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DOSH, Rasha, JORDAN-MAHY, Nikki <http://orcid.org/0000-0001-8617-2636>, SAMMON, Chris <http://orcid.org/0000-0003-1714-1726> and LE MAITRE, Christine L. <http://orcid.org/0000-0003-4489-7107>

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Rasha H Dosh, Nicola Jordan-Mahy, Christopher Sammon, Christine Le Maitre

Rasha H Dosh, Nicola Jordan-Mahy, Christine L Le Maitre, Biomolecular Sciences Research Centre, Sheffield Hallam University, S1 1WB, UK
Rasha H Dosh Department of Anatomy and Histology, Faculty of Medicine, University of Kufa, Kufa, Iraq
Christopher Sammon, Materials and Engineering Research Institute, Sheffield Hallam University, S1 1WB, UK

ORCID number: Rasha H Dosh (0000-0002-2318-6608); Nicola Jordan-Mahy (0000-0001-8617-2636); Christopher Sammon (0000-0003-1714-1726); Christine Le Maitre (0000-0003-4489-7107).

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Correspondence to: Christine L Le Maitre, PhD, Professor in Cell Biology and Tissue Regeneration, Biomolecular Sciences Research Centre, Sheffield Hallam University, S1 1WB, UK. c.lemaitre@shu.ac.uk

Phone +44 (0)114 225 6613

Fax +44 (0)114 225 3064

Abstract

Intestinal stem cells hold great potential in tissue regeneration of the intestine, however there are key limitations in their culture *in vitro.* We previously reported a novel synthetic non-biodegradable hydrogel as a 3D culture model for intestinal epithelium using Caco2 and HT29-MTX cells. Here, we investigated the potential of this system as a 3D scaffold for crypts and single intestinal stem cells to support long-term culture and differentiation.

Intestinal crypts were extracted from murine small intestines and $Lgr5^+$ stem cells isolated by magnetic activated cell sorting. Crypts and stem cells were suspended within Matrigel or L-pNIPAM for 14 days or suspended within Matrigel for 7 days then released, dissociated, and suspended within, or on L-pNIPAM hydrogel for 28 days. Cellular behaviour and phenotype were determined by histology and immunohistochemistry for stem cell and differentiation markers: Lgr5, E-cadherin MUC2 chromograninA and lysozymes.

Isolated crypts and $Lgr5^+$ intestinal stem cells formed enteroids with a central lumen surrounded by multiple crypt-like buds when cultured in Matrigel. In contrast, when crypts and stem cells were directly suspended within, or layered on L-pNIPAM hydrogel under dynamic culture conditions they formed spherical balls of cells, with no central lumen. When enteroids were innitially formed in Matrigel from crypts or single $Lgr5^+$ intestinal stem cells and dissociated into small fragments or single cells and transferred to L-pNIPAM hydrogel they formed new larger enteroids with numerous crypt-like buds. These crypt-like buds showed the presence of mucin-producing cells, which resembled goblet cells, scattered throughout their structures. Immunohistochemistry staining also showed the expression of Lgr5 and differentiation markers of all the main intestinal cell types including: enterocytes, goblet cells, enteroendocrine and Paneth cells. This demonstrated that L-pNIPAM hydrogel supported long-term culture of crypts and $Lgr5^+$ stem cells and promoted intestinal cell differentiation.

Key words: Small intestinal crypts; Small intestinal stem cells; Matrigel; L-pNIPAM hydrogel; *In vitro.*

INTRODUCTION

The intestinal mucosa is composed of columnar epithelia which form intestinal villi and crypts. These crypts are the site of intestinal stem cell (ISC) production[1]. The intestinal epithelium rapidly self-renews every 3-5 days[2]. This is driven by three cell types: intestinal stem cells, crypt base columnar cells (CBCs), which reside at the bottom of the intestinal crypts; and Paneth cells which support and contribute to the stem cell niche[3, 4]. The stem cells within crypts are capable of responding to local environmental changes ensuring that the correct cellular populations are produced within the villi. Hence, the cells within the stem cell niche regulate villus cell proliferation and the stem cell population[5-8]. The stem cell population expresses a leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5), which is a target gene for WNT signalling[2]. Hence a single Lgr5 positive stem cell ($Lgr5^+$) is capable of regenerating all the cell types within the intestinal epithelium[2, 9-11].

It has previously been shown that when small intestinal crypt cells, isolated from mice, are embedded within Matrigel, and cultured in the presence of R-spondin 1, Noggin, and epidermal growth factors, cells expanded in culture without significant genetic or physiologic alterations[12-15]. Under these conditions, cells organised themselves into 3D tissue constructs described as enteroids. These consist of a central sphere-shaped domain, composed mostly of differentiated cells, and numerous bud-like structures similar to that of an intestinal crypt. A noticeable feature of the epithelial enteroid system is its ability to grow cells almost infinitely from a small number of cells, or tiny pieces of tissue[16-18]. Similarly, single isolated $Lgr5^+$ stem cells grown in Matrigel culture also formed enteroids. These enteroids contained crypt and villus domains which recapitulate essential processes of the native intestinal epithelium[19-21]. Wang *et al* (2017) reported that when pre-isolated stem cells or crypts from the human small intestine were cultured within Matrigel they formed cystic-enteroids[22]. These cystic-enteroids formed an enclosed lumen surrounded by a single layer of non-polarized epithelial cells made up of both proliferating cells randomly mixed with differentiated cells. These cystic-enteroids did not reflect the tissue of the intestine *in vivo* and were unsuitable for studying intestinal functions. This led to the development of alternative scaffolds to support the growth of intestinal stem cells. Those which were investigated previously, including type 1 collagen gels, which support long-term (28 days) maintenance and

expansion of enteroids formed from co-culture of human small intestinal crypts with intestinal sub-epithelial myofibroblasts [23]. Similarly, Sachs *et al* (2017) showed that mice intestinal enteroids could be formed from a single $Lgr5^+$ stem cell, when embedded in Matrigel for 3 days. These enteroids, when released from Matrigel and grown in a floating tube of type 1 collagen were shown to align, fuse and self-organize into a hollow tube-like structures which became lined with the stem cells and differentiated into the major types of intestinal epithelial cells[24].

Although these enteroid systems have had a huge impact on intestinal research, the size, structure, and function of epithelial components in these enteroids are not comparable to those seen *in vivo*. For example, differentiated epithelial cells in small intestinal enteroids fail to form finger-like structures, that accurately mimic native intestinal villi [25]. This has led to the use of a variety of bio-fabrication techniques to produce functional intestine which attempts to mimic the villus-crypt architecture of the intestine *in vivo*. A recent study, demonstrated that micro-engineered collagen scaffolds with appropriate extracellular matrix and stiffness (modulus young's = approximately 10 KPa) generated an *in vitro* human small intestinal epithelium with a polarized monolayer which mimicked *in vivo* crypt-villus structure with a suitable cell lineage and an open luminal surface[22]. Although current enteroid culture systems are dependent on animal-derived hydrogels such as Matrigel and collagen gels, these scaffolds are complex and vary in their composition which makes them inappropriate for enteroid expansion for clinical applications. Hence, this has led to the use of synthetic matrices in tissue engineering of the small intestine[26]. Cromeens *et al* (2016) produced tissue-engineered intestine from enteroids using a mixture of synthetic and natural materials. These enteroids were expanded in Matrigel then released and suspended in poly-glycolic acid (PGA) scaffold coated with type 1 collagen. This was then implanted in the peritoneal cavity of immunosuppressed NOD/SCID mice for 4 wks. Tissue-engineered intestine showed the presence of crypts and short villi, which contained all intestinal epithelial cell lineages, however these studies failed to recapitulate the true *in vivo* morphology having only short villi[27]. This study suggested that a synthetic extracellular matrix could provide a suitable scaffold for maintenance and expansion of crypts and intestinal stem cells. Thus, the current study investigated the tissue engineering potential of a novel synthetic L-pNIPAM hydrogel, which has previously

demonstrated the ability to support villus-like structures[28]. Here, we investigate the use of this 3D scaffold to support long-term culture of crypt cells and $Lgr5^+$ intestinal stem cells under dynamic culture conditions. The capacity of these cells to differentiate into intestinal epithelial cells *in vitro* when suspended within or layered on L-pNIPAM hydrogel was also investigated.

