Morphology and Transcriptome Analysis of Intestinal Organoids in Bovine fetus

Juntao Zhang

College of Veterinary Medicine: Henan Agricultural university

Lixin Deng

Henan Agricultural University

Laizeng He

Henan Agricultural University

Yingbo Zhao

Henan Agricultural University

Yake Shi

Henan Agricultural University

zhiping zhang (✉ zhipingzhang@henau.edu.cn)

Henan Agricultural University <https://orcid.org/0000-0002-8414-8859>

Baoyu Zhao

Northwest A&F University: Northwest Agriculture and Forestry University

Research

Keywords: intestine, organoid, morphology, metabolism, Bovine fetus

Posted Date: October 6th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-940188/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Intestinal health is an important guarantee for the healthy development of mammals. Therefore, the research on intestinal development and function continues to be a hot spot. And intestinal organoids are a hopeful alternative for the intestine, especially in large animals. In this study, we selected intestine of fetus calves as a target to study the differences in intestinal development and the development and functions of organoids in vitro, which were less reported in the past. The fetus intestine tissue is considered a preferred source for obtaining organoid because of its sterility and excellent developmental potential.

Methods

We compared the morphology of crypts and epithelial cells from duodenum, jejunum, ileum and colon at 4-6 month age fetus calves by HE staining, immunohistochemistry and transmission electron microscopy. And we cultured organoids from jejunum tissue and analyzed the structure by optical and electronic microscopy, immunological methods. Subsequently, through RNA sequencing, we confirmed the characteristics of gene expression between organoids and crypts.

Results

The results showed the villus of jejunum and ileum was significantly higher than others, but the depth of the duodenum crypt was significantly deeper than the other two ($P < 0.001$). And a large number of fissions were observed in duodenum crypts. In addition, intestinal epithelial stem cell markers LGR⁵ and CTNNB1 were highly expressed in the epithelium, but there was significant difference in villus and crypt of different intestinal segments. And By electron microscopy, we noticed that crypt stem cells were typically long and arranged in single and multiple layers closely, with a big nucleus and a large number of microvilli at the top of the cells. Each cell contained a large number of organelles, such as mitochondria and ribosomes. There are more free ribosomes in jejunum, ileum and colon crypt. Some Paneth cells and goblet cells were interspersed among crypt cells. There are typical tight junctions and adherent junctions (fingers cross pattern) between crypt cells. In addition, we obtained jejunum organoids by tissue culture possessing similar traits, such as microvillus and cell tight junctions. CTNNB1, PCNA and Lyso were highly expressed in the crypt cells around the surface of the organoids which hinted at the prominent capacity of cell proliferation. Furthermore, according to RNA sequencing, we found metabolism-related genes were significantly higher expression in organoids than in crypt stem cells ($Q < 0.001$). On the contrary, immune and disease related genes were significant higher in crypt stem cells ($Q < 0.001$). Finally, we found metabolic genes existed significantly differential ($Q < 0.001$): crypt stem cells were biased towards energy metabolism (fructose and mannose) and vitamin metabolism(retinol), and organoids were biased towards amino acid process(glutathione metabolic process) and cholesterol process. This also implied that enteral nutrition absorption needed to be further processed in myofibroblasts except the crypt epithelium.

Conclusions

In short, different intestinal epithelial morphology, structure and development were different and related to function and organoids from jejunum. The organoids from jejunum were recommended as an ideal model for studying nutrient metabolism in cattle.

Introduction

Cattle are economically important domestic animals in modern stock farming. Digestive system diseases, especially calf diarrhea, often lead to huge economic losses every year. Therefore, it is very important to understand the mechanism of pathogenesis and the function of the healthy intestinal mucosa. Intestinal tissue carries out several physiologic functions, such as immunization, digestion, and absorption of nutrition, etc. And the epithelial cells renew one time every 3-5 days for maintaining the intestinal epithelium integrity and function responding to the challenges of the complex environment in the intestine. Even though all these differentiated epithelial cells were known to derive from intestinal epithelial crypt stem cells located at the base of epithelial crypts and its niche environment to play a key role in forming the homeostasis[1]. When intestinal homeostasis is destroyed by pathogen, toxin, or other abnormal physical and chemical factors, the crypt stem cells could be immediately activated for recovering its function. But many complex mechanisms were unclear. Therefore, it is necessary to establish an ideal model to study it, especially for large animals.

At present, most of the intestinal studies focused on the mouse and human, and Lgr⁵⁺ intestinal stem cells (ISCs) have been isolated and successfully develop intestinal organoids with 3D Matrigel. Several other markers of intestinal crypt stem cells have been identified including Bmi1, Irig1[2-4], which represented the special population of stem cells which differentiate into other cells. And some growth factors are optimized to be used in the cell culture medium containing the stem-cell-niche factors WNT, R-spondin, EGF, Noggin(BMP inhibitor) which can remain ISCs genetically and phenotypically stable[5]. But the systematic comparison of morphology and characteristics was absent on the different parts of the intestine. But above combination had poor efficiency in forming the organoids and in proliferating the ISCs. In addition, in the adult intestine, large numbers of microorganisms restrict to obtain organoids and ISCs. Therefore, it is necessary to study the fetal intestine development and to culture the ISCs or intestinal organoids in vitro easily. It will be of great significance in the study of intestinal pathology and physiology instead of in vivo.

