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IN VITRO DEVELOPMENTAL MODEL OF THE GASTROINTESTINAL TRACT FROM MOUSE EMBRYONIC STEM CELLS

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ABSTRACT

Mouse embryonic stem (ES) cells are pluripotent and retain their potential to form cells, tissues and organs originated from three embryonic germ layers. Recently, we developed *in vitro* organ — gut-like structures — from mouse ES cells. They had basically similar morphological features to a mouse gastrointestinal tract *in vivo* composed of three distinct layers (i.e., epithelium, connective tissue and musculature). Gut-like structures showed spontaneous contractions derived from pacemaker cells (interstitial cells of Cajal) in the musculature. We also examined their formation process and expression pattern of transcription factors crucial for gut organogenesis such as *Id2*, *Sox17*, *HNF3β/Foxa2* and *GATA4*. We found that they mimic the development of embryonic gut *in vivo* and showed a similar expression pattern of common transcription factors. They also maintain their developmental potential after transplantation to a renal capsule. Therefore, gut-like structures are suitable for *in vitro* models of gastrointestinal tracts and their development. In addition, we pointed out several unique features different from gut *in vivo* that provide useful and advantageous tools to investigate the developmental mechanism of the gastrointestinal tract.

Key Words: Gastrointestinal tract, *in vitro* model, development, embryonic stem cells, transcription factors, interstitial cells of Cajal

INTRODUCTION

Gastrointestinal (GI) tracts are composed of cells from three embryonic germ layers (i.e., endoderm, mesoderm, and neural crest originated from ectoderm). GI tracts form long tubes segmented at specific regions from oral to anal ends, and each segment is comprised of at least three layers (epithelium, lamina propria and musculature). Therefore, the development and organogenesis of GI tracts involve complicated spatiotemporal patterning from early embryonic development to late organogenesis. The complexities interfered with researches and the developmental mechanisms are still undefined.^{1,2)} One of the promising solution to the problem is the use of the simpler animal models than mammals, such as birds, amphibians and fish to find mechanisms common to them and mammals.³⁻⁶⁾ Another is to establish *in vitro* model of the

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GI tract using the culture system.⁷⁾ We have recently reported an *in vitro* developmental model of the GI tract (a gut-like structure) from mouse embryonic stem (ES) cells.⁸⁻¹²⁾ ES cells being pluripotent, are able to develop cells and tissues derived from three embryonic germ layers. Several developmental models by ES cells taking basically a similar developmental course to the embryo *in vivo* have been described. In the present review, we summarize the valid *in vitro* developmental model of a GI tract from mouse ES cells and discuss their unique properties.

CULTURE METHODS

The culture system has been described elsewhere.¹²⁾ Mouse ES cells (G4-2; carrying the enhanced green fluorescent protein —EGFP— gene under the control of cytomegarovirus/chicken beta-actin —CAG— promoter) were expanded in ES cell medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum —FBS—) with 1,000 U/mL of leukemia inhibitory factor (LIF). High-quality FBS is very important in preparing the ES cell culture. After expansion of the ES cell population, they were dissociated with 0.25% trypsin and cultured in hanging drops in the absence of LIF. Approximately 500 to 1000 ES cells were incubated in a drop with 15 µL of ES cell medium without LIF for 6 days (the number of ES cells in a drop depends on their growth rate), and the resulting embryoid bodies (EBs) were plated onto dishes for outgrowth. The stage at which EBs were transferred to tissue culture dishes was designated as EB0. EBs and gut-like structures from EB2 to EB28 were made available as an *in vitro* developmental model of GI tract for morphological and molecular biological studies.

