Development and characterization of 2-dimensional culture for buffalo intestinal cells

Nidhi Chaudhary · Himanshu Agrawal · Mamta Pandey · Suneel Onteru · Dheer Singh

Received: 10 December 2016 / Accepted: 4 October 2017 / Published online: 14 October 2017
© Springer Science+Business Media B.V. 2017

Abstract Small intestinal epithelial cells (IEC) play a major role in the absorption of nutrients and toxins. Due to the similarity of genome-wide single copy protein orthologues between cattle and human, establishment of ruminant's primary small IEC culture could be a valuable tool for toxicity studies. Therefore, the current study focused on the development and characterization of buffalo IEC culture, as cattle slaughter is banned in India. The buffalo jejunum fragments were washed consecutively several times in saline, warm phosphate buffered saline (PBS), PBS with 5 mM dithiothreitol, digesting solution and 2% sorbitol in PBS. The cells were cultured on 17 $\mu\text{g}/\text{cm}^2$ collagen coated plates and transwell plates with serum (2% Fetal bovine serum (FBS) and 10% FBS) and serum-free culture conditions. The cells were differentiated into typical epithelial cobblestone morphology from day 5 onwards in 50% successful cultures. The cultured IEC were characterized by gene expression of epithelial cell markers, cytokeratin and vimentin, and enterocyte markers like villin, zonula occluden (ZO1), fatty acid binding protein 2 (FABP2) and small intestinal peptidase (IP). Based on the morphology and gene expression profile, 10% FBS has

been recommended for culturing primary buffalo IEC on collagen coated plates for 10 days. However, 50% of the successful cultures could not show epithelial phenotype on 10% FBS culture conditions even on collagen coated plates. Interestingly, undifferentiated IEC showed an increasing expression of FABP2, IP and ZO1 transcripts compared to differentiated intestinal cells with 10% FBS on collagen plates. Therefore, future studies are needed to understand the role of FABP2, IP and ZO1 in differentiation of buffalo IEC.

Keywords Buffalo · Small intestinal epithelial cells · Collagen-coated plates · Transwell plates · Markers

Abbreviations

IEC	Intestinal epithelial cells
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
DTT	Dithiothreitol
ZO1	Zonula occludens
FABP2	Fatty acid binding protein 2
IP	Intestinal peptidase
2D	Two-dimensional
RNA	Ribonucleic acid
HBSS	Hank's Balanced Salt Solution
DMEM	Dulbecco's Modified Eagle's Medium
ITS	Insulin Transferrin Selenium
cDNA	complementary deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate

N. Chaudhary · H. Agrawal · M. Pandey · S. Onteru · D. Singh (✉)
Molecular Endocrinology, Functional Genomics and Systems Biology Lab, Animal Biochemistry Division, ICAR-National Dairy Research Institute, Karnal, Haryana 132001, India
e-mail: drdheer.singh@gmail.com

qRT-PCR	quantitative real time polymerase chain reaction
eTEM	Experiments that showed typical epithelial morphology
euTEM	Experiments that did not show typical epithelial morphology
RPLP	Ribosomal Protein Lateral Stalk Subunit P1

Introduction

The mammalian digestive system has been designed to be suitable for available food resources in the environment. Ruminants and humans have developed two different types of feeding habits depending on their capability. Thus, they have different digestive systems. Most part of the ruminant digestive system is similar to other mammals, except ruminant stomach. However, the small intestine of ruminants and non-ruminants performs almost similar functions (Hofmann 1989). Hence, the intestinal epithelium from ruminants can simulate the functions of other mammals, including human. Intestinal epithelium is arranged as a single layer covering the luminal side of the digestive tract, and it plays a significant role in the digestive physiology and toxicology. Maximum absorption of nutrients and chemicals takes place in small intestine. The brush border membrane of the intestinal epithelium is mainly involved in the digestion and absorption of ingested food. Therefore, enterocyte cultures are valuable *in vitro* models to assess the toxicity of drugs as well as the molecular mechanisms involved in pathologies caused by infectious agents affecting the intestinal epithelium integrity (Rusu et al. 2005).

Two-dimensional cell culture helps in studies on developmental biology, tissue morphogenesis, disease mechanisms, drug discovery, tissue engineering and regenerative medicine, etc. Intestinal cells can be cultured on solid surfaces, like culture plates or porous surfaces, like transwell plates. Transwell plates are more helpful to study the intestinal cellular functions, such as transport, absorption and secretion. Several investigators have established the culture method for primary intestinal epithelial cells (IEC) of several animal species, like bovine (Follmann et al. 2000; Hoey et al. 2003; Birkner et al. 2004; Rusu et al. 2005;

Kaushik et al. 2008), mouse (Autrup et al. 1978; Pretlow et al. 1978; Kondo et al. 1984; Kedinger et al. 1986; Fukamachi 1992; Macartney et al. 2000), rabbit (Benya et al. 1991; Hata et al. 1993; Reddy et al. 1996), pig (Kaeffer et al. 1993; Velge et al. 1995) and humans (Gibson et al. 1989; Perreault and Beaulieu 1996; Perreault and Beaulieu 1998; Whitehead et al. 1999; Panja 2000). On the basis of the cattle genome project, the repertoire of single protein orthologs is considered to be closer to humans than rats and mice. Hence, the primary cells from cattle can be considered as an alternative animal cell model for toxicity studies. As cattle slaughter is banned in India, cells from buffaloes, the closest species of cattle, can be considered as better cellular models for toxicity studies to avoid health hazards to working personnel. Hence, in the present work, intestinal epithelial cells from buffaloes were considered for the development of two-dimensional (2D) cell culture.

