Reports

Prostaglandin E₂ supports growth of chicken embryo intestinal organoids in Matrigel matrix

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Investigating intestinal physiology in vitro remains challenging due to the lack of an effective primary enterocyte culture system. Recently developed protocols for growing organoids containing crypts and villus from adult mouse intestinal epithelium in Matrigel present an attractive alternative to the classical techniques. However, these approaches require the use of sophisticated and expensive serum-free medium supplemented with epithelial growth factor (EGF), Wnt agonist (R-spondin 1), and bone morphogenetic protein inhibitor (Noggin) in high concentrations. Here we demonstrate that is possible to use an isolated chicken embryonic intestinal epithelium to create such an organoid culture. Structures formed in Matrigel matrix in the first two days following isolation survive and enlarge during ensuing weeks. They have the appearance of empty spheres and comprise cells expressing cytokeratin (an epithelial cell marker), villin (a marker of enterocytes), and Sox-9 (a transcription factor characteristic of progenitors and stem cells of intestinal crypts). With chicken embryonic tissue as a source of organoids, prostaglandin E₂ is as effective as R-spondin 1 and Noggin in promoting sustained growth and survival of epithelial spheroids.

Intestinal epithelium is one of the most rapidly renewing tissues in vertebrate organisms. Intestinal stem cells located at the bottom of the crypts have a profound potential for proliferation. Nevertheless, establishing primary intestinal epithelial cell cultures using standard techniques has met with substantial difficulties, regardless of tissue source (fetal or adult, small or large intestine) and the species of experimental animal used (1,2). The lack of a proper in vitro model system has hampered both the fundamental analysis of gut biology and applied research of intestinal tract physiology.

During the last few years, however, the accumulation of knowledge on crypt stem cell behavior has led to significant progress in the field of intestinal cell culture. It was reported that murine intestinal crypt stem cells immersed in extracellular matrix formed organoids in the presence of Paneth cells producing WNT3 (3,4). The maintenance of self-organizing crypt-villus structures in culture requires a medium supplemented with

epithelial growth factor (EGF; 50 ng/mL), Wnt agonist (R-spondin 1; at least 500 ng/mL), and bone morphogenetic protein (BMP) inhibitor (Noggin; 100 ng/mL) (3,4).

Relatively high concentrations of R-spondin 1 were essential for supporting the Wnt signaling pathway in proliferating cells, a crucial event for the growth and survival of organoids. Noggin is the BMP antagonist produced in vivo at the crypt bottom. It inhibits mesenchyme generated BMP signals that limit stem cell proliferation through modulation of Wnt activity during the process of cell differentiation along the crypt-microvillus axis (5).

A similar approach was used by Ootani et al. who successfully established long-term cultures of intestinal spheres within collagen gel at the air-liquid interface (6). The possibility of getting intestinal tissue by direct differentiation of human pluripotent stem cells using R-spondin 1 containing medium and Matrigel matrix was also recently reported (7).

It is now clear that the proliferation of mammalian intestinal epithelial cells is

only possible in a three-dimensional (3-D) environment, mimicking the situation encountered by stem cells and progenitors in the intestinal crypt niche. As mentioned above, the second condition required to culture this tissue in vitro is the presence of WNT ligand and R-spondin 1. Using nearly micromolar concentration of R-spondin 1 substantially increases the costs and limits the potential applications for long-term organoid cultures. Here, we report the growth of organoids derived from embryonic chicken intestine in Matrigel. We also demonstrate that prostaglandin E₂ (PGE₂) provides a cost-effective alternative to R-spondin 1 and Noggin treatment, as the medium containing PGE₂ supports the growth of embryonic chicken epithelial cell organoids in Matrigel as efficiently as protein Wnt pathway agonists.