MORPHOLOGY AND CHARACTERISTIC FEATURES

Many gut-like structures showed a balloon- or dome-like appearance with a large cavity, and some were flat with a narrow lumen. Tubular structures were rarely observed. Their sizes varied, ranging from 200 to 1500 µm in diameter. Mature gut-like structures contracted spontaneously at a contraction frequency that was temperature-dependent as similar to GI smooth muscles.^{8,13)}

The walls of the gut-like structures were composed of three distinct tissue layers corresponding to the epithelium, connective tissue, and musculature, respectively. The epithelium was comprised of columnar epithelial cells with rare microvilli at their luminal surfaces. Among the columnar epithelial cells, goblet cells and endocrine cells were distributed (Fig. 1). Although



Fig. 1 Cross sections of gut-like structure. Left panel shows semi-thin section stained with toluidine blue. Note that epithelium has neither villi nor crypts. Arrows indicate goblet cells in epithelium. Arrowhead indicates serosa. Right panel shows electron micrograph of goblet cells in epithelium. Bars = 10 µm.

sometimes such an epithelium displays folds in response to contractions of the muscle layer, histological folding such as crypts and villi have never been observed in gut-like structures. A connective tissue layer corresponding to the lamina propria surrounded the epithelium and was separated by a basal membrane in which fibroblasts with collagen fibers were distributed. In the musculature, interstitial cells of Cajal (ICC) scattered among smooth muscle cells were confirmed by electron microscopy and immunohistochemistry for the c-Kit. ICCs played the role of pacemakers in the gut-like structures and produced spontaneous rhythmical contractions as noted above.¹⁴⁾ Neurons corresponding to the enteric nervous system were fewer than those observed in mouse GI tracts. It is curious that outside the gut-like structures there were many neurons with PGP9.5 immunoreactivity, either packed or solitary. Therefore, neurons developed in EB *in vitro*, but their migration into the gut-like structures was incomplete. Ganglia in the gut-like structures were poorly developed. Confocal micrographs showing vascular acetylcholine transporter (VACHT) immunoreactivity demonstrated fine varicose fibers and a small number of ganglia composed of a few neurons. Varicose fibers ran in a disorderly fashion throughout the muscle layer without forming nerve bundles corresponding to an enteric nerve plexus, indicating that the signals required for the migration and/or growth of an enteric nervous system were insufficient.¹⁵⁾ Hematopoiesis and vasculogenesis also failed to occur in the gut-like structures, where neither blood vessels nor blood cells were observed.^{11,16)}

FORMATION PROCESS AND EXPRESSION OF TRANSCRIPTION FACTORS

About 4 days after outgrowth, at EB4, EBs produced several mesenchymal cell clusters with stratified epithelial cells expressing Id2 immunoreactivity and a narrow lumen at the center. They were considered to be the germ of future gut-like structures. Smooth muscles showing α -smooth muscle actin immunoreactivity appeared at the periphery of the cluster at around EB10, and the clusters began to contract weakly as developing gut-like structures. From EB14, the contractions of gut-like structures became strong and regular (Fig. 2). The process shown at EB4, EB10 and

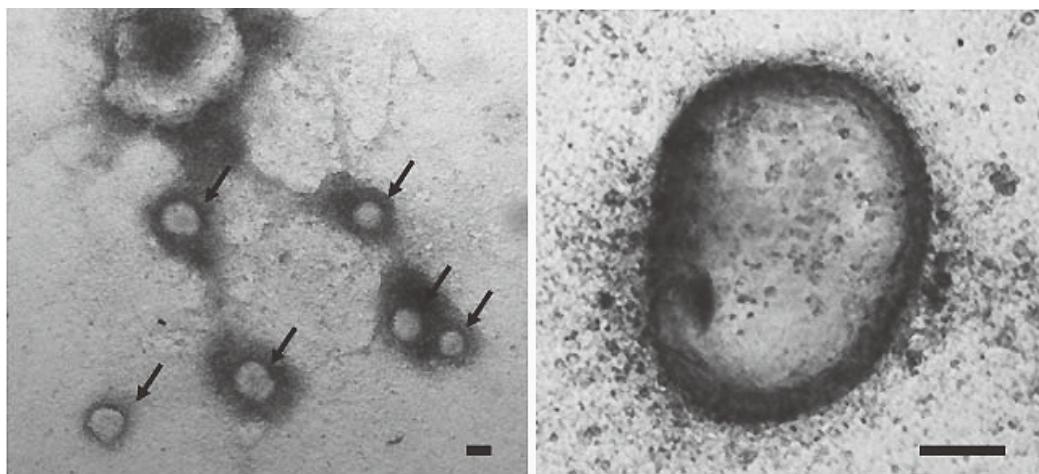


Fig. 2 Embryoid body and gut-like structures. Left panel shows embryoid body (EB) at EB4. Arrows indicate mesenchymal clusters that develop to gut-like structures. Right panel shows a gut-like structure with large cavity at EB17. Bars = 100 μ m.