Every part of the intestinal system performs different functions, such as duodenum performs the chemical digestion, the jejunum is the main site for nutrient absorption, while the ileum absorbs residual nutrients, vitamins, and the main functions of the colon are to absorb water, electrolytes and microbial fermentation products[6]. Therefore, the different morphology and function of every part of the bovine fetus intestine need to be clear, which is helpful for explaining the differentiated intestinal physiology and pathology in bovine. Meanwhile, the development of bovine intestinal organoids represents a great opportunity to substitute efficiently the number of animals used for in vivo experiments. Although some

scientists had reported a reliable procedure to get “mini gut” with Matrigel and organoids growth medium(mouse) from bovine ileal crypts[7]. In order to optimize the cultivation system, more studies are needed to be perfected and be verified. Therefore, it needs to establish an improved method for achieving intestinal organoids in bovine and make it easily to do. and to explore the different functions of every section of the intestine. The authors considered the bovine fetal intestinal tissue is a perfect object for this study. In the article, we compared the morphological characters of the bovine fetal intestine and tried to establish an easy way to obtain organoids without Matrigel and with normal culture medium. And the organoids could be enough for analyzing and evaluating the relation of the bovine intestine with nutrition, microorganism, and drug metabolism, etc. In addition, RNA sequencing was used to determine the difference in gene expression between organoid and crypt stem cells, and to determine the precise application and superiority of organoids in the research field for studying the intestinal digestive function in large animals.

Materials And Methods

Animals

All intestines in this study were obtained from about 5-6-month-old bovine fetuses at local slaughterhouse. The fetal crown-rump length(CR) was measured to estimate fetal age. The fetuses with the whole uterus and intact placenta were kept in the icebox and transported to the lab within 1 hour.

Isolation of different parts of intestinal tissue

In this study, four parts of the intestine were recovered from every fetus, including duodenum, jejunum, ileum and colon. The whole intestine was isolated from the fetal enterocoelia and was washed 5 times with ice-cooled PBS. The mesentery was removed and the enteric cavity was flushed cleanly. Then the intestinal segments were cut 1-cm pieces for the next study.

Organoids Culture and Crypt Isolation

The excised intestinal jejunum segments were washed in PBS containing 0.3 % BSA and penicillin/streptomycin and opened longitudinally. And the subserous layer of intestine was stripped with ophthalmic forceps from the intestinal mucosa layer(Figure 1) was cut into smaller squares measuring 0.2-0.8 mm in diameter. Tissue pieces were transferred into a 4°C PBS solution and washed 3 times to remove single cells and other debris by static settlement for 1min. Following the final wash, the mini intestinal mucosa pieces were placed into a culture medium for 72 h at 5% CO₂ and 38.5 °C. Every 24h, the culture dishes were shocked or pipetted 2 times to prevent pieces from gathering together or adhering to the bottom of the dish. Then the primary organoids with multiple finger-like were picked up under stereomicroscope(XTZ-D) and transferred to the fresh medium for another 72 h. The culture dish was 100 X 20 mm (Easy Dish). The volume of medium was 15 ml and the number of intestinal pieces was adjusted to approximately 200-300 s in 1ml medium. The desired organoids were collected for morphological study and RNA sequencing analysis. The basic medium was DMEM (Hyclone) containing

10% FBS (Gibco), 100 U/ml penicillin, 100 mg/ml streptomycin, 5 µg /ml transferrin, 10 µg /ml insulin, 0.15 mM non-essential amino acid, 1mM sodium pyruvate, 1 µg /ml hydrocortisone.

Crypt stem cells were isolated from developed organoids by pipetting with thin micropipette tip repeatedly until crypt stem cell mass(epithelial stem cell) was completely separated from organoids. Then the crypt stem cell masses were collected under stereomicroscope and washed 3 times with PBS.

H&E staining

For histological analysis, different samples (duodenum, jejunum, ileum and colon) of fetal intestinal pieces were fixed separately in PBS containing 4% (W/V) paraformaldehyde for 24 h. After fixation, the samples were dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 95% and 100%), cleared in xylene, and paraffin-embedded for 24 h and made 5 µm sections on a Leica microtome. After the sections were deparaffinated in xylene, ethanol and water orderly. The sections were stained using standard hematoxylin and eosin protocols. After staining, the slides were mounted with coverslips using Entellan as the mounting medium and were examined under a light microscope.