MATERIALS AND METHODS

Mice

BALB/c mice (Charles River Laboratories) aged 6-12 weeks old were used for the extraction of intestinal crypts and isolation of single $Lgr5^+$ stem cells. No live animals were used in our experiments.

Culture media

The basal culture media (Advanced Dulbecco's Modified Eagle Medium (DMEM/F12) (Life Technologies, Paisley, UK) supplemented with 100 µg/ml penicillin/ streptomycin (Life Technologies, Paisley, UK), 250 ng/ml amphotericin (Sigma, Poole, UK), 2 mM glutamine (Life Technologies, Paisley, UK), and 1 M HEPES buffer (Sigma, Poole, UK)). The crypt basal media was made by supplementing with B-27 (50 x) (Life Technologies, Paisley, UK), N-2 (100 x) (Life Technologies, Paisley, UK), and 0.5 M N-Acetyl-L-cysteine (Sigma, Poole, UK).

Small intestinal crypt extraction

Isolation of small intestinal crypts was performed as described previously[17, 29, 30]. Briefly, the duodenum and jejunum were collected and flushed with ice-cold washing solution (10% (v/v) heat-inactivated foetal bovine serum (FBS) (Life Technologies, Paisley, UK) in PBS without Ca^{2+} and Mg^{2+} (Life Technologies, Paisley, UK)) using a 10 ml syringe fixed with a 18 gauge needle. All luminal content was removed. In order to extract the crypts, dissected intestines were opened lengthwise and villi of intestines were scraped off using a coverslip, and discarded. The rest of the intestine (including the crypts) were washed with washing buffer and cut with scissors into 2-4 mm pieces. These pieces were then transferred into a 50 ml Falcon tube and further washed with ice-cold washing solution (approximately 10 times), and pipetting up and down, until the supernatant was clear. The tissue fragments were transferred into 50 ml falcon tubes and incubated in 25 ml of chelating buffer (2 mM EDTA (Sigma, Poole, UK) in PBS) on ice and shaken gently on an orbital shaker for 15

mins. The tubes were inverted gently, and cell fragments were allowed to settle at the bottom of the tubes. The chelating buffer was carefully removed and the crypts were re-suspended in 20 ml of washing solution for 5 min. Tissues were then passed through a 70 µm cell strainer to remove any remaining tissue debris and the crypt fraction was collected. The fraction (Fraction I) was stored on ice. The remaining tissue fragments were re-suspended in ice-cold washing solution, pipetted 5-10 times and the supernatant was passed through 70 µm cell strainers (Fraction II). The previous step was repeated a second time and a third fraction was collected (Fraction III). The three fractions were pooled and centrifuged at 200 g for 5 min at 4°C. The pellet was re-suspended in 10 ml ice-cold basal culture medium and centrifuged at 300 g for 5 min at 4°C to remove single cells and tissue debris. The number of crypts were counted under the inverted microscope. The crypts were subsequently either directly embedded in either Matrigel or L-pNIPAM hydrogel, or further dissociated into a single $Lgr5^+$ stem cell population.

Suspension of small intestinal crypts within Matrigel

Matrigel (basement membrane matrix, growth factor reduced and phenol red-free) (Corning, UK) was mixed with growth factors (recombinant human R-Spondin 1 (1 µg/ml) (PeproTech, London, UK), recombinant murine Noggin (100 ng/ml) (PeproTech, London, UK) and recombinant murine EGF (50 ng/ml) (PeproTech, London, UK)) on ice. One to five hundred crypts were re-suspended in 50 µl of Matrigel. The suspension was pipetted in the middle of the well of pre-warmed 24 well-plates which had been allowed to cool to room temperature for 2-3 min. The plates were then transferred to 37°C for 15 min to allow complete polymerization of the Matrigel. Five hundred microliters of crypt basal media supplemented with epidermal growth factor (EGF) (50 ng/ml), Noggin (100 ng/ml), and R-Spondin 1 (1 µg/ml) (ENR) was added to each well. Cultures were maintained for up to 14 days, at 37°C, 50 mL/L CO_2 in crypt basal media supplemented with ENR which was replaced every 48 h.

Suspension of small intestinal crypts within L-pNIPAM hydrogel

L-pNIPAM hydrogel was prepared as previously described[28]. One to five hundred crypts were re-suspended in 300 µl of liquid L-pNIPAM hydrogel at 38-39°C. The suspension was added in each well of a 48 well plate and allowed to cool below the lower critical solution temperature of the L-pNIPAM hydrogel to induce gelation. Five

hundred microliters of crypt basal medium supplemented with ENR was added per well. Cultures were maintained for up to 14 days at 37°C, 50 mL/L CO_2 and under dynamic conditions, created using an orbital shaker set at 30 rpm. Media was replaced with fresh crypt basal media supplemented with ENR every 48 h.

Layering of small intestinal crypts on L-pNIPAM hydrogel

Three hundred microliters of L-pNIPAM was added to each well of 48 well plates. Following gelation 300 µl of crypt culture media containing one to five hundred crypts was applied to the surface of hydrogel construct. After a 30 min cell attachment period, 200 µl of crypt basal media was added to each well. All plates were maintained in culture for up to 14 days at 37°C, 50 mL/L CO_2 and under dynamic culture conditions, created using an orbital shaker set at 30 rpm. Fresh crypt basal media supplemented with ENR was replenished every 48 h.

Suspension of small intestinal crypts within Matrigel followed by suspension within or layered on L-pNIPAM hydrogel

The extracted crypt pellet was suspended and cultured in Matrigel for 7 days. The resultant enteroids were released from Matrigel on day 7 and were either suspended within or layered on L-pNIPAM hydrogel. Briefly, to release enteroids completely from Matrigel, the media was removed from the enteroids and 500 µl of cold PBS was added to each well. Matrigel containing enteroids were broken up by pipetting up and down. The suspension was transferred into 15 ml conical tubes and the enteroids were pipetted up and down 50-100 times to mechanically disassociate the enteroids into smaller fragments, and separate them from the matrigel extracelluar matrix. Seven milliliters of cold PBS was added to the tube and pipetted up and down a further 20 times, ensuring complete washing. The suspension was centrifuged at 200 g for 5 min at 4°C. The pellet containing the crypt cells was either suspended within or layered on 300 µl of L-pNIPAM hydrogel and maintained for up to 14 days at 37°C, 50 mL/L CO_2 under dynamic culture, created using an orbital shaker set at 30 rpm. Media was replaced with fresh crypt basal media supplemented with ENR every 48 h.

Isolation of small intestinal stem cells

The crypt pellet was re-suspended with 2 ml of pre-warmed single cell dissociation medium (basal medium supplemented with Y-27632, (10 µM/ml), which is a specific

inhibitor of Rho kinase (ROCK) (Sigma, Poole, UK)) for 30-45 min at 37°C until cells dissociated. During incubation, the crypt suspension was mechanically pipetted with fire-polished pasteur pipette every 5-10 min until the crypts were dissociated into single cells or 2-3 cell clusters. The cell suspension was filtered through 40 µm cell a strainer to remove any remaining cell clumps, then resuspended with basal medium and centrifuged at 300 g at 4°C. The pellet was re-suspended with 1 ml single-cell dissociation medium (basal medium supplemented with Y-27632 (10 µM/ml)), and centrifuged at 500 g for 10 min and the supernatant was completely removed in preparation of Magnetic Activated Cell Sorting (MACS).

