anti-tyrosine hydroxylase (TH; 1:2000, Chemicon), and anti-vesicular acetylcholine transporter (VAChT; 1:1000, Chemicon) antibodies, respectively. Confocal images were taken by a laser confocal microscope (Zeiss LEM 5 Pascal).

Electron microscopy and toluidine blue staining

The gut-like structure and guts from mouse embryos (E10, 12, 17) and neonates were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated, embedded in epoxy resin, and processed for conventional electron microscopy. Semi-thin sections (1 μm thickness) were stained with 0.1% toluidine blue.

RESULTS & DISCUSSION

1) Morphological features of gut-like structures

Dissection microscopy revealed the three-dimensional configurations of gut-like structures, which showed different size and shape including dome-like cysts, tubular forms, flattened spherical shapes, multi-vacuolar shapes. They ranged in size from 200 to 1,500 μm in diameter (Fig. 1).

The gut-like structures were composed of three layers, i.e., epithelium, a connective tissue layer corresponding to lamina propria, and muscularis. Columnar epithelial cells were the main component of the epithelium, and many goblet cells were also observed by electron microscopy and in semi-thin sections stained with toluidine blue. Sometimes enteric endocrine cells with numerous electron-dense granules at the basal layer were observed in the epithelium. The connective tissue layer composed of fibroblasts and collagen fibers was located between and around the muscularis. Smooth muscle cells formed sheets or bundles running in the same direction. ICC showed electron-dense cytoplasm with many mitochondria and caveolae, and were scattered among the smooth muscle cells or in the connective tissue layer. Nerve ganglia were rarely observed in the muscle layer. Neurons were surrounded by glia, like enteric ganglia, but nerve fibers were few both within and outside the ganglia. The gut-like structures were surrounded by a serosa and attached to the outgrowing cellular sheet at

their bases. Although these observations indicated that the structures showed the fundamental feature of the gastrointestinal tract, nerve elements were poorly developed, and vascular systems were lacking (Fig. 2)

2) Formation of gut-like structure

After outgrowth on the plates, EBs grew and expanded rapidly over several days. Many different cell types developed, beating cardiac muscles were readily observed, and clusters comprising of mesenchymal cells were also formed. After about 4 days of outgrowth, each cluster was provided with stratified epithelial cells expressing Id2 immunoreactivity and a narrow lumen at the center.[7,22] Smooth muscles showing α smooth muscle actin immunoreactivity began to differentiate at the periphery of the cluster after 10 days of outgrowth, and the clusters began to contract when they were completed after 14 to 21 days of outgrowth. This process was quite similar to that of the embryonic guts in E10, E17 and in neonates, respectively (Fig. 3). From the morphological view point, we concluded that the formation of gut-like structures mimics the organogenesis of embryonic guts.

3) Development of ICC

Immunohistochemistry using antibodies against c-kit revealed ICC development. C-Kit immunoreactivity appeared at an early stage of outgrowth and became more prominent. They formed clusters that differentiated into either positive or negative cells after 10 days of outgrowth. C-Kit positive cells then constructed cellular networks in the gut-like structures when spontaneous contractions began at 14 to 21 days of outgrowth. Some were similar to ICC at the level of the myenteric plexus of

the mouse GI tracts.[15] The formation process of ICC networks was the same as that at the level of the myenteric plexus in the embryonic gut.[16,18]

4) Development of neurons

Immunoreactivity for PGP9.5 showed only infrequent innervations of gut-like structures, though abundant nerve elements were demonstrated around them (Fig. 2D). Neurons expressed markers indicating several neurotransmitters such as VAChT, NOS, and TH immunoreactivities, confirming that EBs have the potential to develop functional neurons around gut-like structures.[1,24] Immunoreactivity for p75 suggesting neural crest cells with origin was also detected in developing neurons. These findings suggest that factors inducing development of enteric neurons and those attracting migration of neural elements into the gut are different, and gut-like structures either are lacking or reduce the signals driving migration into the wall of the gut-like structure. Recently it has been reported that netrins and DCC work to guide migrating neural crests in developing GI tracts, and GDNF promotes neural crest migration throughout the gut wall.[8,23] Therefore, study of the expression of these factors or elements during development of gut-like structures would provide important information about the mechanisms of neural crest migration.

In conclusion, our results indicated that the fundamental process of formation of gut-like structures from ES cells was similar to that in embryonic gut development *in vivo*. Thus, this system provides a useful model for *in vitro* gut organogenesis and a valuable tool for developmental study, including that of neurons and ICC.

ACKNOWLEDGEMENT

We thank Dr. H. Niwa (Riken) for G4-2 cells, and Dr. T. Yamada (Nara Medical University) for his technical advice on the culture of ES cells. This work was supported by the Ministry of Education, Science, Sports, Cultures and Technology of Japan.

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FIGURE LEGENDS

Fig.1

Shapes of gut-like structures and schematic view of their cross sections.

A: A dome-like structure with an expanded lumen. The wall of the structure is relatively thin as indicated in D. B: Flattened and spherical structure. There are plicae or folds of the epithelium, and its wall is thick as shown in E. C: Multi-vacuolar type structure. The lumen is completely divided into two separate parts, or sometimes connected by a narrow channel. Gut-like structures are composed of three layers, i.e., epithelia indicated by dotted lines, connective tissue layers by gray zones, and the muscularis by black layers in D, E and F. Scale bar = 100 μ m.

Fig. 2

Fine structures of cellular components of gut-like structures.

A: Epithelium (Ep) is composed of columnar epithelial cells including goblet cells (asterisks) as seen in a semi-thin section stained with toluidine blue. They are surrounded by a connective tissue layer and muscularis. Scale bar = 10 μ m. B: The muscularis, demonstrated by electron microscopy, is packed mainly with smooth muscles (Sm) and surrounded by a serosa (Se). Sometimes ICC (ICC) characterized by an electron dense cytoplasm, many mitochondria, and caveolae (arrows) are observed. Scale bar = 1 μ m. C: In toluidine blue semi-thin sections, a few nerve ganglia (G) are observed beneath the epithelium (Ep). Scale bar = 30 μ m. D: Immunohistochemistry for anti-PGP9.5 antibody in the whole mount preparation demonstrates that many nerve elements including ganglia surrounds the gut-like structures, shown by a broken line, and that only a few of them intrude into the gut-like structures. Scale bar = 50 μ m

Fig. 3

Comparisons between embryonic gut development and formation of gut-like structures by semi-thin sections stained with toluidine blue.

A: In the embryonic gut at E10 epithelium (Ep) is composed of stratified epithelial cells and surrounded by mesenchymal cells. Scale bar = 10 μ m applied in all panels. B: In neonates the muscle layer (M) developed at the periphery and epithelial cells differentiate into several cell types including goblet cells. C: In the gut-like structure stratified epithelial cells with a narrow lumen appeared first at the center of the mesenchymal cells after 4 days of outgrowth. D: The muscle layer surrounds well developed epithelium in the gut-like structure after 14 days of outgrowth when it begins to contract spontaneously.