Immunohistochemical staining

The intestinal sections were processed following the above protocols. After successively incubating with citric acid antigen retrieval solution (PH6.0) and 3 % H₂O₂ for 25 mins, the slides were rinsed with PBS (PH7.4) three times, each for 5 mins and nonimmune binding of antiserum was blocked by incubating sections in PBS supplemented with 3% BSA for 30 mins. After pre-treatment, the slides were incubated with the primary antibody (1:50) (LGR5/GPR49 Rabbit Monoclonal Antibody, AF1582, Beyotime. Anti-beta Catenin Rabbit pAb, GB11015, Servicebio) overnight at 4°C and were rinsed with PBS (PH7.4) 3 times, each for 5 mins. Sections were incubated with corresponding secondary antibodies (HRP conjugated, GB23303) for 50 min. The sections were visualized with DAB. Finally, sections were counterstained with hematoxylin for 3 min and dehydrated and mounted with Pertex. The slides were then examined and photographed, and the data was collected and analyzed with Image J software. Immunofluorescence staining were carried out to detect Expression of target proteins in organoid following the above methods. The primary antibodies were selected Anti-beta Catenin Rabbit pAb(GB11015, Servicebio), Anti-PCNA Rabbit pAb(GB11010), Anti-Lysozyme Rabbit pAb(GB11345), respectively. Secondary antibody was Cy3-labeled Goat Anti-Rabbit IgG (GB21303, Servicebio). Then DAPI was then used for nuclear staining.

Transmission electron microscopy (TEM)

Every part of intestinal tissue or organoids fixed on 2.5% glutaraldehyde were washed 4 times in PBS, each time for 15 min at 4°C. Then, tissue was postfixed with 1 % osmium tetroxide (OsO₄) for 1 h at 4°C. Tissue blocks were then rinsed in PBS 4 times, each time for 15min and dehydrated through a graded series of alcohol (50, 70, 80, 95, 100 and 100%, each time for 15 min). Tissue blocks were infiltrated with 100 % acetone 2 times, each for 15 min, and with Epon812:acetone (1:1 for 1.5 h, 2:1 overnight). With

Spurr embedding media (Ladd Research,) followed by 3 changes in 100% Epon812 for 12 h each. Finally, tissue blocks were embedded overnight at 60°C in fresh 100% Epon812 using flat embedding molds. Tissues were trimmed with razor blades and sectioned at 50 to 70 nm on a diamond knife using an Ultramicrotome(Leica EM UC7). Sections were stained on a grid with saturated uranyl acetate for 20 min at room temperature in the dark, followed by 3 water rinses, and 5 min in lead citrate with 3 water rinses. Grids were analyzed using a JEM-1400 transmission electron microscope (Jeol Ltd, Tokyo, Japan) at 100 kV accelerating voltage(TEM Center of Henan University of Chinese Medicine) [8].

RNA sequencing

RNA was extracted from Organoids and crypts using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The lysate was loaded into an RNase-free centrifuge tube and RNA-seq (DNBSEQ-500 analysis platform) was performed by Huada Gene Technology Co., Ltd (Wu han). Three samples were used in each group for RNA-sequencing assay. Every sample contained approximately 30 organoids or 300 crypts. Reference genome (Bos_taurus) version is ARS-UCD1.2_GCF_002263795.1. Cytoscape 3.8.2 (<https://cytoscape.org/>) and ClueGO app were applied to data analysis and mapping.

Statistical analysis

The results were analyzed using Student's two-tailed t-test for paired data or one-way ANOVA complemented with a post-test for multiple values where appropriate. A confidence interval of 95% with $p \leq 0.05$ was considered statistically significant.

Results

Morphological construction of the intestinal epithelium

Villus and crypts of bovine fetus intestine had different heights and depths. Villus of the jejunum and ileal were higher than of duodenum and colon($P<0.05$), villus of the colon were the shortest than others($P<0.01$)(Figure 3). Crypts of duodenum were nearly two times the depth of others($P<0.01$)(Figure 4). In addition, duodenum crypts contained numerous goblet cells and fissions. By comparison, a lot of goblet cells were developed on the villus of ileum and colon, and cell nucleus of these villi was distributed at the bottom of columnar epithelial cells which demonstrated these cells were differentiated cells. And there was a mass of metabolite product in the ileal and colon cavity, which suggested metabolic level of whole intestinal epithelial cells was prompt at fetus period. At the crypts, most the cells possessed a large nucleus and arranged multiple layers, especially in the jejunum. So these crypt cells are target cells for researching intestinal stem cells(Figure 2).

Immunohistochemical assay of intestinal tissue

Given this Lgr5 is a typical mark intestinal crypt stem cell and CTNNB1 is the core of WNT pathway which is essential to the development and differentiation of intestinal crypt stem cells. Lgr5 and CTNNB1 were expressed in stem cells of the different intestines. In this study, we found villus and crypts expressed both

of them significantly. Lgr5 and CTNNB1 at all crypts were expressed significantly stronger than at villus. And Lgr5 expressions gradually decreased from duodenum, jejunum, ileum to colon($P<0.05$). But the expression of CTNNB1 was the highest at jejunum crypts($P<0.05$). Therefore, different segments of intestine gene expression exists various.