Materials and methods

Tissue isolation

Epithelial tissue was obtained from embryonic chicken intestine using the method developed

by Barker et al. This method was originally used to isolate crypts and villi fractions of adult mouse intestine, and we adapted it for chicken tissue (8). Embryonated eggs from Ross 308 hens were obtained from a local professional hatchery (Krak-Drób, Ściejowice near Kraków, Poland). For each isolation, material was pooled from four chicken embryos. Small intestines were isolated from 18-day-old embryos immediately following decapitation and washed in a Petri dish filled with ice-cold phosphate buffered saline containing Mg^{2+} and Ca^{2+} [PBS (Ca^{2+}/Mg^{2+})]. Then the intestine was separated from loose mesenchymal tissue and visible blood vessels, transferred to a new dish with cold PBS (Ca^{2+}/Mg^{2+}), cut into approximately 2-cm-long fragments, and dissected longitudinally with surgical forceps. Open pieces of the intestine were moved to a precooled 15-mL tube filled with cold PBS lacking Mg^{2+} and Ca^{2+} , but containing 2.5 mM EGTA and 0.5% glucose (PBS-EGTA-Glu), and incubated with shaking at 4°C for 15 min. The tube was then shaken vigorously 10 times, allowed to stand for 2 min on ice, and the supernatant decanted and discarded. The fresh portion of PBS-EGTA-Glu (10 mL) was added, and the tissue was again incubated with shaking for 45 min from the initial transferring into PBS-EGTA-Glu. At that moment, the shaking step was repeated, the tissue placed back on the shaker with a third portion of PBS-EGTA-Glu, and incubated for the next 45 min.

Organoid culture

The cells and the epithelial tissue fragments released between 45 and 90 min of incubation in PBS-EGTA-Glu were used for the subsequent culture. First, they were filtered through a 100- μ m nylon cell strainer (BD Bioscience, San Diego, CA, USA) and then washed twice in cold DMEM by centrifugation ($1000\times g$ for 5 min at 4°C). Twelve-well plate cell culture inserts (0.4- μ m pore size, transparent; BD Biosciences) were coated with 5 μ L Matrigel (BD Matrigel-hESC-qualified matrix; BD Bioscience). The medium used to culture the organoids consisted of DMEM/F12 (PAA Laboratories GmbH, Pasching, Austria) with antibiotic-antimycotic solution (Zell Shield; Minerva Biolabs, Berlin, Germany), ITS Premix (BD Biosciences) diluted 100 times, and 25 ng/mL epidermal growth factor (EGF) with or without 250 ng/mL R-spondin 1, 25 ng/mL Noggin (both from R&D Systems, Minneapolis MN, USA) or with or without 5 μ g/mL PGE₂ (Cayman Chemical, Ann Arbor, MI, USA). The pellet was then resuspended in a small amount of culture medium (50 μ L), mixed with equal amount of Matrigel, and poured into inserts. Cell culture medium (750 μ L) was added to the lower chamber, and

Figure 1. Formation of intestinal organoids in Matrigel matrix. Bright field images of live unstained samples of intestinal epithelium forming organoids in a Matrigel layer on cell culture inserts. Tissue fragments were cultured in serum-free DMEM/F12 supplemented with 25 ng/mL EGF, 250 ng/mL R-spondin 1, and 25 ng/mL Noggin. In the first 6 h, irregular pieces of epithelium closed and formed numerous crypt like structures (black arrow). In the following hours, the organoids transformed into regular spheres or more elongated tubes. After 3 days, organoids were still enlarging, some reaching a diameter of 0.5 mm. Scale bar, 50 μ m.

the tissue-Matrigel mixture was allowed to solidify for 30 min at 37°C, before 250 μ L cell culture medium was added on the top. In the first week, 50 μ L medium were added every second day to the upper chambers of the inserts. For long-term cultures, the medium was exchanged in both upper and lower compartments at 3-day intervals.

Image acquisition, morphometric measurements, and statistical analysis

The bright field images of live organoid cultures were taken with a Moticam 1000 digital color camera (Motic, Xiamen, China) mounted on an inverted microscope TC5400 Meiji (Meiji Techno, Saitama, Japan). The measurement of the projected organoid area was performed with Moticplus 2000 software (Motic, Xiamen, China). The statistical significance of differences in the number and projected area of organoids maintained in various conditions was calculated by analysis of variance (ANOVA) with post-hoc Tukey's test, using Statistica 9 software.

Time-lapse recording of growing organoids was performed with a Leica DFC 360 FX digital camera mounted on an inverted microscope (Leica DMI6000B; Leica Microsystems GmbH, Wetzlar, Germany) equipped with a CO₂, humidity, and temperature controlled incubator using the AF6000 software for image acquisition and analysis.