Magnetic Activated Cell Sorting (MACS)

Following the manufacturer's protocol the cell pellet was resuspended up to 1 x 10^6 nucleated cells in 45 µl of degassed MACS buffer solution (PBS plus 0.5% (w/v) bovine serum albumin (BSA), and 2 mM EDTA). This MACS buffer solution was prepared by diluting 1 ml MACS BSA stock solution (Miltenyi Biotec, UK) with 20 ml autoMACS rinsing solution (Miltenyi Biotec, UK). Five microlitres of FcR blocking reagent (Miltenyi Biotec, UK) was added to the suspension to block unwanted binding of Lgr5 antibody to mouse cells expressing Fc receptors (e.g. B cells, monocytes or macrophages); mixed well and refrigerated for 10 min at 2-8°C. Cells were labeled by adding 5 µl of biotinylated Lgr5 antibody (Miltenyi Biotec, UK), mixed well and refrigerated for 10 min. Cells were then washed by adding 1-2 ml of degassed MACS buffer and centrifuged at 500 g for 10 min. The supernatant was removed and cell pellet re-suspended in 70 µl of degassed MACS buffer. Cells were magnetically labeled by adding 20 µl of anti-biotin microbeads (Miltenyi Biotec, UK), mixed well and refrigerated for 15 min. The cells were washed in 1-2 ml of degassed MACS buffer and centrifuged at 500 g for 10 min. The supernatant was removed and cell pellet re-suspended in 500 µl of degassed MACS buffer and magnetically separated.

The MACS MS column (Miltenyi Biotec, UK) was inserted into the column holder on the MACS separator. The column was rinsed with 500 µl of degassed buffer and the effluent was discarded. The magnetically labeled cell suspension was loaded onto a MACS MS column; unlabelled cells were flushed through the column with 3 x 500 µl degassed MACS buffer. The unlabelled cell fraction was collected and a new

collection tube was placed under the column. The column was removed from the magnetic holder, 1 ml of degassed MACS buffer was pipetted onto the column and plunger applied causing the magnetically labeled Lgr5 positive ($Lgr5^+$) cells to be released and collected from the column. To increase the purity of the magnetically labeled fraction, the $Lgr5^+$ fraction was applied onto a freshly prepared MS column as described above, and MACS sorting repeated. The number of $Lgr5^+$ cells/ml were counted following sorting and cell suspensions were centrifuged at 500 g for 5 min and the supernatant discarded. The cell pellet was used for subsequent culture experiments.

Cytospin of $Lgr5^+$ sorted cells

A sample of the $Lgr5^+$ sorted cells was fixed in 40 g/L formaldehyde (Sigma, Poole, UK) for 20 min. The cells were centrifuged at 300 g for 5 min and resuspended in PBS at a cell density of 300 cells/µl. One hundred microliters of cell suspension was cytospun by centrifugation at 1000 rpm for 3 min (Shandon cytospin 3, Thermo Scientific, Loughborough, UK). Slides were then air-dried and stored at 4°C until needed for immunohistochemical investigation.

Suspension of $Lgr5^+$ stem cells within Matrigel

Matrigel was mixed with growth factors: Notch ligand Jagged-1 peptide (1 µM/ml) (Eurogentec, Southampton, UK), and ENR on ice. One to two hundred $Lgr5^+$ stem cells were re-suspended in 10 µl of Matrigel and pipetted into the middle of the well of a pre-warmed 96 well-plate. Following 2-3 min at room temperature, they were transferred to 37°C for 15 min to allow complete polymerization of the Matrigel. One hundred microliters of fresh crypt basal media supplemented with 2.5 µM/ml GSK3β inhibitor (CHIR99021) (Sigma, Poole, UK) (to activate β-catenin and other survival pathways), ROCK activity inhibitor (2.5 µM/ml Thiazovivin) (Sigma, Poole, UK) to decrease anoikis by increasing the stability of E-cadherin) (both of these supplements were added during the first 2 days culture only, as previously described[31]), and ENR was added to each well. To reduce apoptosis and promote the survival of single stem cells during the first 2 days of culture a Notch agonist Jagged-1 peptide (1 µM/ml) was also added to the cell culture media. Cultures were maintained for up to 14 days at 37°C, 50 mL/L CO_2, and the media was replaced with fresh crypt basal media supplemented with ENR every 48 h.

Suspension of Lgr5$^+$ stem cells within Matrigel followed by suspension within or layered on L-pNIPAM hydrogel

Isolated Lgr5$^+$ stem cells were suspended in Matrigel, the enteroids which formed from Lgr5$^+$ stem cells were released from Matrigel at day 7. Briefly, media was removed and 100 µl of cold PBS was added to each well. Matrigel was broken up by pipetting up and down to mechanically disassociate the enteroid into single cells. The suspension was transferred into 1.5 ml Eppendorf and centrifuged at 300 g for 5 min. The pellet was either suspended within or layered on L-pNIPAM hydrogel and maintained for 1 to 4 weeks at 37°C, in 50 mL/L CO_2 under dynamic culture conditions as described above. Media was replaced with fresh crypt basal media supplemented with ENR every 48 h. Jagged-1 peptide (1 µM/ml) was added to the cell culture media during the first 2 days of culture.

Characterisation of crypts and Lgr5$^+$ stem cells

Bright field images of crypts and Lgr5$^+$ stem cells

Crypts and Lgr5$^+$ stem cells were suspended within Matrigel for up to 14 days. Bright field images were taken using an inverted microscope (Olympus IX8, UK) and images captured using Cell-F software.

Histological assessment

Crypt cells either suspended within or layered on L-pNIPAM hydrogel together with those Lgr5$^+$ stem cells suspended in Matrigel for 7 days released from Matrigel and suspended within or layered on L-pNIPAM hydrogel were assessed. Triplicate samples were fixed in 40 g/L formaldehyde/PBS for 24 h prior to washing in PBS and processed to paraffin wax in a TP1020 tissue processor (Leica Microsystem, Milton Keynes, UK). Four-micron sections were cut and mounted onto positively charged slides (Leica Microsystem Milton Keynes, UK). Sections were stained with Haematoxylin and Eosin or Alcian Blue/Periodic acid Schiff's (PAS) as described previously [28].

Immunohistochemical Assessment

Immunohistochemistry was performed as described previously[28] to investigate: Adhesion junction protein (using anti-E-cadherin antibody (1:200 mouse monoclonal, heat antigen retrieval) (ab76005, Abcam, Cambridge, UK); MUC2 production using

anti-MUC2 antibody (1:100 rabbit polyclonal, heat antigen retrieval) (Santa Cruz, Heidelberg, Germany); Chromogranin A production as an indication of enteroendocrine cell presence using anti-Chromogranin A antibody (1:100 rabbit polyclonal, heat antigen retrieval) (ab15160, Abcam, Cambridge, UK); and lysozyme production as an indication of Paneth cell presence (using anti-Lysozyme antibody (1:2000 rabbit monoclonal, heat antigen retrieval) (ab108508, Abcam, Cambridge, UK).

RESULTS

Morphological assessment of crypts isolated from the small intestine suspended within Matrigel, L-pNIPAM hydrogel or layered on L-pNIPAM hydrogel

When isolated crypts were suspended in Matrigel, they formed enteroid-like structures within 1 day (Figure 1A). After 2 days, these structures began to develop crypt-like buds. Larger mature buds made up of several well-defined cells were seen by day 6 to 7, suggesting the presence of stem cells within the crypt buds. Between day 7 and 14, the enteroids enlarged with increasing number of crypt-like buds (Figure 1A).