Transmission electron microscope(TEM) imaging

From TEM intestinal crypt ultrastructure, several structure characteristics were discovered in different intestinal segments including cell junctions, organelles, crypt stem cells, Paneth cells and goblet cells, and so on. First, the ultrastructure of crypts exhibits significantly various. In the crypts of the duodenum, numerous protein-containing vesicles (black) were generated in Paneth cells, which secrete a variety of small proteins from vesicles against pathogens. Other types of cell were intestinal stem cells with small nucleus distributed among Paneth cells. In the crypts of jejunum, stem cells were arranged tightly and multi-layered in some places. The cells were squeezed to a long strip shape with microvillus close to intestinal crypt lumen and cell nucleus were moved lightly to the bottom of the cells. At the peripheral of crypts, there was a layer of stroma cells with a small nucleus and cell volume. Ileum crypts had similar features with the jejunum and secretory cells were inserted among intestinal stem cells. At the colon crypts, except for all the tightly arranged stem cells, many goblet cells were observed at the bottom of the colon, in which were occupied by mucous granules and had very small cell nucleus. At all of crypts, tight junctions of cells were formed by several desmosomes close to the lumen which effectively made the intestinal epithelium intact. In addition, numerous cross-fingers like membrane structure strengthened the cell adherent's junctions between crypt cells. At the same time, abundant organelles were scattered around the cytoplasm of the crypt cells. In addition, in duodenum crypt cells, there is more rough endoplasmic reticulum than others. A mass of free ribosomes was clustered in crypt cell cytoplasm of jejunum, ileum, and colon.

Comparison of CTNNB1 expression in crypts of fetus and adult bovine

Comparing the CTNNB1 expression in the crypt, we found adult and fetus crypts had significantly different. All the intestinal crypt cells were highly expressed CTNNB1, but fetal intestinal villus also had a higher level of CTNNB1 than adults, which showed the fetal intestinal epithelium powerfully dynamic.

Jejunum organoids and crypt stem cells imaging

In the study, we could culture and obtained Jejunum's organoids effectively, appearing in different shapes and villus-like structures. Organoids were packaged tightly by intestinal epithelial cells. The body cavity of organoids was filled with plenty of matrix cells (myofibroblasts) and scaffolds supporting the crypt epithelial cell development. Crypts developed round-shaped cell clusters in a very short time and occurred visually vesicles(Figure 8).

Immunofluorescent staining of organoid

According to the immunofluorescent staining of organoid, we found the epithelial cells highly expressed PCNA, CTNNB1, and lysosome, displaying the epithelial cells proliferative potential and vitality. Particularly, CTNNB1 was specific in the epithelial cells, meaning the trait of stem cells. And myofibroblasts under the crypt cell layer were filled in the organoids, expressing PCNA and lysosome, which displayed these cells potential roles in support of the development of the organoids(Figure 9).

Ultrastructure of the organoid

From the ultrastructure of the organoid, microvillus were observed to arrange at the surface of the crypt epithelial cells, and desmosomes junctions between the cells were clear. Epithelial cell had a large nuclear, abundant mitochondria and ribosomes, the characteristic of the cells were similar with in vivo intestinal crypt epithelial cells. But the shape of epithelial cells changed from flat to columnar gradually depending on its space of development. And the junction of cells was looser than in vivo crypt stem cells(Figure 10).

Information of RNA sequencing in organoids and crypts(stem cells)

Organoids and crypts showed significant difference in gene expression(3744 vs 3738 $Q < 0.05$; 1453 vs 1751, $Q < 0.0001$). By KEGG pathways enrichment, there were totally different pathways. In organoids(Figure 11), most of the high expressed genes were related to almost all metabolism pathways, such as Glutathione metabolism, mineral absorption, and lysosome, etc. And most of them shared key genes and affected each other forming networks. These demonstrated the organoids carried out the complex function of digestion and transport as a mini digestion organ. And some genes (LRP2, WNT2B, WNT11) were beneficial to organ formation. In addition, HOX genes (such as HOXA5, HOXA6, HOXB3, HOXB4, HOXB5 and HOXD4, $Q < 0.001$), which are key in organ development and regulation, were active in organoids. Further, mitochondria development-related genes (MRPL58, MRPS33, MRPS25, MRPS24, MRPS34, MRPL34, $Q < 0.05$) were significantly expressed in organoids Implementing complex functions(Figure 11). Conversely, in crypts, there were numerous genes related to cell junctions, Inflammation, stem cell development, immunity and cancers. And some classically key driver genes were involved, such as Jun, NFKB1, MAPK8, CTNNB1 and IL6 etc. Cell cycle-regulation genes including CCND1, CDK6, CDKN1A were significantly high in expression which implied active in the proliferation and senescence of the crypt cells. It also conformed to the demand of intestinal epithelial cell fast renewal. It was said that Wnt signaling pathway was critical to forming the homeostasis of the intestinal stem cells. But its relevant genes existed [inconformity](#). In crypts, almost of genes including key CTNNB1 were positive to Wnt signaling pathway. In organoids, several high expressed genes such as SFRP2 and SFRP5 were prohibitive to these pathways. It suggested matrix cells and crypt cells are different. But Gene RSPO2 and ROR2 were positive to the pathway, explaining the RSPO2 and ROR2 protein secreted from matrix cells and supporting the crypt or epithelial stem cell development(Figure 12).

