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Mini Review

Novel intestinal stem cell culture system

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The intestinal epithelium is the most rapidly self-renewing tissue in adult mammals. In the past, extensive efforts have been made to establish primary small intestinal culture systems. However, no defined, reproducible and robust culture system has been developed. We have identified essential growth factors to maintain intestinal stem cells *in vitro* and established a novel culture system for mouse and human small intestinal and colonic epithelial stem cells. In this culture system, isolated crypts form "organoid structures" with a histological hierarchy that recapitulates the *in vivo* intestinal epithelium. We further applied the organoid culture system to diseased epithelia, such as adenoma, adenocarcinoma and Barrett's epithelium. The organoid culture system provides a versatile platform to study intestinal epithelial cell biology and the mechanisms of gastrointestinal disease.

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Introduction

Intestinal stem cells (ISCs) are defined as cells showing long-term self-renewal and multi-lineage differentiation (namely enterocytes, goblet cells, enteroendocrine cells and Paneth cells). The existence of ISCs was predicted by Charles Leblond in the 1970s[1], but their location was not determined until 2007, when a genetic lineage tracing experiment showed that Lgr5+ ISCs gave rise to stem cells and all other intestinal epithelial cells for more than a year[2].

More recently, +4 cells located just above Paneth cells, that is, those cells at the +4 position from the bottom of the crypts, were functionally demonstrated to be ISCs[3]. In fact, these two locations were proposed to be ISC positions in the 1970s and 1980s[1, 4, 5]; however, it remains controversial whether the two stem cells are equal or are different types of stem cells in the intestine[6].

Intestinal epithelial cells are considered to be resistant to *in vitro* culture. Quanoli et al. first established cell lines from non-cancerous rat embryonic intestinal epithelium, and many researchers have used these cell lines as models of normal intestinal epithelium[7]. Christopher Potten's group established the first primary intestinal epithelial cell culture system[8] in which isolated crypts, including mesenchymal cells, are grown on collagen type 1 coated plates. They failed to culture pure epithelial crypts and postulated that mesenchymal cells are essential to maintain the intestinal crypt culture. In this culture system, crypt epithelial cells are vulnerable to passage and unable to be cultured for long periods of time.

Establishment of mouse small ISCs

We failed to observe Lgr5+ stem cells in the Potten primary culture system and concluded that the culture conditions were not sufficient to maintain ISCs. To establish a new culture system for ISCs, we tested combinations of growth factors that were selected by genetic evidence of ISC self-renewal, differentiation and carcinogenesis. Subsequently, we found that a combination of three growth factors, epidermal growth factor (EGF), noggin and R-spondin, is essential to maintain ISCs *in vitro*[9]. Interestingly, each growth factor signal controls a signaling pathway that is frequently mutated in human colorectal cancer[10]: EGF activates KRAS signaling and activating mutations occur in KRAS; noggin blocks BMP (Bone morphogenic protein) signaling, leading to loss-of-function mutations occurring in BMPRIA or SMAD4; and R-spondin activates the Wnt pathway, in which activating mutations occur in APC or CTNNB1. Intestinal epithelial cells also require basement membrane-derived extracellular matrix (Matrigel) as a scaffold for three-dimensional growth.

In the culture system, crypts form "organoids" that mimic crypt-villus structures. The organoids are composed of a central cyst structure and surrounding crypt-like budding structures. ISCs and Paneth cells reside at the bottom of the crypt-like structures, while post-mitotic enterocytes migrate toward the central cyst structure and end up being shed into the lumen. The organoids produce all four types of mature cells, and, interestingly, their frequency and localization patterns are preserved. It was believed that ISC self-renewal and differentiation were regulated by subepithelial mesenchymal niche cells. However, we clearly showed that a single ISC forms crypt-villus structures *in vitro* without the mesenchymal cellular niche[9]. Therefore, the intestinal epithelium organizes morphologically, controls differentiation, and self-renews autonomously.