When isolated crypts were directly suspended within or layered on L-pNIPAM hydrogel under dynamic culture conditions, H&E staining showed that crypts failed to form true enteroid structures by day 10 to 14, with no appearance of crypt-like buds (Figure 1B). However, alcian Blue/PAS staining showed the presence of mucin-producing goblet cells scattered throughout their structures, and the presence of an adherent mucus layer by day 14 (Figure 1B).

Morphological assessment of the enteroids when released from Matrigel, dissociated and suspended within or layered on L-pNIPAM hydrogel

When enteroids were formed in Matrigel for 7 days, and dissociated into small fragments and then transferred into or onto L-pNIPAM hydrogel they formed new enteroids within 10 days with numerous crypt-like buds (Figure 1C), containing mucin-producing cells (Figure 1C).

Phenotypical assessment of the enteroids derived from crypts suspended directly in L-pNIPAM hydrogel or in Matrigel for 7 days then dissociated and re-suspended in L-pNIPAM

Immunohistochemistry staining was used to determine the cellular composition of the enteroids derived from crypts suspended within L-pNIPAM hydrogel following 10 days culture (Figure 2A-E) or derived from dissociated enteroids re-suspended and cultured within L-pNIPAM hydrogel (Figure 2F-J). The stem cell marker Lgr5 expression was within the crypt-like structure of all enteroids; this was expressed alongside markers for all terminally differentiated epithelial cell lineages which identified the presence of enterocytes, goblet cells, enteroendocrine cells and paneth cells by day 10.

Stem cell marker Lgr5 was expressed at the base of the crypt-like buds of the enteroids (Figure 2A and F). Cell-cell adhesion marker: E-cadherin was expressed under all culture conditions, but was most highly expressed in the Matrigel to L-pNIPAM enteroids (Figure 2B and G). Goblet cell marker MUC2 showed the presence of goblet cells and an adherent mucus layer covering the enteroids (Figure 2C and H). The enteroendocrine cell marker: chromogranin A, showed immunopositive cells scattered throughout the enteroid structure (Figure 2D and I). Paneth cell marker for lysozymes, was seen at the base of the crypt-like buds, but only in those tissues cultured directly into L-pNIPAM hydrogel (Figure 2E); whilst enteroids which were grown in Matrigel and then moved to L-pNIPAM hydrogel showed a scattered distribution of lysosome positive cells throughout the enteroid (Figure 2J).

Morphological assessment of the isolated Lgr5$^+$ small intestinal stem cells suspended within Matrigel, or suspended in Matrigel for 7 days then dissociated and re-suspended in or layered on L-pNIPAM

Single Lgr5$^+$ intestinal cells (6.89 ± 0.56%) suspended within Matrigel formed enteroids after 5 days in culture. Between day 10 and 14, the enteroids formed numerous crypt-like buds (Figure 3). When Lgr5$^+$ cells were grown in Matrigel for 7 days, released, dissociated and cultured within or on L-pNIPAM hydrogel, they proliferated and started to form crypt-like buds. There was differentiation of these cells, and by day 7 goblet cells were seen, and by day 21 to 28 the enteroids were surrounded by an adherent mucus layer (Figure 4A & B).

Immunohistochemistry staining for differentiation markers of isolated $Lgr5^+$ stem cells prior to L-pNIPAM 3D culture showed strong immunopositivity for Lgr5 and E cadherin, plus some low level chormogranin A and lysozome. There was no immunopositivity for MUC2 (Figure 5A). However, following culture in L-pNIPAM Lgr5 was expressed throughout the entire 28 day study period (Figure 5B & C). There were many $Lgr5^+$ cells with the base of crypt-like buds at day 21 to 28. Cell-cell adhesion marker E-cadherin was expressed at the later time points at day 21 and 28. (Figure 6A & B). $Lgr5^+$ intestinal stem cells were differentiated into mucin-producing goblet cells. However, the expression of MUC2 was time-dependent, with the highest levels of MUC2 immunopositivity being observed at day 21 and 28 (Figure 6C & D). Chromogranin-A immunopositivity which is indicative of the presence of enteroendocrine cells, was not consistantly observed and was effected by the culture conditions. When $Lgr5^+$ intestinal stem cells were suspended within L-pNIPAM hydrogel chromogranin-A expressed occurred at day 21 to 28 (Figure 7 A). However, when $Lgr5^+$ intestinal stem cells were layered on L-pNIPAM hydrogel Chromogranin-A expression occurred at day 14 (Figure 7B). Lysozyme expression was seen between 7-28 days, indicating the differentiation of Paneth cells when suspended within or layered on L-pNIPAM hydrogel (Figure 7 C & D). However the highest expression was observed in the enteroids when layered on L-pNIPAM hydrogel (Figure 7 D).

Discussion

In vivo, intestinal stem cells are positioned at the base of the intestinal crypts and have the ability differentiate into multiple epithelial progenies and initiate mucosal regeneration[32]. Thus, intestinal stem cells hold great potential in tissue engineering and researchers worldwide are using these stem cells in conjunction with a variety of biomaterial and biological approaches in order to grow intestinal mucosa. A number of biomaterials including Matrigel, collagen type 1, PGA, PLA, PLGA have been investigated as potential cellular scaffolds [33-38]. Here we investigate the potential of a synthetic porous L-pNIPAM hydrogel as a 3D scaffold for enteroid development from crypts and $Lgr5^+$ intestinal stem cells under dynamic culture conditions, when suspended within or layered on our hydrogel.

3D culture system for intestinal crypts

Matrigel is a naturally derived 3D scaffold, rich in laminin and collagen, and is commonly used in *in vitro* culture. Its use in intestinal epithelial culture is well known[38] and it was the first system used to culture intestinal stem cells. Its high laminin content is thought to encourage cellular differentiation of intestinal crypt cells and supports villus formation[41]. Sato *et al.* (2009) developed an *in vitro* culture system capable of developing intestinal enteroids from isolated crypts and single $Lgr5^+$ stem cells. These enteroids showed the presence of a central core lined with villus-like domains surrounded by crypt-like buds after only 14 days in culture. These crypt-like buds maintained the basic crypt morphology; however, the central core of the enteroid at the top of the crypts became filled with dead cells which were shed from the crypt villus domain[38]. This enteroid structure and accumulation of dead cells has been subsequently observed in a number of studies and is described as a cystic-enteroid [15, 18, 38]. Despite the potential advantages of Matrigel, particularly the similarity to native extracellular matrix; there are also a number of disadvantages, including limited stability for long term culture, low mechanical stiffness and high batch variation[42, 43]. Furthermore Matrigel is inappropriate for clinical applications as it is sourced from cancerous tissues (Engelbreth-Holm-Swarm mouse sarcoma) [42, 43].

Hence, these limitations have led to the use of synthetic scaffolds with well-defined biochemical and biophysical properties as a scaffold for intestinal tissue engineering [44-46]. This current study demonstrated that the synthetic highly porous L-pNIPAM hydrogel facilitated crypt cell seeding and adhesion. However, crypt-like buds were not observed when crypts were directly layered on the L-pNIPAM hydrogel surface. This suggests that there is some degree of *in vitro* conditioning required to support enteroid formation on the surface of the hydrogel. Nevertheless, when crypts were directly placed into the L-pNIPAM hydrogels, immunohistochemical staining did demonstrate the presence of $Lgr5^+$ stem cells, enterocytes, goblet cells, enteroendocrine cells and Paneth cells; suggesting the ability of our hydrogel to support cellular survival and differentiation. Of note, when crypts were suspended within Matrigel for 7 days prior to re-suspension within or layered on L-pNIPAM hydrogel for 10 days under dynamic culture conditions the enteroids had multiple crypt-like buds, containing mucin-secreting goblet cells. This suggests that isolated crypts require extracellular matrix signals to form enteroids with crypt-like buds. This

extracellular matrix may have orginiated from Matrigel culture, and despite substantial washes was still retained by the crypts; or alternatively was produced by the crypts during the 10 day culture period. Previous studies have also shown the requirement of an extracellular matrix such as Matrigel[45] or collagen type I[27] when using synthetic scaffolds to support intestinal crypt cultures [47] Similarly, Matrigel was used to coat PLGA villus-moulds to support enteroid formation of co-cultured intestinal crypts, myofibroblasts and macrophages[45], Here, the cells were shown to differentiate into all the intestinal cell lineages, however they did not form true-villi, but instead produced an enteroid in the shape of the villus-mould [45]. In contrast, our previous work has shown that monocultured or co-cultured human colon carcinoma cell lines Caco-2 and HT29-MTX cells grown on our L-pNIPAM hydrogel showed spontaneous formation of villus-like structures [28, 48].