EB14 was quite similar to that of the embryonic guts on embryonic days (E)10, E17, and in neonates, respectively.⁹⁾ In the mouse embryo, a definitive endoderm at E10 expressed Id2, and muscle layer showed α -smooth muscle actin immunoreactivity at E17, and regular spontaneous contractions at birth. From the morphological and physiological viewpoints, we became satisfied that the formation of gut-like structures mimicked the organogenesis of embryonic guts.

The expression patterns of crucial transcription factors in embryonic gut and gut-like structures were demonstrated and compared by *in situ* hybridization. *Sox17*, appearing in the definitive endoderm at early embryonic stages E8 to E13, was strongly expressed in the epithelium of the gut-like structures at EB5, but declined at later stages. Inhibitory transcription factor *Id2* was detected in the epithelium of the gut from E10 to newborn, and in all gut-like structures throughout developmental stages. In the mid gestation embryos, *HNF3 β /Foxa2* was strong in the foregut but weak in other regions. Although it was expressed in the epithelium of the gut-like structures, the expression became heterogeneous after EB7 and was lost in most gut-like structures at EB14. Another foregut marker, *GATA4*, was demonstrated heterogeneously in the gut-like structures, suggesting that gut-like structures mimicked molecular mechanisms *in vivo*.¹⁰⁾

TRANSPLANTATION OF GUT-LIKE STURCTURES

Gut-like structures at different stages (from EB6 to EB21) were transplanted under the renal capsule of severe combined immunodeficiency (SCID) mice to evaluate their developmental potential. Explants were identified as EGFP-positive grafts under a fluorescent dissection microscope (Fig. 3). Three weeks after transplantation, gut-like structures became 3 to 5 times as large as their original size. They continued to develop in the renal capsule and took on a mature appearance after three weeks. They can survive for longer periods in the renal capsule than in the culture medium without any appearance of degradation. Gut-like structures have supplied blood circulation through newly synthesized vasculature, and extrinsic nerve fibers originated from a host animal. Their histological features revealed no sign of teratoma. Transplantation of undifferentiated ES cells, however, quickly developed various teratoma-like tissues. Therefore, ES cell lineages were already decided at EB6, and they sustained their lineages after transplantation.¹¹⁾ Further, gut-like structures themselves did not inhibit either vasculogenesis or innervation.¹⁶⁾



Fig. 3 Gut-like structure transplanted to renal capsule of a SCID mouse. Left panel shows gut-like structure (arrow) and a host kidney. Right panel shows fluorescent dissection micrograph of that same area. Grafted gut-like structure (arrow) was defined by EGFP and supplied with blood vessels. Bar = 1 mm.

CONCLUSION AND REMARKS

Our findings on the gut-like structures indicated that they had basically similar morphological features and mimicked the development of embryonic gut *in vivo*. Gut-like structures are, therefore, a basic model of gut organogenesis and provide useful tools to study cell differentiation of the GI tract. However, they also showed some defects. Their appearance, for example, was balloon-like rather than assuming a tubular structure. Epithelium was equipped with the characteristic cell population of GI tracts, but they had neither villi nor crypts. Although numerous neurons differentiated outside the gut-like structures, ganglion cells within the structures were few and blood vessels never developed. Some signal molecules were different from those of the embryonic gut *in vivo*. These defects or differences in gut-like structures clarify the molecular mechanisms essential for the normal development, and provide unique and advantage option for the future investigations.

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