The objective of this paper is to develop and characterize a prototype of 2D cell culture for buffalo intestinal epithelial cells. To the best of our knowledge, earlier studies on differential levels of intestinal ribonucleic acid (RNA) markers between the differentiated, which attained epithelial shape, and undifferentiated, which did not attain epithelial shape, primary intestinal cells are very scanty. Towards this goal, the present paper also describes the transcriptional difference between differentiated and undifferentiated primary intestinal cells cultured in media with different concentrations of serum and serum free conditions on collagen coated cell culture dishes and transwell plates.

Materials and methods

Intestinal epithelial cell isolation

Intestinal epithelial cells were isolated using the established protocol (Rusu et al. 2005) with a few modifications. The jejunum part of buffalo small intestine was collected from a slaughter house (Delhi, India), and it was washed 9–10 times with saline supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) (Penicillin–Streptomycin, Gibco, Grand Island, NY, USA), gentamycin (40 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) and amphotericin B (2.5 µg/ml) (Sigma-Aldrich). Further, it was

washed with warm (37 °C) 1 × phosphate buffered saline (PBS) supplemented with 2.7 mg/ml of D-Glucose (Sigma-Aldrich), 4 mM L-Glutamine (Sigma-Aldrich). Later, intestinal fragments were filled with 5 mM Dithiothreitol (DTT, Sigma-Aldrich), their ends were closed with a thread, and were shaken in a shaker for 10 min to remove mucus. This process was repeated once again. Then, the fluid was replaced with a digesting solution of Ca^{+2} and Mg^{+2} containing Hank's Balanced Salt Solution (HBSS) (Gibco), supplemented with collagenase-II (0.1 mg/ml) (Sigma-Aldrich) and Dispase-II (0.1 mg/ml) (Sigma-Aldrich), and incubated for 15 min in a shaker at 37 °C. Using the same digesting solution, a second digestion step was performed for 45 min at the same conditions. Then, each fragment was longitudinally wide opened and pre-digested epithelium from the digestive mucosa was scraped by using a sterile surgical blade. The scraped material was incubated at 37 °C in PBS, containing 1 mg/ml Dispase-II, for 10 min whilst doing active pipetting movements. Later, the intestinal cells were pelleted by centrifugation at 140×g for 3 min. The supernatant was discarded. The cell pellet was suspended in PBS containing 2% sorbitol (Sigma-Aldrich), and the contents were centrifuged at 140×g for 3 min. This pellet washing step was repeated for 5 times to obtain a clear cell pellet of intestinal epithelial cells without fibroblasts. The Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) was used for suspending the pellet and cell viability was determined by trypan blue staining. Finally, the intestinal cells suspended in DMEM medium were used for culturing on collagen coated plates.

Cell culture

The intestinal epithelial cells were cultured in 24 well collagen I (17 $\mu\text{g}/\text{cm}^2$) (Collagen Rat tail 1, Gibco) coated plates and 6 well collagen coated transwell plates (0.4 μm) (Cat No. 3491, Corning, Corning, NY, USA) at a concentration of 6×10^5 and 12×10^5 viable cells/ml, respectively in high glucose DMEM, supplemented with 4 mM Glutamine (Sigma-Aldrich), 1 mg/ml BSA (Sigma-Aldrich), 500 $\mu\text{g}/\text{ml}$ hydrocortisone (Sigma-Aldrich), 20 ng/ml Epidermal Growth Factor (Sigma-Aldrich), 20 nM Triiodothyronine (Sigma-Aldrich), 3× Insulin Transferrin Selenium (ITS) (Sigma-Aldrich), 1% nonessential amino

acids (Sigma-Aldrich), 1× Penicillin Streptomycin solution (Gibco), 2.5 $\mu\text{g}/\text{ml}$ of Amphotericin B (Sigma-Aldrich), 40 mg/ml of Gentamycin (Sigma-Aldrich) either with Fetal bovine serum (FBS) (2 or 10%) (Sigma-Aldrich) or without FBS (serum free). The cultures were maintained at 37 °C in 5% CO_2 and 95% humidity. The first medium change was done after 20 h and then the medium was changed after every 48 h till 10 days. The cells were observed after media change on the 1st day, 3rd day, 5th day, 7th day and 10th day for their viability and attachment under an inverted microscope (Nikon ECLIPSE Ti Microscope), and photography was done at 100×.

RNA isolation and complementary deoxyribonucleic acid (cDNA) synthesis

Total RNA was isolated using miRNeasy Mini kit (Qiagen GmbH, Hilden, Germany, Cat no 217004). The concentration and purity of isolated RNA samples were determined using Nanophotometer (IMPLEN Nanophotometer, Munich, Germany). The cDNA was synthesized by using Revert Aid First-Strand cDNA synthesis kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania, Cat no. K1622). The reaction mixture contained 300 ng of total RNA, 1 μl of random hexamer (0.2 $\mu\text{g}/\mu\text{l}$), 1 μl of oligonucleotide and nuclease free water up to 12 μl . The contents were mixed gently and incubated at 65 °C for 5 min and then 2 min at room temperature. The reagents further added were 4 μl of 5× reaction buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM MgCl_2 , 50 mM DTT), 1 μl of RNase inhibitor (20 IU), 1 μl of (deoxyNucleotide Tri Phosphate) dNTPmix (10 μM) and 2 μl of Moloney murine leukemia virus reverse transcriptase (200 IU) to make a final volume 20 μl . The contents were incubated at 25 °C for 10 min, 42 °C for 30 min, and 95 °C for 3 min.