In the current study, our cultures demonstrated the early stages of enteroid formation and cellular differentiation and maintenance of cultures for upto the 28 days. It remains to be determined if longer term cultures would result in spontaneous villi formation, or whether transplantation of the L-pNIPAM enteroids in *in vivo* models may induce villi formation. This effect has been observed previously when enteroids grown on a PLGA scaffolds were transplantation in to *in vivo systems* [37, 49, 50].

LGR-5 Stem cell Culture

Similar to crypt cultures, Matrigel has been used as a 3D culture system for intestinal stem cells [51]. The current study, found that the isolated $Lgr5^+$ stem cells formed enteroids with multiple crypt-like buds when grown in Matrigel. These data agrees with a number of studies where enteroids were generated in Matrigel from single $Lgr5^+$ stem cells after 5 days[18, 20, 29, 38, 52]. When enteroids were initially formed in Matrigel, then dissociated into single cells and suspended within or layered on L-pNIPAM hydrogel, the stem cells generated crypt-like buds within 21 days, and expressed markers of enterocytes, mucus producing goblet cells, enteroendocrine, and Paneth cells, together with a residing population of $Lgr5^+$ stem cells. This suggests that these $Lgr5^+$ stem cells can be differentiated into the four main types of intestinal epithelial cells when suspended within or layered on L-pNIPAM hydrogel. However, it is unknown whether the cells released from the enteroid cultures grown in the Matrigel, were stem cells or differentiated intestinal cells. It has been shown

previously that enterocyte progenitors can become proliferative stem cells and Paneth-like cells during crypt regeneration, to replenish stem cell loss[53]. However *in vivo* enterocyte progenitors not only can dedifferentiate to stem cells, but also secretory progenitors, which derive from $Lgr5^+$ stem cells and generate goblet cells, enteroendocrine cells and Paneth cells, which can then dedifferentiate back to stem cells, to replace lost stem cells[53]. Thus, the cells derived from the dissociated Matrigel enteroids could have been stem cells, or differentiated cells which then dedifferentiated to an earlier progenitor cell which then went on to form later enteroids in L-pNIPAM hydrogels, this requires further investigation.

Gjorevski *et al* reported the first synthetic matrix for the expansion of mouse and human intestinal stem cells. They tested the effect of matrix mechanical properties on the stem cell survival, proliferation, expansion and differentiation. It was shown that varying stiffness of polyethylene glycol (PEG) hydrogel and extracellular matrix components were required for stem cell expansion and enteroid formation. Fibronectin based adhesion was required for intestinal stem cell survival and proliferation, whilst stem cell expansion required high matrix stiffness. Thus a soft matrix and laminin-based adhesion were required for both intestinal stem cell differentiation and enteroid formation[26]. The relationship between matrix stiffness and the intestinal crypt and stem cell behaviours can be seen in this study. The Matrigel which is relatively soft, having a young's modulus < 500 Pa, supported enteroid formation, but not crypt-like buds or villus formation. Whilst the L-pNIPAM hydrogel which is much stiffer with a storage modulus of 0.18-2.8 MPa[28] supported intestinal crypts and stem cells survival, differentiation and enteroid formation under dynamic culture conditions.

These findings are in agreement with our previous study which demonstrated the potential of L-pNIPAM hydrogel to maintain, differentiate, and stimulate Caco-2 and HT29-MTX cells to form villus-like structures compared to low stiffness alginate, where the cells only formed spheroid-like structures under dynamic culture conditions[28]. Of note L-pNIPAM hydrogels were able to maintain enteroid cultures for the whole 28 days investigation period; this is problematic for Matrigel cultures due to low long term stability. Of particular interest would be the potential development of our synthetic L-pNIPAM hydrogel as a microcapsule delivery system similar to that reported for scalable production of organoids utilising a shell structure

of Matrigel and alginate using bioreactor systems for mass culture[54]. Due to the longer stability of our hydrogel the culture beyond the 6 days investigated in this prior study[54] would be important to investigate.

Conclusion

L-pNIPAM hydrogel was a suitable scaffold to support the formation of enteroids *in vitro* following an initial priming culture in Matrigel from small intestinal crypts and $Lgr5^+$ stem cells isolated from mice and supported appropriate cell differentiation. Further studies are now required to investigate the ability of L-pNIPAM hydrogel as a scaffold for crypts and $Lgr5^+$ stem cells isolated from human small intestine and colon.

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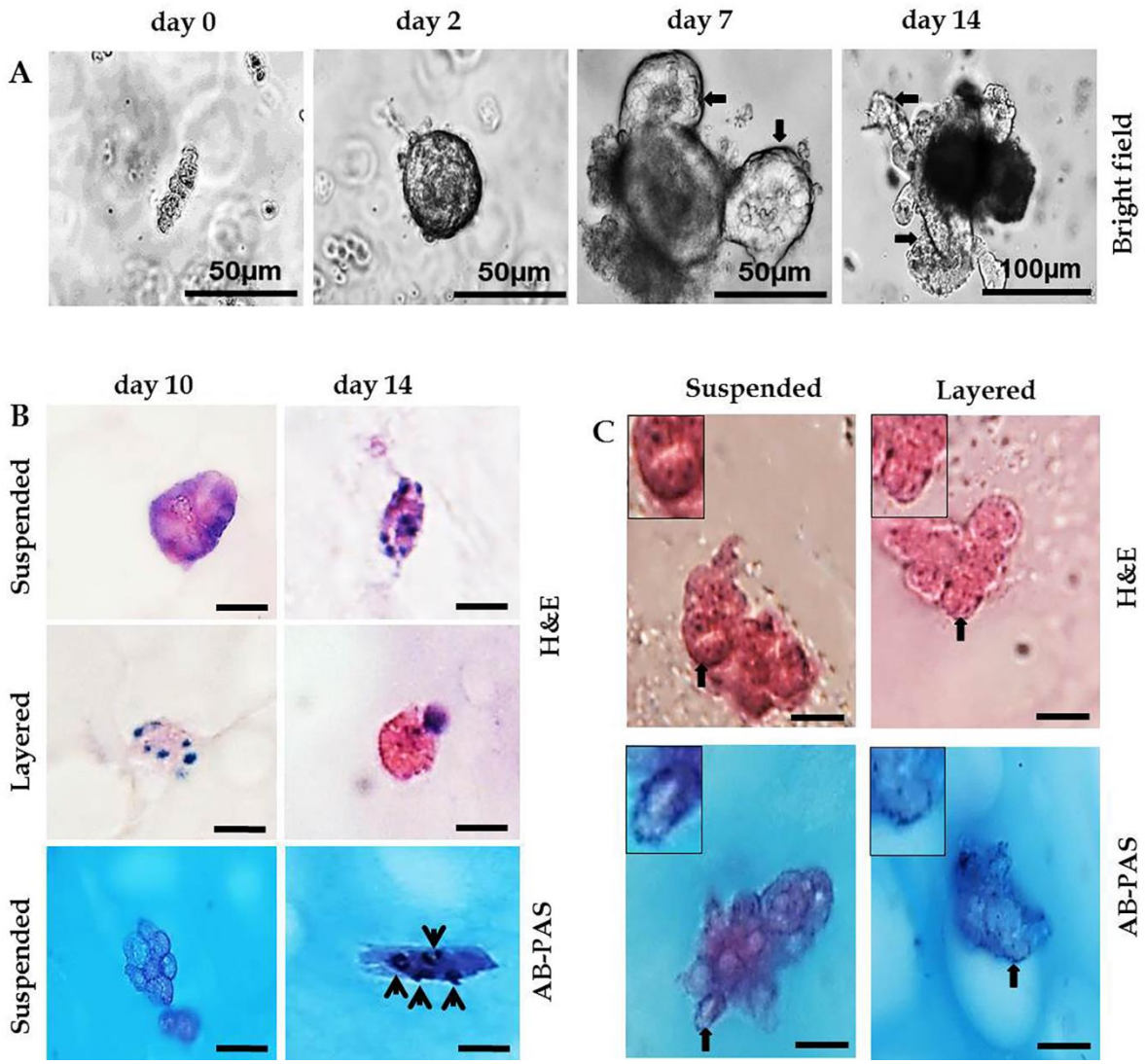