Histochemistry

To reveal the structure of observed spheroids, the cells immersed in Matrigel were fixed in 3.7% buffered formaldehyde, embedded in paraffin, and processed according to standard histological protocols. The paraffin blocks were cut into 5- μ m-thick sections, deparaffinized, and stained with hematoxylin and eosin. For observing the histological sections, a Moticam 1000 camera mounted on a Nikon Eclipse E600 inverted microscope (Nikon, Melville, NY, USA) was used.

Immunoblotting

To evaluate the differentiation levels of cells that built the organoids walls, immunoblotting was performed to detect the epithelial marker, cytokeratin, and the enterocyte marker, villin. The Matrigel layer was washed with cold PBS, scrubbed in Matrisperse (BD Biosciences) and incubated in Matrisperse on ice for 30 min. The released organoids and cells were centrifuged ($1000\times g$ for 5 min), and pellets were lysed with lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, supplemented with protease inhibitor cocktail, pH 7.5), and 10 μ g total cellular protein were subjected to PAGE in 10% polyacrylamide gels and transferred to the PVDF membrane. The membranes

Figure 2. Organoid appearance on day 4 of culture in Matrigel. (A) Cells were maintained in serum-free DMEM/F12 supplemented with 25 ng/mL EGF (Con); with 25 ng/mL EGF 250 ng/mL R-spondin 1, 25 ng/mL Noggin (Rspo1, Nog, respectively); or in serum-free DMEM/F12 supplemented with 25 ng/mL EGF and 5 μg/mL PGE₂ (PGE₂). Note that PGE₂ is as effective as Rspo1 in supporting the growth of organoids. (B) Immunoblotting of whole organoid protein extracts shows the presence of both cytokeratin and villin. Immunoprecipitation with Sox-9 antibody shows the presence of Sox-9 transcription factor in some cells forming the organoid wall. Total protein extracted from 9-day-old chicken embryos (D9) were used for the positive control. (C) Histological staining of thin paraffin sections of organoids revealed a lumen containing some apoptotic cell debris (arrow) enclosed by a single layer of epithelial cells (arrowhead). M, the porous membrane of cell culture insert. (D) Bright field images of the intestinal organoids cultured for 3 and 5 weeks in the presence of 25 ng/mL EGF and PGE₂. Scale bar, 100 μm.

were blocked in 5% fat-free milk in PBS-T (0.05% Tween) for 2 h, incubated overnight at 4°C with a monoclonal antibody against chicken villin (MCA292; AbD Serotec, Raleigh, NC, USA), diluted 1:2000 in 5% fat-free milk in PBS-T, or against cytokeratin (monoclonal anti-cytokeratin, pan mixture) C2562 from Sigma-Aldrich (St. Louis, MO, USA), 1:1000, followed by a 2-h incubation in rabbit HRP conjugated anti-mouse IgG secondary antibody (dilution 1:2000 in 5% fat-free milk in PBS-T; Dako Cytomation, Carpinteria, CA, USA). To visualize bands, a Metal-Enhanced 3,3'-Diaminobenzidine

(DAB) kit (Thermo Scientific, Waltham, MA, USA) was used. Immunodetection of β-actin was performed on the same membrane using an anti-β-actin monoclonal antibody (A2888; Sigma-Aldrich) diluted 1:2000.

Immunoprecipitation

Samples containing 5 μg organoid protein extracts were incubated for 3 h at 4°C on the shaking platform with 1 μL rabbit polyclonal anti-SOX-9 (PAB12736; Abnova, Taipei City, Taiwan). Afterwards, 4 μL Protein A/G Plus Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to each

sample, and they were incubated overnight at 4°C, with gentle agitation. Complexes were washed three times with radioimmunoprecipitation buffer as described elsewhere (9). Subsequently, 10 μL Western blot analysis sample buffer were added to each pellet, the samples were denatured for 5 min at 98°C, separated by 10% SDS-PAGE, transferred to PVDF membrane, and subjected to the regular Western blot analysis procedure [blocking in 5% fat-free milk in PBS-T for 2 h, incubating in the first antibody against SOX-9 diluted 1:3000 and with goat polyclonal anti rabbit IgG HRP-conjugated antibody diluted 1:20,000 (Dako Cytomation) for 2 h at room temperature]. To visualize bands, a West Pico chemiluminescent kit (Thermo Scientific) was used.