Quantitative real time polymerase chain reaction (qRT-PCR) for intestinal RNA markers

The mRNA expression of the intestinal RNA markers (Table 1) was quantified by relative quantification method. The PCR reaction mixture consisted of 5 μl of cDNA, 0.2 μM of forward and reverse primers, 5 μl of SsoFast™ Evagreen® Super Mix (Bio Rad Laboratories, Hercules, CA, USA) and nuclease free water to make the reaction volume up to 12 μl . The qPCR

Table 1 List of primers for selected transcript markers

Gene name	Primer sequence 5'–3' F-forward, R-reverse	Expected PCR product size (bp)	Accession number
Cytokeratin	F: GGACCCCTGGCTTCAACTAC R: ACCGCAAGAGCCTTTCACCT	148	NM_001033610.1
Vimentin	F: CGTCAGCAGTATGAGAGCGT R: ACTCGTTAGTCCCTTTGAGCG	199	NM_173969.3
Villin	F: GTGGAGATGAGCGGGAGATG R: TTTCTCTGCAGCCTCTTG	153	NM_001013591.1
ZO1	F: GTTTCTGAGGGAAAGGCGGA R: CGCCTTCTGTGTCTGTGTCT	154	XM_005196202.2
FABP2	F: ATAGCCTCGCAGATGGAACCT R: CCTCTTGGCTTCCACACCTTC	170	NM_001025332.1
IP	F: GGGGATCATTGTGGGTCACTT R: GAGTTCTCTAAGGTTCTCCCG	151	NM_001100308.1

reactions were performed in MJ Mini BIO-RAD thermocycler by heating the contents at 95 °C for 5 min (pre-incubation), followed by 40 cycles of 95 °C for 10 s (denaturation), 60 °C for 30 s (annealing), 72 °C for 30 s (extension). Subsequently, melting curve analysis was done by incubating the reaction mixture between 65 and 95 °C for 5 s. Melting peaks were analysed to ensure correct amplification and thus generation of single products. For relative quantification (Ct) of transcript within a sample, Ct value of Ribosomal Protein Lateral Stalk Subunit transcript (RPLP) was used as a normalizer to nullify any possible experimental error. Relative quantification of marker transcripts among different culture systems was performed by calculating fold change through standard $2^{-(Ct)}$ method, considering the Ct value of each transcript from fresh cells as a control.

Statistical analysis

Non parametric Kruskal–Wallis statistic test with Dunn's Multiple Comparison test was used to compare the statistical differences in fold change among different culture systems and fresh cells. $P < 0.05$ and $P < 0.01$ were considered as significant. On the basis of mean fold change values, a heat map was prepared by in-house scripts implemented in R software.

Results and discussion

Isolation and 2D culturing of buffalo primary intestinal cells

In vitro culturing of mammalian primary intestinal cells has been observed as a difficult task (Weng et al. 2005). Particularly, no method is currently available for culturing buffalo primary intestinal cells. Therefore, a method has been standardized for the isolation and culturing of buffalo primary intestinal epithelial cells in this study. Particularly, the cells were cultured on collagen-coated plates and collagen-coated transwell plates for 10 days. To attain this goal, we adapted a previously established method for 2D cell culture of bovine primary small intestinal epithelial cells on collagen-coated ($17 \mu\text{g}/\text{cm}^2$) plates (Rusu et al. 2005). In the present study, mucus removal was the major problem during the initial isolation steps of small intestinal epithelial cells. This problem was resolved by washing the intestinal lumen two times for 10 min at each time with 5 mM DTT in $1 \times$ PBS containing antibiotics. The 5 mM DTT was chosen as this concentration was reported to provide higher purity and increased viability than 2 mM DTT (Goodyear et al. 2014). Similarly, two time washing for 10 min at each time was observed better than one time washing for 20 min, as suggested for bovine intestinal cell culture (Rusu et al. 2005; Goodyear et al. 2014) (Fig. 1).

Fig. 1 Buffalo intestinal epithelial cells isolation. **a** Fragment of buffalo intestine tissue, **b** fragment filled with 5 mM DTT in PBS, **c** intestinal fragment longitudinally wide opened

The isolated buffalo small intestinal epithelial cells were cultured on 24 well collagen coated plates ($17 \mu\text{g}/\text{cm}^2$) under different conditions, like serum free medium, medium with 2% FBS and medium with 10% FBS. Similarly, the cells were cultured on collagen-coated transwell plates under serum-free and serum-containing medium (2 and 10% FBS). The serum-free medium was chosen because of the reported contradictory effects of FBS on intestinal cell proliferation in earlier studies on other species (Kedinger et al. 1987; Weng et al. 2005; Chopra et al. 2010). For example, FBS at 2–20% concentration was found to severely inhibit intestinal epithelial cell proliferation, although FBS supported the proliferation of fibroblast and colonic adenocarcinoma cells (Fukamachi 1992). On the contrary, overgrowth of mesenchymal cells was also found in those cultures if FBS concentration was greater than 2.5% (Evans et al. 1994; Macartney et al. 2000; Chopra et al. 2010). In addition, bovine epithelial intestinal primo culture used 2% FBS in earlier studies (Rusu et al. 2005). Therefore, 2% FBS was used in the present study. Furthermore, it is well known that Caco-2 cells, a commonly used colon cancer cell line, are usually maintained on transwell plates with a medium containing 10% FBS (Lechanteur et al. 2017; Rani et al. 2017; Vashisht et al. 2017; Vij et al. 2016). Hence, 10% FBS was also chosen in this study.