Figure 1 (A) Bright-field morphology of the isolated small intestinal crypts suspended within Matrigel for 0-14 days. Representative organoids with black arrows indicating crypt buds. (B) Morphology of the isolated small intestinal crypts when suspended directly within or layered on L-pNIPAM hydrogel under dynamic culture conditions for 14 days. The cells stained with H&E AB-PAS. Black arrows indicate mucin-producing goblet cells. (C) Morphology of the isolated small intestinal crypts when suspended within Matrigel for 7 days then the derived organoids were released from Matrigel and dissociated into small fragments and then re-suspended within or layered on L-pNIPAM hydrogel under dynamic culture conditions for 10 days. Stained with H&E: the black arrows indicate the crypt-like buds and AB-PAS: the black arrows indicate mucin-producing goblet cells. Scale bar = 20 µm.

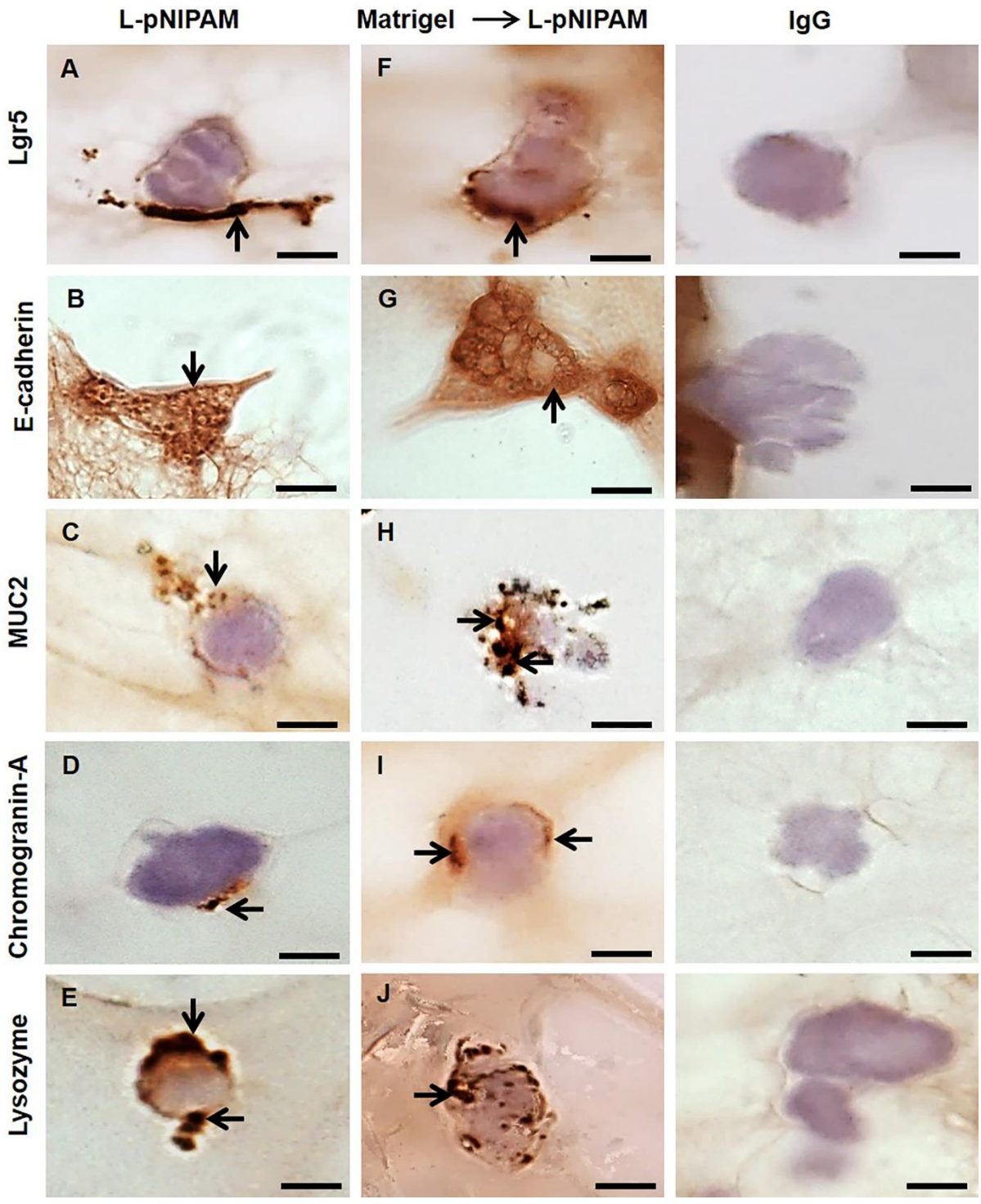

Figure 2 Immunohistochemistry staining (black arrows) for Lgr5, E-cadherin, MUC2, Chromogranin-A, and Lysozyme of the isolated small intestinal crypts: A-E suspended directly within L-pNIPAM hydrogel under dynamic culture conditions for 10 days; F-J suspended within Matrigel for 7 days then the organoids were released from Matrigel and dissociated into small fragments then suspended within L-pNIPAM hydrogel under dynamic culture conditions for 10 days. Cell nuclei were stained with haematoxylin (blue). IgG as negative controls. Scale bar = 20 μm.