To explore the metabolism pattern, we compared the genes of KEGG pathways in the metabolism between organoids and crypts to sum up the difference. The results displayed its features. In organoids, GGT1(1.9 folds) and GGT5(3 folds) were expressed highly related with amino acid and peptide catabolic

process. And some key genes in glutathione metabolic processes was highly, such as GSTM1(2.2folds), GSMT2 etc. Another high expressed gene clusters gathered in steroid biosynthesis and cholesterol metabolic process($Q < 0.001$). Crypts groups showed distinct traits in metabolism. Fatty acid degradation and fat-soluble vitamin metabolism(like retinol) related genes were involved as CYP26A1(6.3 folds), CYP1A1(2.4folds) etc. ($Q < 0.001$). Another area in crypts were energy metabolism process genes(PFKP , PGD and HKDC1) concerned fructose, mannose, glucose and pentose etc. Nucleosidase activity(Loc534181 8.9 folds, NT5C2 1.0 folds) and inositol phosphate metabolism (PI4K2B, 1.4folds, PLCE1, 1.3 folds)were highly expressed in crypt cells(Figure 13).

Discussion

The digest system is one of the most complicated developmental systems to carry out at least four major functions: digestion of food, absorption of nutrients, excretion of hormones, and defense against pathogens[9]. There were a lot of research outputs in experimental animals and human intestinal epithelial cells, and it continues to be top issue on intestinal stem cells for Intestinal development and pathology, particularly in crypt stem cells and in vitro organoids. Few references are available in the larger animals, so it was worthy to exploit it. These studies described the characteristic of epithelium structure in the bovine fetuses and cultured intestinal organoids by a simple method without Matrigel and special supplements. And from RNA sequencing, we analyzed the intestinal organoids at gene expression patterns.

Intestinal function and its structure have a close relationship. We found the duodenum revealed a deeper crypt with a few fissions and a thicker intestinal layer in the bovine fetus. The villus height was greater in jejunum and ileum segment than other two. The enteric and absorptive capacity could be related to villus height, volume, and density of the mucosa surface. The ileum showed the greatest number of goblet cells. The mucins are synthesized and secreted by exocytosis by the goblet cells into the gut lumen compounding a mucus layer, lubricating and protecting the intestinal epithelium [10].

Intestinal epithelial cells metabolize very rapidly and numerous exfoliated cells and villi were observed in the bovine fetus intestine. Although a marked similarity in surface membrane antigens was revealed between fetal intestinal epithelial cells and adult crypt cells [11]. We found LGR⁵ and CTNNB1, showing the cell's differentiation and stem ability, expressed in the villus less than in crypts. And the villus epithelial cells had very limited regenerative potential and the most of cells showed a cell nucleus close to the bottom of the columnar epithelial cell and the rate of nucleus and cytoplasm was lower. But in the crypt cells, the nucleus is located in the central region and the rate of nucleus and cytoplasm was utmost higher. These indicate that the cell amplification capacity is completely different between villus and crypt at the fetus stage of bovine. It implied the villus cells began to differentiate into functioning cells even in the womb. And the crypt cells exhibited excellent peculiarity of amplification in comparison to the villus cells in vitro. Other researchers also concluded that the growth ability of intestinal stem cells in vitro was closely related to tissue maturation in vivo[12]. Therefore, the crypt cells are our target cells achieving intestinal development and organoids. Jordi had reported that villus and intervillus (crypts) cells are

equipotent and have the same regenerative potential, which were isolated from the small intestine of mice at E16.5[13]. The conflicting conclusion may be of different species or developmental stages. Hence, it suggests only the crypt cells are used to isolate the intestinal stem cells in bovine fetus.

Intestinal organoids are complex three-dimensional structures that mimic the cell-type composition and tissue organization of the intestine by recapitulating the self-organizing ability of cell populations and provide in vitro model system for pathways, and mechanisms involved in epithelial damage and repair[14, 15]. Here, we have established a simple method for isolating and developing intestinal organoids and crypt stem cells without supplement of special growth factors and Matrigel. Although the 3D culture system was popular for developing intestinal organoids in vitro, its efficiency is still extremely lower for develop more organoids.

In the human intestine, colon cancer is the most serious disease of the digestive system. So lots of efforts were devoted to colon-relevant studies about physiology and pathology. And using colon cancer stem cells and Matrigel, the 3D organoids were established and applied to research the cancer mechanism and anticancer drug screening. On the contrary, in livestock like bovine, scientists mainly concentrate on intestinal absorption and abnormal symptoms (Diarrhea, Hematochezia) which have a worse effect on their health, growth or milk production. From these results in bovine fetus, the structure of intestine appeared the significant distinction in different intestinal segments. And jejunum is the main part for absorbing nutrition and is the longest segment. Therefore, jejunum crypt cells in bovine are prime targets that may be used to research the absorption of nutrition and diarrheal pathology by virus, bacteria and toxins, etc. And in these studies, we succeed to obtain the crypt cells and organoids from bovine fetal jejunum, the morphology and development of its organoid displayed similar features as in vivo intestine. The epithelial cells arranged on the surface of organoids and there is numerous microvillus on the top of the cell. And every epithelial cell has a large nucleus and abundant organelles. Between the epithelial cells exist tight desmosomes junction and membrane crimp links. At the same time, epithelial cells expressed strongly positive CTNNB1 playing a key role in crypt stem cell of organoid development in WNT signal pathway. Except in the crypt stem cell, Gene PCNA and Lyso also were positive in the matrix cells, illustrating the strong capacity of the cells in proliferation and metabolism. It was consistent with organoids RNA sequencing. So the methods of isolating and establishing organoid can be dependable to get high-quality organoids as bovine jejunum in vitro models. Furthermore, it needs to be clear the inner of organoid which are full of collagen and matrix cells, which were often thought hollow in the published reports. Certainly, It is necessary to figure out the crypt and organoid roles in Intestinal function and development.