Buffalo intestinal cells showed a typical epithelial cobblestone morphology after differentiation. The cells were observed on the 1st day, 3rd day, 5th day,

7th day and 10th day after media change. After 24 h (1st day), the cells started attaching to collagen-coated plates and gave rise to various circular proliferating foci, which further attained a typical epithelial cobblestone like structure on the 5th day in the culture containing serum (Fig. 2). The epithelial cells maintained this characteristic shape up to the 10th day in serum containing medium compared to serum free medium. Buffalo epithelial cells could not acquire an epithelial shape in serum free culture, even though the cells were able to maintain viability and expression of marker transcripts similar to fresh cells. Hence, the current study supports the use of FBS in buffalo intestinal culture without affecting the proliferation and differentiation to an extent. The appearance of epithelial morphology in the present study was similar to the morphology of the intestinal epithelial cells observed in previous studies for other species (Follmann et al. 2000; Rusu et al. 2005). However, such a typical epithelial shape was observed in 50% of culture experiments in the present study. The remaining 50% of culture experiments failed to attain such a typical epithelial shape in spite of the maintenance of viability and the expression of intestinal epithelial transcripts in 10 days of culture (Fig. 3). This could be due to the differences in intestine samples obtained from slaughter house. This observation further leads to an assumption of expecting the molecular differences between the epithelial cells having attained cobblestone morphology (differentiated) and those having failed to attain the cobblestone morphology

Fig. 2 Morphological observation of buffalo small intestinal epithelial cells differentiated into a typical epithelial shape in the cultures on collagen coated plates. **a** Intestinal epithelial cells get attached to collagen coated plates after 24 h, **b** intestinal epithelial cells on the 3rd day of the culture, **c** the cells started to

become circular proliferating foci and attained cobblestone shape on the 5th day, **d** and **e** indicate the confluent form of intestinal epithelial cells on the 7th and 9th day, respectively. Magnification is at $\times 100$ and the size of the scale bars is 100px

(undifferentiated). Therefore, gene expression data were analysed separately for differentiated and undifferentiated intestinal epithelial cells.

In the current research work, transwell plates were also used for culturing buffalo primary intestinal cells. Transwell plates simulate *in vivo* conditions by providing an environment for polarization of cells. Particularly, the transwell plates allow cells to take up and secrete molecules on both the basal and apical surfaces, thus enabling the cells to carry out metabolic activities in a more physiological fashion. Moreover, coated transwell plates can facilitate cell growth and differentiation at higher levels (Holtkamp et al. 1998). Surprisingly, the buffalo intestinal cells cultured on collagen-coated transwell plates did not attain a typical epithelial shape (Fig. 4) and failed to attain a confluent monolayer. This could be due to direct usage

of primary intestinal cells, which have a general property of clumping, on transwell plates without prior expansion of intestinal stem cells. A recent study reported that the monolayer of primary intestinal cells can only be obtained on transwell plates with prior expansion of intestinal stem cells in 3D cultures, and use them later on transwell plates for obtaining a higher number of viable cells (Moon et al. 2014).

Growing pure intestinal epithelial cells in a culture system was considered as a hard task in previous studies, which used either neonatal or adult intestinal tissues, because of the higher proliferation of fibroblasts in serum containing media (Kedinger et al. 1987). The intestinal epithelial cell growth was almost always obtained from the cultures consisting of epithelial and non-epithelial cell populations (Quaroni and May 1980). Hence, it was recommended that the existence

Fig. 3 Morphological observation of undifferentiated buffalo small intestinal epithelial cells in cultures on collagen coated plates. **a** Intestinal epithelial cells get attached to collagen coated plates after 24 h, **b** intestinal epithelial cells on the 3rd day of the culture, **c** intestinal epithelial cells on 5th day of the

culture, **d** and **e** indicate the live undifferentiated buffalo intestinal epithelial cells on the 7th and the 9th day of the culture. Magnification is at $\times 100$ and the size of the scale bars is 100px

of heterogeneous cell types may be essential for intestinal epithelial proliferation (Evans et al. 1992). Since growing colonies of epithelium have been observed in the cultures of the present study, fibroblast support would have been helpful for functional differentiation of intestinal epithelial cells. It can be noted that 2% sorbitol washing was performed in this study to remove fibroblasts from the intestinal epithelial cells. However, the earlier studies indicated that the addition of fibroblasts or conditioned medium supported primary epithelial cell growth (Hague and Paraskeva 1996; Perreault and Beaulieu 1996, 1998; Kaeffer 2002). Similarly, some other studies also indicated the requirement of non-epithelial cell types to support growth and viability of primary epithelial cells (Evans et al. 1992; Booth et al. 1994; Macartney et al. 2000). In addition, a few studies showed that extracellular matrix and mesenchymal cells are

required to maintain differentiation of the intestinal epithelium (Sanderson et al. 1996). Therefore, serum and fibroblast support might be needed for better differentiation of primary buffalo intestinal epithelial cells for culturing on collagen coated plates.