Identification of the "ISC niche"

The fact that intestinal stromal cells are dispensable for the maintenance of ISCs in the culture system suggested the existence of niche cells in the intestinal epithelium. In the *Drosophila* genital stem cell niche system, a firm attachment between stem cells and niche cells is essential to maintain stemness. Therefore, we specifically focused on Paneth cells, which intimately adhere to ISCs. We found that CD24 expression is upregulated in Paneth cells, which enabled us to isolate live Paneth cells[11]. The microarray analysis of Paneth cells and ISCs revealed that the ISC growth-promoting factors, including EGF, Notch ligand and Wnt ligand, are exclusively expressed in Paneth cells, and their ligands are expressed in ISCs. To confirm the stem cell niche function at the single cell level, we sorted doublets composed of Paneth cells and ISCs or non-Paneth cells and ISCs and examined their organoid forming efficiency. Using our culture system, Paneth-ISC doublets formed organoids more efficiently than non-Paneth-ISC doublets. Furthermore, genetically modified mice with reduced numbers of Paneth cells or completely lacking Paneth cells possessed proportionally decreased numbers of ISC in their intestines. These data strongly indicated that Paneth cells provide niche signals to ISC[11].

Establishment of human intestinal epithelial cell culture

As is the case with other stem cell culture systems, human ISCs were more vulnerable to *in vitro* culture stress compared with mouse ISC. Most human intestinal crypts die within one week under the culture conditions used for mouse colon epithelial cells. This failure is not due to species differences in growth factors used in the culture. We screened various hormones, vitamins and small molecule inhibitors and found that an addition of three reagents (nicotinamide, p38 inhibitor and Alk (activin-like kinase) 4/5/7 inhibitor) drastically improved the culture efficiency of human intestinal crypts[12]. Using these culture conditions, we succeeded in forming organoids from a single colonoscopic biopsy sample (Fig.1). Taking advantage of the establishment of a culture system, we sorted EphB2-expressing (as a surrogate marker of Lgr5) cells from colon epithelium and examined their stemness. As a result, EphB2+ cells efficiently formed organoids containing all lineages of colonic epithelial cells. The EphB2+ cell-derived organoids showed unimpeded growth over the long term. This result provided the first evidence of the identity of human ISCs[13].

Application of organoid culture system to diseased epithelium

Many colorectal cancer cell lines have been established and used for biological analyses. Although these cell lines are useful to investigate the response of cells to various stimuli because of their easy handling and reproducibility, they often lose the biological characteristics of their paren-

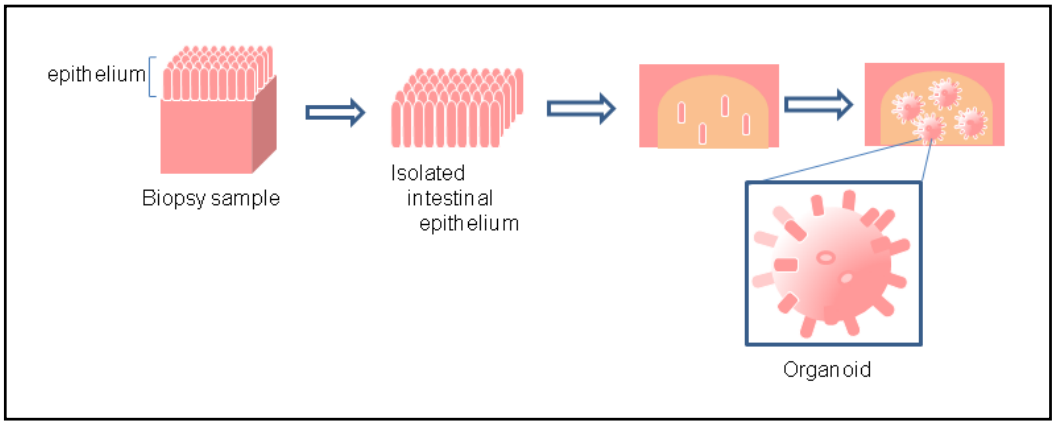

Fig.1 A single colonoscopic biopsy sample efficiently forms organoid structures in the optimized culture condition

EDTA chelation releases intestinal epithelium from biopsy samples. The isolated intestinal crypts are embedded in Matrigel covered by optimized culture medium. The crypts form stereotypic structures called "organoid".

tal tumors. Many researchers have tried to establish robust primary colon cancer cell culture systems; however, the success rate has been 10-45%. We recently applied the organoid culture system to colorectal cancer cells and established a novel colon cancer culture system with an efficiency of greater than 90%[12]. This colon cancer organoid culture system will be useful to predict the therapeutic efficacy of chemotherapy or molecular targeting therapy.