RNA isolation and RT-PCR

Organoids released from Matrigel were lysed with TRI-reagent (Sigma-Aldrich), and total RNA was isolated according to the manufacturer's instruction. One microgram RNA was transcribed into cDNA using a Transcriptor Reverse Transcriptase kit (Roche, Indianapolis, IN, USA) with oligo(dT) primers. PCR was performed with specific primers for chicken *cdxA* (forward 5'-TAGGT-TGCCCCAG-AGGGGCCG-3', reverse 5'-CTCCTGTGTCCCAGCACGCC-3') and *cdxB* (forward 5'-AACAAGTTCCC-TGTTCCCACCAC-3', reverse 5'-GCAGCAGCACG-AACTC-CCTGA-3') genes.

Enzymatic assay

Sucrase-isomaltase is a brush border enzyme typical of differentiation state. Its sucrase activity was measured in 1-week-old organoids according to Messer and Dahlqvist (10). The organoids released from Matrigel with Matrisperse were centrifuged (600×g for 5 min) and resuspended in 200 μL modified Tyrode solution containing 5 mM sucrose instead of glucose and sonicated for 30 s on ice. After incubation for 3 h in 37°C, the samples were centrifuged (2000×g for 5 min), and the amount of glucose produced by cells was measured in the supernatants by using glucose oxidase (GOX from *Aspergillus niger*, G6125; Sigma-Aldrich), peroxidase, and orto-dianisidine (11). The enzyme activity is expressed as milliunits per milligram (mU/mg) of protein. One unit is defined as the activity that hydrolyzes 1 μmol substrate/min at 37°C.

Results and discussion

Until now, a method for culturing intestinal epithelial cells as organoids has not been used for nonmammalian cells. In avian embryo and adult birds, proliferation of intestinal epithelial cells is not restricted to the crypt bottom

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Figure 3. Organoids were cultured for 4 days, and bright field images were captured with a digital camera. The number of organoids was counted (A) and the projected organoids per field were calculated (B). For each experimental condition, seven microscopic fields were analyzed. Bars represent mean $\pm$ SD. Hash marks (#) indicate samples that were statistically different from the “negative” control of only EGF in medium. Asterisk marks (*) indicate samples statistically different from the “positive” control of medium supplemented with EGF, Noggin, and R-spondin 1.

(12). Therefore, we tested the methodology of organoid culture applied previously to adult mouse intestine for embryonic chicken tissue expecting to obtain a substantial amount of differentiated cells. We observed the growth of large organoids during the first week of culture in medium containing EGF, R-spondin 1, and Noggin (Figure 1).

Time-lapse video recording enabled us to track the behavior of embryo-derived epithelium in culture. From this observation, we conclude that the chicken organoid growth pattern is different from mouse. During the first 6 h, the planar epithelial fragments closed into 3-D structures and formed crypt-like protrusions. Next, they evolved into regular spheres with expanding lumen, enclosed by a thin layer of cells. The organoid lumen appears to be more alkaline than the surrounding medium as determined by the reddish color

of the media. The difference in pH between the inside and outside of the spheres indicates that they are covered by a nonleaking layer of tightly bound differentiated cells. Some of the spheres enlarge and fuse with neighboring organoids or change shape to move inside the Matrigel matrix. Organoids remained viable for several weeks, but formation of crypt-like structures was seldom observed at later stages of culture. They were also hardly visible on the histological section (Figure 1, Figure 2, C and D, and Supplementary Video S1).

Various culture conditions showed that some small spheres were also formed in serum-free medium supplemented only with EGF, but they were obviously less numerous and did not reach the same size as in the Wnt-agonist- and BMP-inhibitor-supplemented medium. The presence of fetal calf serum (FCS), even at 1% concentration, substantially slowed down the

Figure 4. Transcript abundance of *cdxA* and *cdxB* genes expressed in cells building organoids kept in various culture conditions as compared with β -actin. Cells were maintained in serum-free DMEM/F12 supplemented with 25 ng/mL EGF (Con); with 25 ng/mL EGF 250 ng/mL R-spondin 1, and 25 ng/mL Noggin (Rspo1, Nog, respectively); or in serum-free DMEM/F12 supplemented with 25 ng/mL EGF and 5 μ g/mL PGE₂ (PGE₂) or in control medium with 1% FCS.

process of organoid formation, regardless of R-spondin 1 and Noggin presence (Figure 3). In the medium supplemented only with EGF, spheroids deteriorated when kept in Matrigel longer than 1 week (data not shown).