Characterization of intestinal epithelial cells by RNA markers

The cultured buffalo intestinal cells were characterized by a total of six transcripts, two of them (cytokeratin and vimentin) are general epithelial cell markers in general, and the remaining four of them (villin, ZO1, FABP2 and IP2) particularly pertain to the intestinal epithelial cells. As 50% of the successful culture experiments showed differentiation of the cultured cells into a typical epithelial shape and the remaining 50% of the successful cultures did not show

Fig. 4 Morphological observation of buffalo small intestinal epithelial cells cultured on transwell plate. **a** After 24 h, the cells attached to the collagen coated transwell plate, **b** intestinal epithelial cells on the 3rd day of the culture; **c–e** indicate that

small intestinal epithelial cells did not generate confluent layers, but maintained their cellular polarity till the 10th day on transwell plate. Magnification is at $\times 100$ and the size of the scale bars is 100px

such a typical epithelial shape, the cells in these culture systems were considered as good models to study the transcriptional differences between the differentiated and undifferentiated buffalo primary intestinal cells. Therefore, these culture systems resulting into differentiated and undifferentiated intestinal cells were considered as separated groups for transcript analyses. Taken together, the groups were FS: Fresh cells used for the experiments that showed typical epithelial morphology (eTEM); SFS: serum free culture of eTEM; S2% FBS-2% FBS used in eTEM; S10%FBS-10% FBS used in eTEM; FNS—fresh cells used for the experiments which did not show typical epithelial morphology (euTEM); SFNS: Serum free culture of euTEM; NS2%FBS: 2% FBS used in euTEM; NS10%FBS: 10% FBS euTEM; NSTW2%FBS: 2% FBS used euTEM on transwell plates; NSTW10%FBS: 10% FBS used in euTEM on

transwell plates; NSTWSF- Serum free culture of euTEM on transwell plates.

Gene expression studies of general epithelial markers revealed that 2% FBS in the medium promoted the upregulation of cytokeratin and vimentin in differentiated primary buffalo intestinal epithelial cells. Specifically, the expression of these genes was significantly ($P < 0.05$) higher in S2%FBS than SFNS (Fig. 5a, b). However, their cellular expression in the remaining culture systems did not differ significantly from fresh cells, indicating the culture systems in the current study could maintain the epithelial markers similar to fresh isolated cells. Significantly increased expression of vimentin, a specific marker of fibroblast, in S2%FBS might indicate that 2% FBS might be promoting the growth of fibroblasts along with intestinal epithelial cells (Macartney et al. 2000; Rusu et al. 2005). Cytokeratin

Fig. 5 Relative expression of **a** vimentin, **b** cytokeratin, **c** ZO1, **d** villin, **e** IP, **f** FABP2 genes in cultured buffalo intestinal epithelial cells. Buffalo intestinal cells were cultured for 10 days and the selected genes were quantified by q-PCR. The values on Y-axis are Mean $\pm$ S.E.M. of relative fold change of gene expression in different culture systems than in fresh cells in four independent experiments. The alphabets a, b indicate the significant expression of the gene at $P < 0.05$ in cultures of buffalo intestinal epithelial cells as determined by non parametric Kruskal–Wallis statistic test with Dunn’s Multiple Comparison test. **g** Hierarchical clustering heat map for gene expression profiling of buffalo small intestinal epithelial cells in different culture conditions. The intensity of the color in panels

0–40 indicates the fold change of gene expression relative to fresh cells. Green colour indicates up regulation and red indicates down regulation. FS—fresh cells used for the experiments which showed typical epithelial morphology (eTEM); SFS—serum free culture in eTEM; S2%FBS-2% FBS in eTEM; S10%FBS-10% FBS in eTEM; FNS-Fresh cell used for the experiments which did not show typical epithelial morphology (euTEM); SFNS-serum free in euTEM; NS2%FBS-2% FBS in euTEM; NS10%FBS-10% FBS euTEM; NSTW2%FBS-Transwell 2% FBS in euTEM; NSTW10%FBS-Transwell 10% FBS in euTEM; NSTWSF-Transwell serum free in euTEM. (Color figure online)

is a part of epithelial cell cytoskeletal complex required for epithelial cell differentiation. Hence, presence of cytokeratin determined the purity of epithelial cell culture (Chandrasekaran et al. 1990; Schlage et al. 1998; Kaushik et al. 2008). The increased expression of cytokeratin confirms that the cells in these culture systems were of epithelial type (Sun et al. 1979; Weng et al. 2005). Thus, we concluded that the cells in the culture systems were

primarily of epithelial in nature, as most of the fibroblast cells might have removed during sorbitol wash.

Among the four intestinal specific genes analysed in this study, the ZO1 gene encodes a tight junction protein. In general, its expression confirms the epithelial nature of culture (Weng et al. 2005). In the present study, its expression was statistically ($P < 0.05$) higher at NS10%FBS than SFS, but there

was no difference among other groups. However, an increasing trend of its expression in undifferentiated cells either with 2 or 10% FBS indicated that there could be a higher expression of truncated ZO1 rather than intact ZO1 in undifferentiated rounded buffalo primary intestinal epithelial cells. It was reported that an increased expression of truncated ZO1 lead to the transition of epithelial cells from cobblestone morphology to rounded and slightly elongated shape in corneal epithelial cells (Ryeom et al. 2000). Further studies are needed to confirm the expression of the truncated ZO1 in undifferentiated rounded buffalo primary intestinal epithelial cells in the culture system.

It is well known that villin is a cytoskeletal protein of enterocytes associated with morphology and motility of epithelial cells as well as actin bundling in microvilli (Arpin et al. 1988; Friederich et al. 1989; Pringault et al. 1991; Tomar et al. 2004, 2006; Athman et al. 2005). Lower expression of this enterocyte marker was observed in ulcerative colitis and Crohn's disease (Kersting et al. 2004). Further, it was found to play a role in antiapoptosis and homeostasis of intestinal epithelial cells through the activation of PI3Kinase/Akt pathway (Wang et al. 2008). In the current study, the expression of this enterocyte marker was more or less similar in all culture systems and fresh cells, except in serum free cultures. Particularly, there was a significant difference between SFS and SFNS (Fig. 5d). The lower expression of villin in SFNS depicted a probable higher apoptosis in undifferentiated buffalo primary intestinal epithelial cell culture under serum free conditions. Further mechanistic studies are required to prove the antiapoptotic role of villin in buffalo intestinal cells.