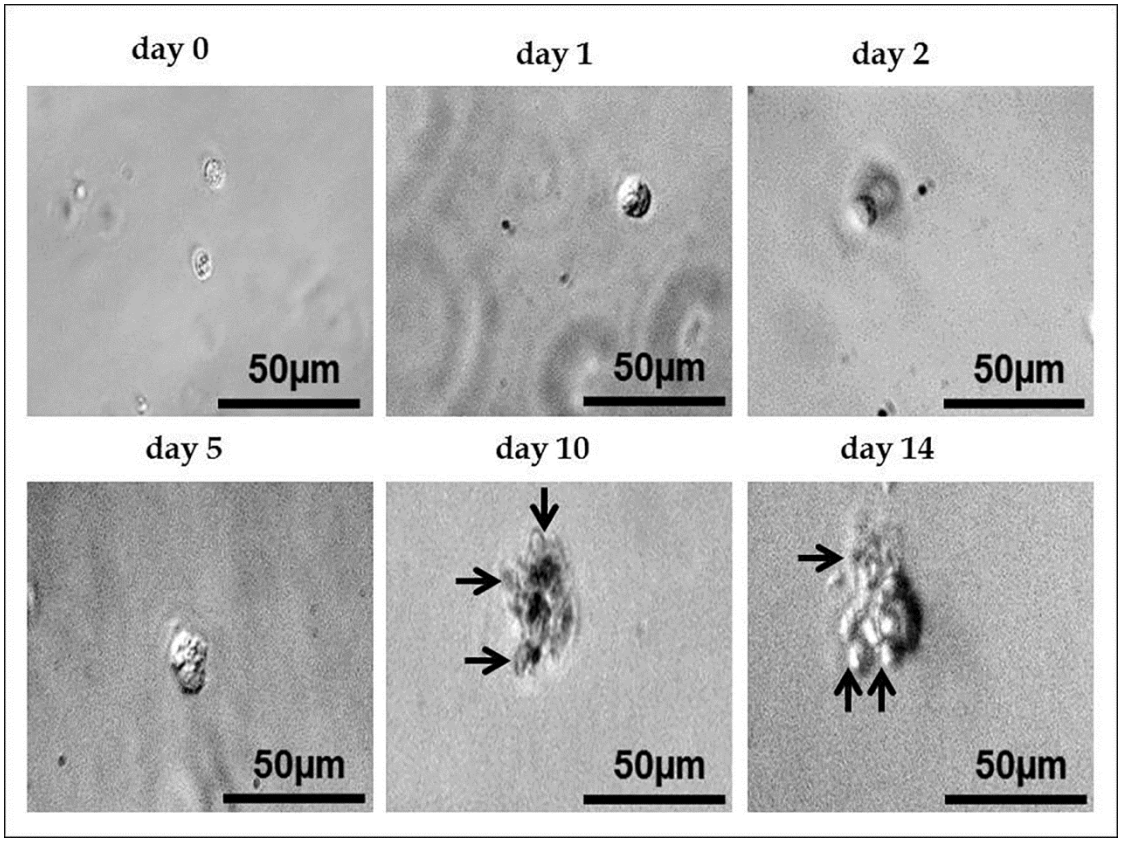

Figure 3 Bright-field morphology of the isolated $Lgr5^{+}$ intestinal stem cells suspended within Matrigel from day 0 to 14. Black arrows indicate organoid buds. Scale bar= 50 μm.

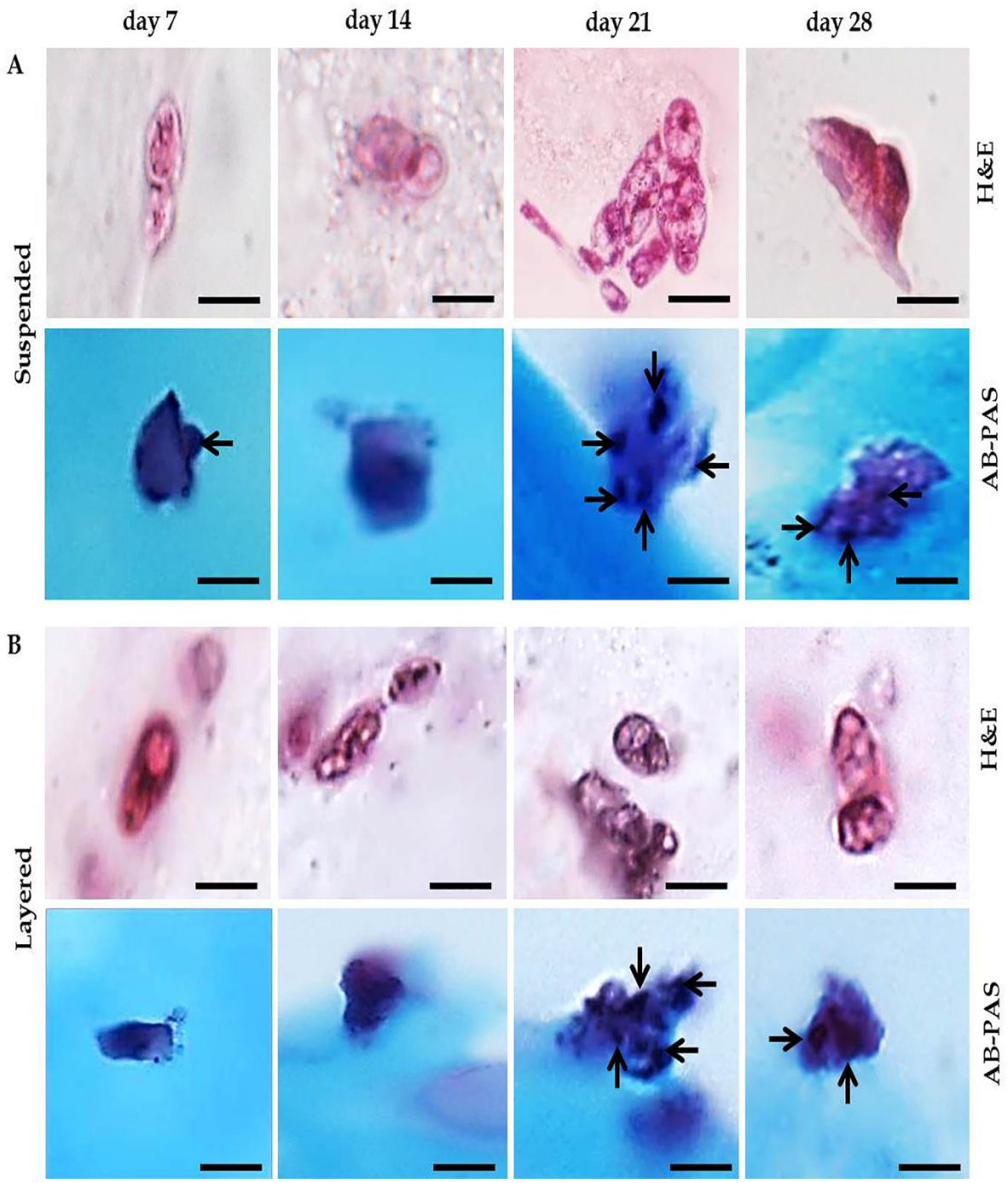

Figure 4 Morphology of the isolated Lgr5^{+} intestinal stem cells suspended within Matrigel for 7 days then the organoids were released from Matrigel and dissociated into single cells and (A) were suspended in L-pNIPAM hydrogel or (B) were layered on L-pNIPAM hydrogel under dynamic culture conditions for 7 to 28 days. Cells were stained with H&E and AB-PAS. The black arrows indicate goblet cells. Scale bar = 20 μm.