According to the results of RNA sequencing, Organoids and crypts had significant difference in gene expression patterns. Organoid's genes relative to the metabolism pathway were highly expressed. Jonathan reviewed that metabolic pathways are the core of life and numerous discoveries have shed light on how metabolic pathways determine cellular fate and function[16]. Such as genes Kegg enrichment in glutathione metabolism, mineral absorption, Lysosome pathways, etc., which are closely related to the intestine function. Glutathione plays an important cytoprotective role in the gut and is one

of the main intracellular reducing compounds for optimizing the intestinal Ca^{2+} absorption and depleting drugs such as menadione or vitamin K_3 , sodium deoxycholate or diets enriched in fructose, which induce several features of the metabolic syndrome, produce inhibition of the intestinal Ca^{2+} absorption[17]. The lysosome pathway is the major route for clearance of aberrant components to maintain protein homeostasis and normal cellular function[18]. And Increasing lysosome enzyme activities help to clear pathological cellular waste. In the bovine intestinal organoids, numerous genes were enriched in the lysosome pathway indicating the organoid's complicated function. In addition, genes involved in the vascular smooth muscle contract pathway were highly expressed, which made the organoid have a similar character with bowel movements. Other metabolism pathways on purine, pyruvate, histidine, etc. were positive. mTOR and PPAR signaling pathways were also discovered superior in the bovine intestinal organoids. mTOR pathway is nutrient-sensing pathway playing a central role in the synthesis of proteins. PPAR pathway can regulate various metabolic processes such as fatty acid metabolism[19]. HOX genes and mitochondria development-related genes (MRPL58, MRPS33, etc.) were highly expressed in organoids. One is in charge of the development of organs, the other is beneficial to mitochondrial function. Both of them suggested that organoids possessed organ specialty and high metabolic levels. In brief, the development of the organoids from bovine jejunum can be used as a model for metabolism studies, in which few reports were published on intestinal organoids.

The intestinal epithelium is the most rapidly renewed tissue and its renewal is strictly controlled by intestinal stem cells, which forms a highly dynamic and selective barrier that controls the absorption of fluid and solutes while restricting pathogen access to underlying tissues. Barrier properties are achieved by intercellular junctions[20]. And diverse localization of epithelial intercellular junction proteins along the intestinal tract may correlate with differences in paracellular permeability and adhesion along the intestinal tract[21]. These studies agreed with the conclusion. We compared the gene expression between crypt stem cells and organoids. Distinguished from the Kegg pathway enrichment on metabolism in organoid, crypt stem cells showed the gene enrichment characteristics in cell junction, immunization and disease pathways. And the enriching genes in TNF/FoxO/NF-kappaB/PI3K-AKT signaling pathways formed a network in the crypt stem cells closely related to immunity and inflammation. And most genes involved Cellular senescence/Apoptosis/cancer signaling pathways. Moreover, ample genes in Wnt signaling pathway and regulating pluripotent stem cells fully showed the various points of crypt stem cells. Such as CTNNB1 was the one of key drive genes in the crypts and It's consistent with our IHC results, distinguishing from Wnt signaling pathway in organoids. Because The intestinal epithelium has one of the highest turnover rates, with 10^{11} epithelial cells (~200 g) being lost every day in humans. This indicates an immense proliferative process driven by the stem cell compartment located in the crypt[22].

To compare the genes in metabolism between organoid and stem cell, we found there was [biases](#) in signaling pathways. Such as crypt stem cells had priority in fat-soluble vitamin metabolic process, energy metabolism and fatty acid degradation. Ilya Lukonin also reported that retinoic acid metabolism of organoids in initiating transcriptional programs that guide the cell-fate transitions of intestinal epithelium from the regenerative state and driving enterocyte differentiation[23]. We found the retinoic acid

metabolism was restricted in crypt stem cells. Relatively, organoids possessed the capacities in amino acid (peptide) catabolic process and steroid (metabolic) process. It implied matrix cells also play a digestive cooperative role except for the work of epithelial (crypt) cells in nutrition metabolism.

Conclusion

In conclusion, studying the intestinal development of fetal cattle, we have learned the structure and morphology of different developmental segments of the intestine, and the development characteristics of duodenum, jejunum, ileum and colon are different. Moreover, due to its high developmental potential and without contamination by intestinal microbial, we can easily develop organoids from jejunum epithelial tissue. According to transcriptome analysis, using in vitro crypt stem cells is competent in detecting Immune inflammation, cancer, intestine development and even Interaction mechanism with gut microbiota. By comparison, intestinal organoids are more compatible and closer to in vivo intestines, particularly for evaluating metabolism in large animals.