The expression of small intestinal peptidase (IP), a jejuncocyte marker, was not significantly different among all the culture systems, except among SFNS, 2%FBS and NS2%FBS. Its expression was significantly higher in SFNS than 2%FBS and NS2%FBS (Fig. 5e). This indicates that the cells in SFNS might have stimulated the general amino acid control (GACC) pathway and upregulation of the amino acid transporters for balancing the autophagy during serum starvation (Chen et al. 2014). As IP is an essential protein for the function of neutral amino acid

transporters (Fairweather et al. 2012), its expression might have been enhanced in the buffalo intestinal cells cultured in SFNS.

Like other intestinal cell specific markers, the expression of FABP2, another jejuncocyte marker (Sacchettini et al. 1990) was not significantly different among different culture systems, except between NS10%FBS and NSTW10%FBS (Fig. 5f). This observation indicated that the cultured buffalo intestinal cells could maintain the fatty acid metabolism similar to the fresh cells. The higher expression of FABP2 in NS10%FBS could be a cause of reduced growth and undifferentiation inspite of the presence of serum. The FABP2 is an intracellular fatty acid binding protein involved in cellular uptake, transport and intracellular metabolism of long chain fatty acids in enterocytes (Gomez et al. 2007; Kishida et al. 2009). The upregulation of FABP2 was found to reduce the growth and proliferation of enterocytes due to its competition with FABP1 for binding to linoleic acid, a component of culture media (Darimont et al. 2000).

In order to obtain a broad picture of the selected intestinal transcripts, mean fold change of their expression in different culture methods relative to fresh cells is depicted on a heat map (Fig. 5g). The intensity of the colour from red to green in heat map indicates fold change from decreasing trend (red) to increasing trend (green) compared to the fresh cells. It appears that the cells cultured in 10% FBS showed a similar or higher expression of transcript markers than the fresh cells. Therefore, 10% FBS might be better to culture buffalo intestinal cells to obtain in vivo molecular phenotype on collagen coated plates. However, the cells in 50% of the culture systems containing 10% FBS media did not show the epithelial phenotype. Nevertheless, these undifferentiated cells maintained a transcript profile on par with the differentiated cells cultured with 10% FBS in the remaining 50% culture systems. Interestingly, undifferentiated cultured intestinal epithelial cells in 10% FBS media showed an increasing trend of higher expression (green colour) of FABP2, ZO1 and IP genes, indicating a clue for the role of these genes during differentiation. Such functional role needs to be validated for buffalo intestinal cells in future studies.

Conclusion

The intestinal epithelial cells showed typical epithelial morphology on day 5 of the 10 days culture system on collagen coated plates. However, this differential epithelial shape was observed in only 50% of the successful cultures. As the current study used primary intestinal cells for cell culturing, the intestinal cells did not differentiate into epithelial cells on transwell plates. In all the culture systems used, 10% FBS maintained all the selected intestinal transcript markers at higher levels than for the fresh intestinal cells. Hence, 10% FBS rather than 2% FBS is better for the buffalo intestinal cell culture on collagen coated plates. The undifferentiated intestinal epithelial cells showed an increasing trend of gene expression for FABP2, IP and ZO1 genes in comparison to the differentiated epithelial cells. Further functional role of these genes in the differentiation of primary buffalo intestinal epithelial cells needs to be studied. Overall, high glucose DMEM with 10% FBS has been recommended for two dimensional culturing of buffalo primary intestinal cells on collagen coated plates for 10 days.

Acknowledgements The authors are thankful to the Director, ICAR-NDRI for providing infrastructure to carry out the present study. This research work was financially supported by Department of Biotechnology, Ministry of Science and Technology, India (Grant No. 102/IFD/SAN/3670/2014-15).