Cohn and collaborators previously reported that PGE₂ promotes stem cell growth and survival after intestine radiation-induced injury (13). It was also suggested that the interaction of Wnt and PGE₂ pathways is a master regulator of vertebrate hematopoietic stem cell proliferation during regeneration and recovery (14). PGE₂ was already used to stimulate the growth of organoids in the case of human colonic stem cell culture as an additive to a medium containing Wnt3a, R-spondin 1, Noggin, gastrin, and vitamin nicotinamide (15). Therefore, we set out to determine whether PGE₂ was able to substitute for R-spondin 1 and Noggin in organoid supporting medium. It emerged that this prostaglandin is very effective in sustaining survival and promoting growth of epithelial spheres in a Matrigel matrix

(Figure 2A and Figure 3). Long-term culture confirmed that epithelial spheroids were still viable and visible in Matrigel after 5 weeks (Figure 2D). Immunoblot analysis proved that chicken organoids comprise cells expressing epithelial intermediate filaments (cytokeratin) and markers of differentiating and differentiated enterocytes (villin). The expression level of these proteins in organoids remains stable regardless of cell culture conditions (Figure 2B). The presence of Sox-9, a stem/progenitor population marker and member of the Sry-box transcription factor family, was detected by immunoprecipitation followed by immunoblotting. In mammals, Sox-9 is expressed at the bottom of the crypts both in stem cells and in Paneth cells; however, Paneth cells are thought not to exist in bird intestine (16). Therefore, we anticipated that the amount of Sox-9 protein in chicken tissue would be proportional to the number of intestinal stem cells. In organoids grown in the presence of PGE₂, Sox-9 protein is as abundant as in the R-spondin 1- and

Noggin-treated cells. In cultures treated with EGF alone, Sox-9 protein expression was dramatically lower (Figure 2B).

Similar to mammals, the expression of caudal family transcription factors (*cdx*) in birds is restricted to the intestine of adult animals, and these genes are presumed to be responsible for activating a differentiation program in enterocyte progenitors. A study by Geyra et al. concluded that chicken *cdxA* gene is a homolog of mammals' *cdx2* and is preferentially expressed in the epithelium of the villi. The chicken *cdxB* expression pattern indicates that it is present in all intestinal epithelial cells (17). Semiquantitative RT-PCR showed that both *cdxA* and *cdxB* are expressed in cultured organoids. Organoids cultured in the presence of R-spondin 1 and Noggin or PGE₂ are characterized by a higher *CdxA* mRNA level. This indicates that in those conditions, SOX-9 positive cells exist in the proliferative compartment as well as on the intestinal differentiation pathway (Figure 4). Sucrase activity measurements show that organoids cultured in the presence of Wnt-agonists or PGE₂ express high levels of this brush border hydrolase activity (not shown), indicating the presence of mature enterocytes. Cells cultured for 1 week in the presence of PGE₂ demonstrated the activity of 4.1 mU/mg protein $\pm$ 2.5 mU/mg, and in the presence of Wnt-agonists, cells demonstrated the activity of 2.1 mU/mg protein $\pm$ 2.5 mU/mg (mean $\pm$ SD, n = 3, the difference is not significant).

The high cost of media required to culture intestinal organoids hampers use of the model in large-scale industrial and laboratory applications. Therefore PGE₂ is an attractive and much less expensive alternative to the recombinant Wnt agonists. The effectiveness of this method remains to be investigated in mammalian models. However, our method of chicken organoid culture could be potentially used as a source for differentiated epithelial cells. This opens up new opportunities for studies on avian intestine physiology, investigations of the mechanisms of drugs and feed absorption, and research on gut immunity.

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Competing interests

The authors declare no competing interests.

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