Abbreviations

HE: hematoxylin-eosin staining

LGR5: Leucine Rich Repeat Containing G Protein-Coupled Receptor 5

CTNNB1: β -catenin

PCNA: proliferating-cell nuclear antigen

Lyso: lysosome

Declarations

Availability of data and materials

The datasets produced and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

The authors thank the TEM Center of Henan University of Chinese Medicine for collaboration and technical assistance. And authors thank Ms. Z Liu in Servicebio company with the help of morphological test.

Funding

Supported by China Agriculture Research System of MOF and MARA(CARS-37) and by Natural Science Foundation of Henan Province(212300410355)

Author information

Affiliations

1. College of Veterinary Medicine, Northwest A & F University, Yangling, Shaanxi, China,712100

Juntao Zhang, Baoyu Zhao

1. College of Veterinary Medicine, Henan Agricultural university, Zhengzhou, Henan, China, 450046

Juntao Zhang, Lixin Deng, Laizeng He, Yingbo Zhao, Yake Shi & Zhiping Zhang

Contributions

JtZ and LxD performed all Intestinal histological analysis. JtZ, HIZ,YbZ and YkS cultured the intestinal organoids. ZpZ and ByZ designed the study and participated in its coordination. JtZ and ZpZ performed statistical analysis of the sequencing reads, including the following bioinformatic analysis. JtZ and ZpZ wrote the main draft of the manuscript. All authors read and approved the final manuscript.

Corresponding authors

Correspondence to Zhiping Zhang or Baoyu Zhao.

Ethics declarations

Ethics approval and consent to participate

All studies and regulatory licenses were approved by Henan Agricultural University's ethics committees.

Consent for publication

Not applicable.

Competing interests

The authors declare that have no competing interests.

References

1. Kastl AJ, Terry NA, Wu GD, Albenberg LG. The Structure and Function of the Human Small Intestinal Microbiota: Current Understanding and Future Directions. Cellular and Molecular Gastroenterology and Hepatology 2020; 9(1): 33-45. doi: <https://doi.org/10.1016/j.jcmgh.2019.07.006>.
2. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 2007; 449(7165): 1003-07. doi: 10.1038/nature06196.

3. Sangiorgi E, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. *Nature Genetics* 2008; 40(7): 915-20. doi: 10.1038/ng.165.
4. Powell Anne E, Wang Y, Li Y, Poulin Emily J, Means Anna L, Washington Mary K, et al. The Pan-ErbB Negative Regulator Lrig1 Is an Intestinal Stem Cell Marker that Functions as a Tumor Suppressor. *Cell* 2012; 149(1): 146-58. doi: <https://doi.org/10.1016/j.cell.2012.02.042>.
5. Drost J, van Jaarsveld RH, Ponsioen B, Zimmerlin C, van Boxtel R, Buijs A, et al. Sequential cancer mutations in cultured human intestinal stem cells. *Nature* 2015; 521(7550): 43-47. doi: 10.1038/nature14415.
6. Beaumont M, Blanc F, Cherbuy C, Egidy G, Giuffra E, Lacroix-Lamandé S, et al. Intestinal organoids in farm animals. *Veterinary Research* 2021; 52(1): 33. doi: 10.1186/s13567-021-00909-x.
7. Hamilton CA, Young R, Jayaraman S, Sehgal A, Paxton E, Thomson S, et al. Development of in vitro enteroids derived from bovine small intestinal crypts. *Veterinary Research* 2018; 49(1): 54. doi: 10.1186/s13567-018-0547-5.
8. Abal P, Louzao MC, Fraga M, Vilariño N, Ferreira S, Vieytes MR, et al. Absorption and Effect of Azaspiracid-1 Over the Human Intestinal Barrier. *Cellular Physiology and Biochemistry* 2017; 43(1): 136-46. doi: 10.1159/000480331.
9. Karmakar S, Deng L, He XC, Li LJAJoP-G, Physiology L. Intestinal epithelial regeneration: Active versus reserve stem cells and plasticity mechanisms. 2020; 318(4): G796-G802.
10. Nordi W, Moretti DB, Lima A, Pauletti P, Susin I, Machado-Neto RJCJoAS. Intestinal histology of newborn goat kids fed lyophilized bovine colostrum. 2013; 58(5): 232-41.
11. Quaroni A. Fetal characteristics of small intestinal crypt cells. 1986; 83(6): 1723-27. doi: 10.1073/pnas.83.6.1723 %J Proceedings of the National Academy of Sciences.
12. Kondo Y, Rose I, Young GP, Whitehead RH. Growth and differentiation of fetal rat small intestinal epithelium in tissue culture: Relationship to fetal age. *Experimental Cell Research* 1984; 153(1): 121-34. doi: [https://doi.org/10.1016/0014-4827\(84\)90454-3](https://doi.org/10.1016/0014-4827(84)90454-3).
13. Guiu J, Hannezo E, Yui S, Demharter S, Ulyanchenko S, Maimets M, et al. Tracing the origin of adult intestinal stem cells. *Nature* 2019; 570(7759): 107-11. doi: 10.1038/s41586-019-1212-5.
14. Serra D, Mayr U, Boni A, Lukonin I, Rempfler M, Challet Meylan L, et al. Self-organization and symmetry breaking in intestinal organoid development. *Nature* 2019; 569(7754): 66-72. 2019/04/26. doi: 10.1038/s41586-019-1146-y.
15. Blutt SE, Klein OD, Donowitz M, Shroyer N, Guha C, Estes MK. Use of organoids to study regenerative responses to intestinal damage. *Am J Physiol Gastrointest Liver Physiol* 2019; 317(6): G845-g52.