References

- Arpin M, Pringault E, Finidori J, Garcia A, Jeltsch JM, Vandekerckhove J, Louvard D (1988) Sequence of human villin: a large duplicated domain homologous with other actin-severing proteins and a unique small carboxy-terminal domain related to villin specificity. *J Cell Biol* 107:1759–1766
- Athman R, Fernandez MI, Gounon P, Sansonetti P, Louvard D, Philpott D, Robine S (2005) *Shigella flexneri* infection is dependent on villin in the mouse intestine and in primary cultures of intestinal epithelial cells. *Cell Microbiol* 7:1109–1116
- Autrup H, Stoner GD, Jackson F, Harris CC, Shamsuddin AKM, Barrett LA, Trump BF (1978) Explant culture of rat colon: a model system for studying metabolism of chemical carcinogens. *In vitro* 14:868–877
- Benya RV, Schmidt LN, Sahi J, Layden TJ, Rao MC (1991) Isolation, characterization, and attachment of rabbit distal colon epithelial cells. *Gastroenterology* 101:692–702
- Birkner S, Weber S, Dohle A, Schmahl G, Follmann W (2004) Growth and characterisation of primary bovine colon epithelial cells in vitro. *Altern Lab Anim* 32:555–571
- Booth C, Patel S, Bennion GR, Potten CS (1994) The isolation and culture of adult mouse colonic epithelium. *Epithel Cell Biol* 4:76–86
- Chandrakasani G, Hwang CB, Ryder M, Bhatnagar RS (1990) Keratin expression in cultures of adult human epidermal cells. *Cell Mol Biol* 37:847–852
- Chen R, Zou Y, Mao D, Sun D, Gao G, Shi J, Liu X, Zhu C, Yang M, Ye W, Hao Q, Li R, Yu L (2014) The general amino acid control pathway regulates mTOR and autophagy during serum/glutamine starvation. *J Cell Biol* 206:173–182
- Chopra DP, Dombkowski AA, Stemmer PM, Parker GC (2010) Intestinal epithelial cells in vitro. *Stem cells Dev* 19:131–142
- Darimont C, Gradoux N, Persohn E, Cumin F, Pover AD (2000) Effects of intestinal fatty acid-binding protein overexpression on fatty acid metabolism in Caco-2 cells. *J Lipid Res* 41:84–92
- Evans GS, Flint N, Somers AS, Eyden B, Potten CS (1992) The development of a method for the preparation of rat intestinal epithelial cell primary cultures. *J Cell Sci* 101:219–231
- Evans GS, Flint N, Potten CS (1994) Primary cultures for studies of cell regulation and physiology in intestinal epithelium. *Annu Rev Physiol* 56:399–417
- Fairweather SJ, Broer A, O'Mara ML, Broer S (2012) Intestinal peptidases form functional complexes with the neutral amino acid transporter BoAT1. *Biochem J* 446:135–148
- Follmann W, Weber S, Birkner S (2000) Primary cell cultures of bovine colon epithelium: isolation and cell culture of colonocytes. *Toxicol In Vitro* 14:435–445
- Friederich E, Huet C, Arpin M, Louvard D (1989) Villin induces microvilli growth and actin redistribution in transfected fibroblasts. *Cell* 59:461–475
- Fukamachi HI (1992) Proliferation and differentiation of fetal rat intestinal epithelial cells in primary serum-free culture. *J Cell Sci* 103:511–519
- Gibson PR, Van De Pol E, Maxwell LE, Gabriel A, Doe WF (1989) Isolation of colonic crypts that maintain structural and metabolic viability in vitro. *Gastroenterology* 96:283–291
- Gomez LC, Real SM, Ojeda MS, Gimenez S, Mayorga LS, Roque M (2007) Polymorphism of the FABP2 gene: a population frequency analysis and an association study with cardiovascular risk markers in Argentina. *BMC Med Genet* 8:1
- Goodyear AW, Kumar A, Dow S, Ryan EP (2014) Optimization of murine small intestine leukocyte isolation for global immune phenotype analysis. *J Immunol Methods* 405:97–108
- Hague A, Paraskeva C (1996) The intestinal epithelial cell. In: Harris A (ed) *Epithelial cell culture*. Cambridge University Press, New York, pp 25–41
- Hata Y, Ota S, Nagata T, Uehara Y, Terano A, Sugimoto T (1993) Primary colonic epithelial cell culture of the rabbit producing prostaglandins. *Prostaglandins* 45:129–141
- Hoey DE, Sharp L, Currie C, Lingwood CA, Gally DL, Smith DG (2003) Verotoxin 1 binding to intestinal crypt epithelial cells results in localization to lysosomes and abrogation of toxicity. *Cell Microbiol* 5:85–97