Another application is metaplastic esophageal epithelium or Barrett's epithelium (BE). We first established defined long-term culture conditions for BE[12]. Although BE histologically mimics small intestinal epithelium, BE organoids require an additional growth factor, FGF10. This finding is consistent with a recent report suggesting that BE has a residual embryonic cell origin[14]. In contrast to normal esophageal squamous epithelium, BE cells show active Notch signaling. Notch inhibitor (gamma secretase inhibitor) treatment depleted cycling BE cells and induced goblet cell differentiation in a mouse model of BE[15]. We verified the differentiation promoting effect of the Notch inhibitor in human BE organoids, indicating that the Notch signal is a feasible target for Barrett's esophagus.

Conclusion

The intestinal organoid culture system based on Matrigel with EGF/Noggin/R-spondin is an ideal platform to investigate intestinal stem cell function. The culture system has three advantages that have not been accomplished in other tissue culture conditions. First, the organoids show stem cell self-renewal and multipotential differentiation in a single culture condition, while other culture conditions need two separate culture conditions for stem cell self-renewal and differentiation. Second, the incidence of each differentiated cell type in the organoids is similar to that of *in vivo* intestine. These two features enable us to analyze how stem cells switch their fates, self-renew and differentiate. Third, organoids can self-renew indefinitely *in vitro*. In other human tissue stem cell culture conditions, stem cell proliferation is restricted by the Hayflick Limit, in which human somatic cells are limited in the number of times they can divide. By contrast, the organoids can readily achieve more than 100 population doublings in our optimized culture conditions, which exceeds the Hayflick limit. The fact that human intestinal organoids underwent growth arrest in mouse colon culture conditions supports the notion that "culture stress" in inadequate culture conditions leads to the Hayflick limit.

In this culture system, intestinal epithelial cells grow in the absence of mesenchymal cells, which gives insight into the epithelial cell-autonomous regulation of stem cells. We identified Paneth cells as an ISC niche, which provide a growth environment for ISC by expressing Wnt-3, EGF and Notch ligand. This is the first proof of a stem cell niche at a single-cell level for mammalian tissue stem cells. In the colon, there are no Paneth cells, although we found CD24+ cells between the colon Lgr5+ stem cells. We speculate the CD24+ Paneth-like cells function as the colon stem cell niche. Apparently, the colon CD24+ Paneth-like cells

are incapable of producing sufficient amounts of Wnt ligand to maintain stem cells in the current culture conditions. Further studies are warranted to identify the source of Wnt ligand in the colon epithelium.

The clinical importance of the organoid culture system is two-fold: regenerative medicine and drug screening. Taking advantage of normal stem cell features recapitulated in the organoid, the organoids can be applied for regenerative therapy. In contrast to iPS/ES cells or direct reprogramming, the organoid system does not produce concerns related to exogenous vector usage or teratoma formation. Furthermore, the digestive organs are easily accessible, which facilitates the development of organoids from a patient's tissue samples via endoscopy. We believe that tissue stem cell therapy is the best regenerative therapeutic option within the digestive system. Of note, we recently succeeded in regenerative therapy using organoids in a mouse model of inflammatory bowel disease, which supports the clinical applications of organoid therapy. With regard to drug screening, organoids possess nearly normal responses to chemical compounds. We have already verified that Gamma secretase inhibitors showed pro-secretory differentiation effects on organoids, as observed *in vivo* in the intestine, suggesting that we can predict the effect of drugs on digestive organs using an organoid culture system. We envision that organoid technology will achieve clinical benefits in the future.

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Disclosure of conflict of interests

No conflict of interests.

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