2019/10/08. doi: 10.1152/ajpgi.00346.2018.

16. Muri J, Kopf M. Redox regulation of immunometabolism. *Nature reviews Immunology* 2021; 21(6): 363-81. 2020/12/20. doi: 10.1038/s41577-020-00478-8.
17. Moine L, Rivoira M, Díaz de Barboza G, Pérez A, Tolosa de Talamoni N. Glutathione depleting drugs, antioxidants and intestinal calcium absorption. *World journal of gastroenterology* 2018; 24(44): 4979-88. 2018/12/05. doi: 10.3748/wjg.v24.i44.4979.
18. Saftig P, Haas A. Turn up the lysosome. *Nat Cell Biol* 2016; 18(10): 1025-7. 2016/09/30. doi: 10.1038/ncb3409.
19. Oh HYP, Visvalingam V, Wahli W. The PPAR-microbiota-metabolic organ trilogy to fine-tune physiology. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2019; 33(9): 9706-30. 2019/06/27. doi: 10.1096/fj.201802681RR.
20. Garcia-Hernandez V, Quiros M, Nusrat A. Intestinal epithelial claudins: expression and regulation in homeostasis and inflammation. *Ann N Y Acad Sci* 2017; 1397(1): 66-79. 2017/05/12. doi: 10.1111/nyas.13360.
21. Gumber S, Nusrat A, Villinger F. Immunohistological characterization of intercellular junction proteins in rhesus macaque intestine. *Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie* 2014; 66(9-10): 437-44. 2014/08/26. doi: 10.1016/j.etp.2014.07.004.
22. Karmakar S, Deng L, He XC, Li L. Intestinal epithelial regeneration: active versus reserve stem cells and plasticity mechanisms. 2020; 318(4): G796-G802. doi: 10.1152/ajpgi.00126.2019.
23. Lukonin I, Serra D, Challet Meylan L, Volkmann K, Baaten J, Zhao R, et al. Phenotypic landscape of intestinal organoid regeneration. *Nature* 2020; 586(7828): 275-80. 2020/10/07. doi: 10.1038/s41586-020-2776-9.

Figures

Figure 1

Mucosa pieces isolation of fetal calf jejunum tissues for culturing the intestinal organoids, Intact epithelium with immature villus (A); Intestinal tissues without mesenterium (B); Subserosa stripped from intestinal tissues (C); Pieces of intestinal epithelium layers (D).

Figure 2

Different bovine fetus intestinal segments HE staining including duodenum, jejunum ileum and colon. (Left, villus. Right, crypts. the green dotted lines mark the location of the crypts)

Figure 3

Intestinal villus height

Figure 4

Intestinal crypt depth

Figure 5

A. Immunohistochemical analysis of different intestines for detecting LGR5 and CTNNB1 B. Relative level of LGR5 and CTNNB1 (There are significant differences between different stars)

Figure 6

TEM Ultrastructure of different intestinal segments observed by TEM imaging. The first line graphs showing normal crypt morphology. The second line and third line graphs are evident tight junctions and adherents junctions between cells. The last line graphs were organelles including mitochondria, endoplasmic reticulum, ribosome etc. Abbreviations of the cell structures: mitochondria (M), microvilli (Mv), nucleus (N), lumen(Lu), tight junction (white triangle), rough endoplasmic reticulum(rER) , electron-dense secretory granules (SG), mucous granules(MG)

Figure 7

CTNNB1 expression in the crypt cell (up fetus; down adult)

Figure 8

Cultured organoids from jejunum(left) and Crypts pipetted off from organoids (right)

Figure 9

Cultured organoid immunofluorescent staining for detecting PCNA, CTNNB1 and Lyso

Figure 10

Cultured organoid TEM imaging A. showing normal intestinal epithelial cell morphology on the organoid, B. intestinal epithelial cell(crypt stem cell) with a large nucleus(N), C. tight junctions and microvilli(Mv), D. a pool of mitochondria(M) at the bottom of crypt stem cell. E. secretory vesicles from the bottom side of cell, F. rough endoplasmic reticulum and numerous free ribosomes.

Figure 11

Significantly high expressed genes in KEGG pathways in Organoids (Left) and differentially expressed genes enrichment in KEGG pathways(Right)

Figure 12

Significantly high expressed genes in KEGG pathways in Crypts (Left) and differentially expressed genes enrichment in KEGG pathways(Right)

Figure 13

The difference of metabolism KEGG pathways between Organoids and crypts. High expressed key Genes in metabolism in organoids (right red) and in crypts (right, green)