- Hofmann RR (1989) Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system. *Oecologia* 78:443–457
- Holtkamp GM, Rossem MV, Devos AF, Willekens B, Peek R, Kijlstra A (1998) Polarized secretion of IL-6 and IL-8 by human retinal pigment epithelial cells. *Clin Exp Immunol* 112:34–43
- Kaeffer B (2002) Mammalian intestinal epithelial cells in primary culture: a mini-review. *In Vitro Cell Dev Biol Anim* 38:123–134
- Kaeffer B, Bottreau E, Velge P, Pardon P (1993) Epithelioid and fibroblastic cell lines derived from the ileum of an adult histocompatible miniature boar (d/d haplotype) and immortalized by SV40 plasmid. *Eur J Cell Biol* 62:152–162
- Kaushik RS, Begg AA, Wilson HL, Aich P, Abrahamsen MS, Potter A, Babiuk LA, Griebel P (2008) Establishment of fetal bovine intestinal epithelial cell cultures susceptible to bovine rotavirus infection. *J Virol Methods* 148:182–196
- Kedinger M, Simon-Assmann PM, Lacroix B, Marxer A, Hauri HP, Haffen K (1986) Fetal gut mesenchyme induces differentiation of cultured intestinal endodermal and crypt cells. *Dev Biol* 113:474–483
- Kedinger M, Haffen K, Simon-Assmann P (1987) Intestinal tissue and cell cultures. *Differentiation* 36:71–85
- Kersting S, Bruewer M, Schuermann G, Klotz A, Utech M, Hansmerten M, Krieglstein CF, Senninger N, Schulzke JD, Naim HY, Zimmer KP (2004) Antigen transport and cytoskeletal characteristics of a distinct enterocyte population in inflammatory bowel disease. *Am J Pathol* 165:425–437
- Kishida K, Aoyama M, Masaki M, Shidoji Y (2009) The Ala54Thr polymorphism in the fatty acid-binding protein 2 gene leads to higher food intake in Japanese women. *Mol Psychiatry* 14:466–467
- Kondo Y, Rose I, Young GP, Whitehead RH (1984) Growth and differentiation of fetal rat small intestinal epithelium in tissue culture: relationship to fetal age. *Exp Cell Res* 153:121–134
- Lechanteur A, Almeida A, Sarmiento B (2017) Elucidation of the impact of cell culture conditions of Caco-2 cell monolayer on barrier integrity and intestinal permeability. *Eur J Pharm Biopharm* 119:137–141. doi:[10.1016/j.ejpb.2017.06.013](https://doi.org/10.1016/j.ejpb.2017.06.013)
- Macartney KK, Baumgart DC, Carding SR, Brubaker JO, Offit PA (2000) Primary murine small intestinal epithelial cells, maintained in long-term culture, are susceptible to rotavirus infection. *J Virol* 74:5597–5603
- Moon C, VanDussen KL, Miyoshi H, Stappenbeck TS (2014) Development of a primary mouse intestinal epithelial cell monolayer culture system to evaluate factors that modulate IgA transcytosis. *Mucosal Immunol* 7:818–828
- Panja A (2000) A novel method for the establishment of a pure population of nontransformed human intestinal primary epithelial cell (HIPEC) lines in long term culture. *Lab Invest* 80:1473–1475
- Perreault N, Beaulieu JF (1996) Use of the dissociating enzyme thermolysin to generate viable human normal intestinal epithelial cell cultures. *Exp Cell Res* 224:354–364
- Perreault N, Beaulieu JF (1998) Primary cultures of fully differentiated and pure human intestinal epithelial cells. *Exp Cell Res* 245:34–42
- Pretlow TP, Stinson AJ, Pretlow TG (1978) Cytologic appearance of cells dissociated from rat colon and their separation by isokinetic and isopycnic sedimentation in gradients of Ficoll. *J Natl Cancer Inst* 61:1431–1438
- Pringault E, Robine S, Louvard D (1991) Structure of the human villin gene. *Proc Natl Acad Sci USA* 88:10811–10815
- Quaroni A, May RJ (1980) Establishment and characterization of intestinal epithelial cell cultures. *Methods Cell Biol* 21:403–427
- Rani P, Vashisht M, Golla N, Shandilya S, Onteru SK, Singh D (2017) Milk miRNAs encapsulated in exosomes are stable to human digestion and permeable to intestinal barrier in vitro. *J Funct Foods* 34:431–439
- Reddy PM, Sahi J, Desai G, Vidyasagar D, Rao MC (1996) Altered growth and attachment of rabbit crypt colonocytes isolated from different developmental stages. *Pediatr Res* 39:287–294
- Rusu D, Loret S, Peulen O, Mainil J, Dandridge G (2005) Immunochemical, biomolecular and biochemical characterization of bovine epithelial intestinal primocultures. *BMC Cell Biol* 6:1
- Ryeom SW, Paul D, Goodenough DA (2000) Truncation of mutants of the tight junction protein ZO-1 disrupt corneal epithelial cell morphology. *Mol Biol Cell* 11:1687–1696
- Sacchettini JC, Hauft SM, Van Camp SL, Cistola DP, Gordon JI (1990) Developmental and structural studies of an intracellular lipid binding protein expressed in the ileal epithelium. *J Biol Chem* 265:19199–19207
- Sanderson IR, Ezzell RM, Kedinger M, Erlanger M, Xu ZX, Pringault E, Leon-Robine S, Louvard D, Walker WA (1996) Human fetal enterocytes in vitro: modulation of the phenotype by extracellular matrix. *Proc Natl Acad Sci USA* 93:7717–7722
- Schlage WK, Bulles H, Friedrichs D, Kuhn M, Teredesai A (1998) Cytokeratin expression patterns in the rat respiratory tract as markers of epithelial differentiation in inhalation toxicology I. Determination of normal cytokeratin expression patterns in nose, larynx, trachea, and lung. *Toxicol Pathol* 26:324–343
- Sun TT, Shih C, Green H (1979) Keratin cytoskeletons in epithelial cells of internal organs. *Proc Natl Acad Sci USA* 76:2813–2817
- Tomar A, Wang Y, Kumar N, George S, Ceacareanu B, Hassid A, Chapman KE, Aryal AM, Waters CM, Khurana S (2004) Regulation of cell motility by tyrosine phosphorylated villin. *Mol Biol Cell* 15:4807–4817
- Tomar A, George S, Kansal P, Wang Y, Khurana S (2006) Interaction of phospholipase C- γ_1 with villin regulates epithelial cell migration. *J Biol Chem* 281:31972–31986
- Vashisht M, Rani P, Onteru SK, Singh D (2017) Curcumin encapsulated in milk exosomes resists human digestion and possesses enhanced intestinal permeability in vitro. *Appl Biochem Biotechnol*. doi:[10.1007/s12010-017-2478-4](https://doi.org/10.1007/s12010-017-2478-4)
- Velge P, Kaeffer B, Bottreau E, Langendonck N (1995) The loss of contact inhibition and anchorage-dependent growth are key steps in the acquisition of *Listeria monocytogenes* susceptibility phenotype by non-phagocytic cells. *Biol Cell* 85:55–66
- Vij R, Reddi S, Kapila S, Kapila R (2016) Transepithelial transport of milk derived bioactive peptide VLPVPQK. *Food Chem* 190:681–688

- Wang Y, Srinivasan K, Siddiqui MR, George SP, Tomar A, Khurana S (2008) A novel role for villin in intestinal epithelial cell survival and homeostasis. *J Biol Chem* 283:9454–9464
- Weng XH, Beyenbach KW, Quaroni A (2005) Cultured monolayers of the dog jejunum with the structural and functional properties resembling the normal epithelium. *Am J Physiol Gastrointest Liver Physiol* 288:G705–G717
- Whitehead RH, Demmler K, Rockman SP, Watson NK (1999) Clonogenic growth of epithelial cells from normal colonic mucosa from both mice and humans. *Gastroenterology* 117:858–865