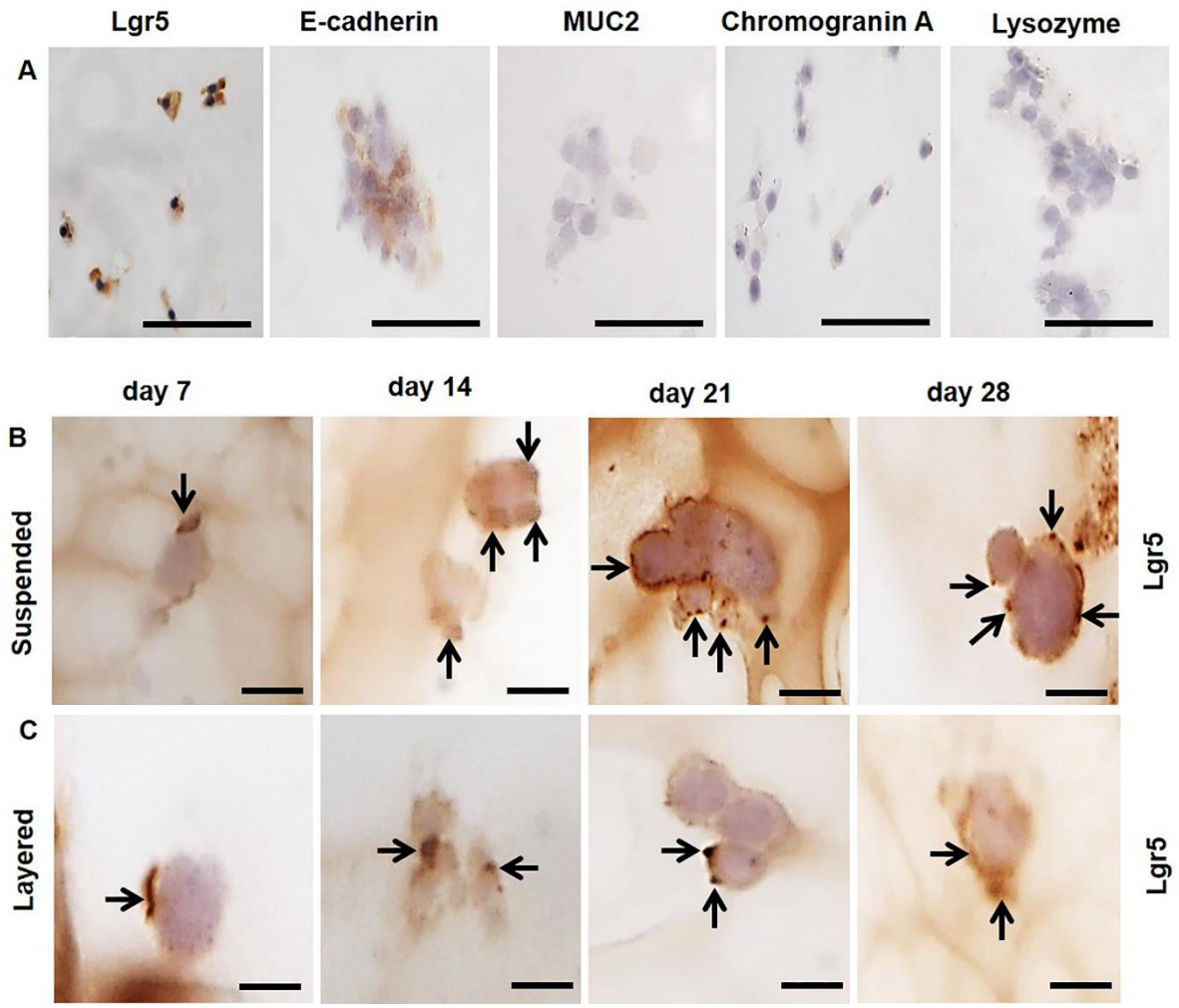

Figure 5 Immunohistochemistry staining for (A) Lgr5; E-cadherin; MUC2; Chromogranin-A and Lysozymes in cytospin of the isolated $Lgr5^{+}$ intestinal stem cells. (B) Lgr5 expression in isolated $Lgr5^{+}$ intestinal stem cells when suspended within Matrigel for 7 days then the organoids were released from Matrigel and dissociated into single cells then suspended within L-pNIPAM hydrogel or layered on L-pNIPAM hydrogel under dynamic culture conditions for 7 to 28 days. Cell nuclei were stained with haematoxylin (blue). Scale bar = 20 μm. Cell nuclei were stained with haematoxylin (blue). Scale bar (A) 100 μm, (B& C) 20 μm.

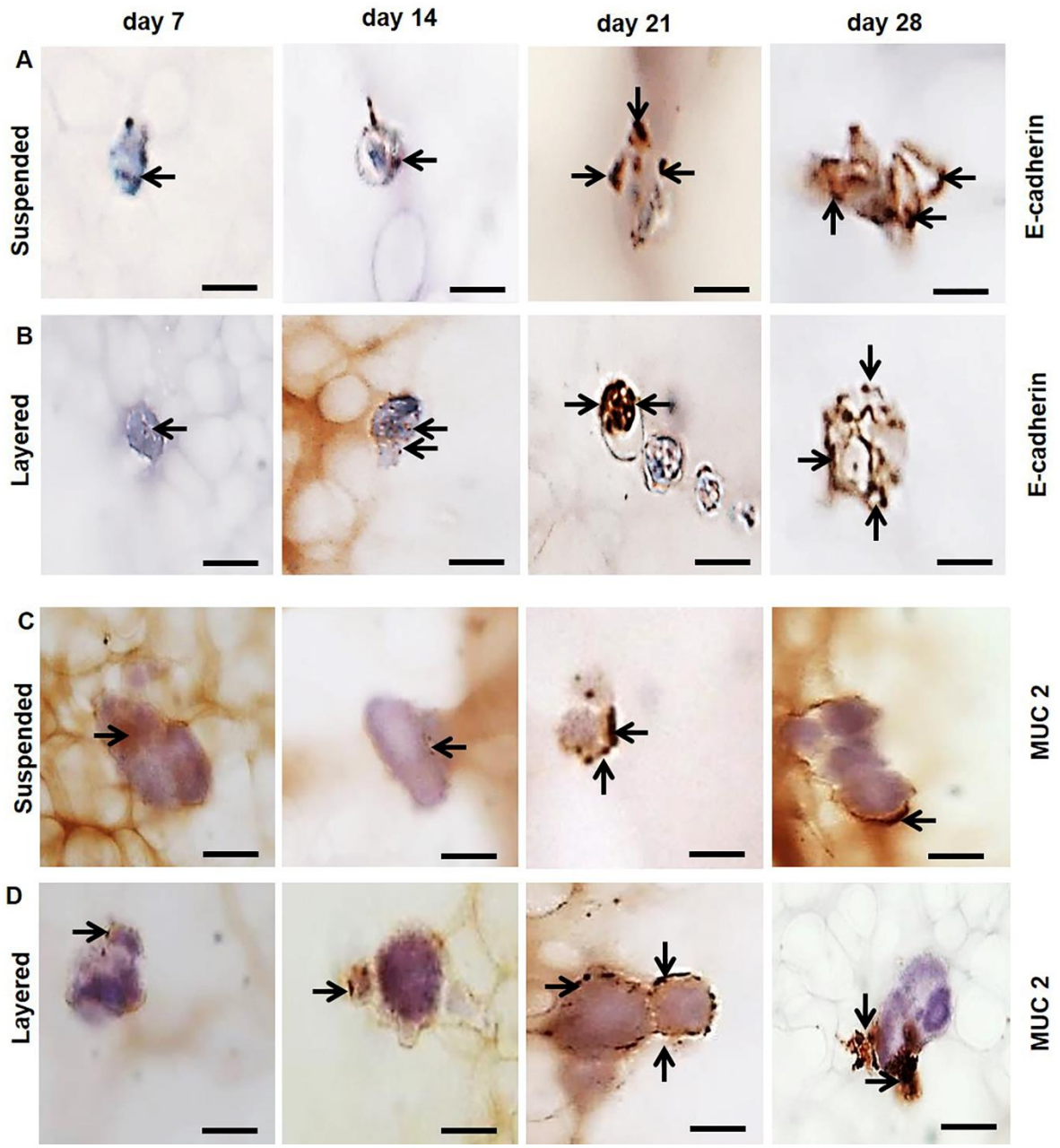

Figure 6 Immunohistochemistry staining (black arrows) for E-cadherin (A&B) and MUC2 (C&D) of the isolated $Lgr5^{+}$ intestinal stem cells suspended within Matrigel for 7 days then the organoids were released from Matrigel and dissociated into single cells and suspended within L-pNIPAM hydrogel or layered on L-pNIPAM hydrogel under dynamic culture conditions for 7 to 28 days. Cell nuclei were stained with haematoxylin (blue). Scale bar = 20 μm.

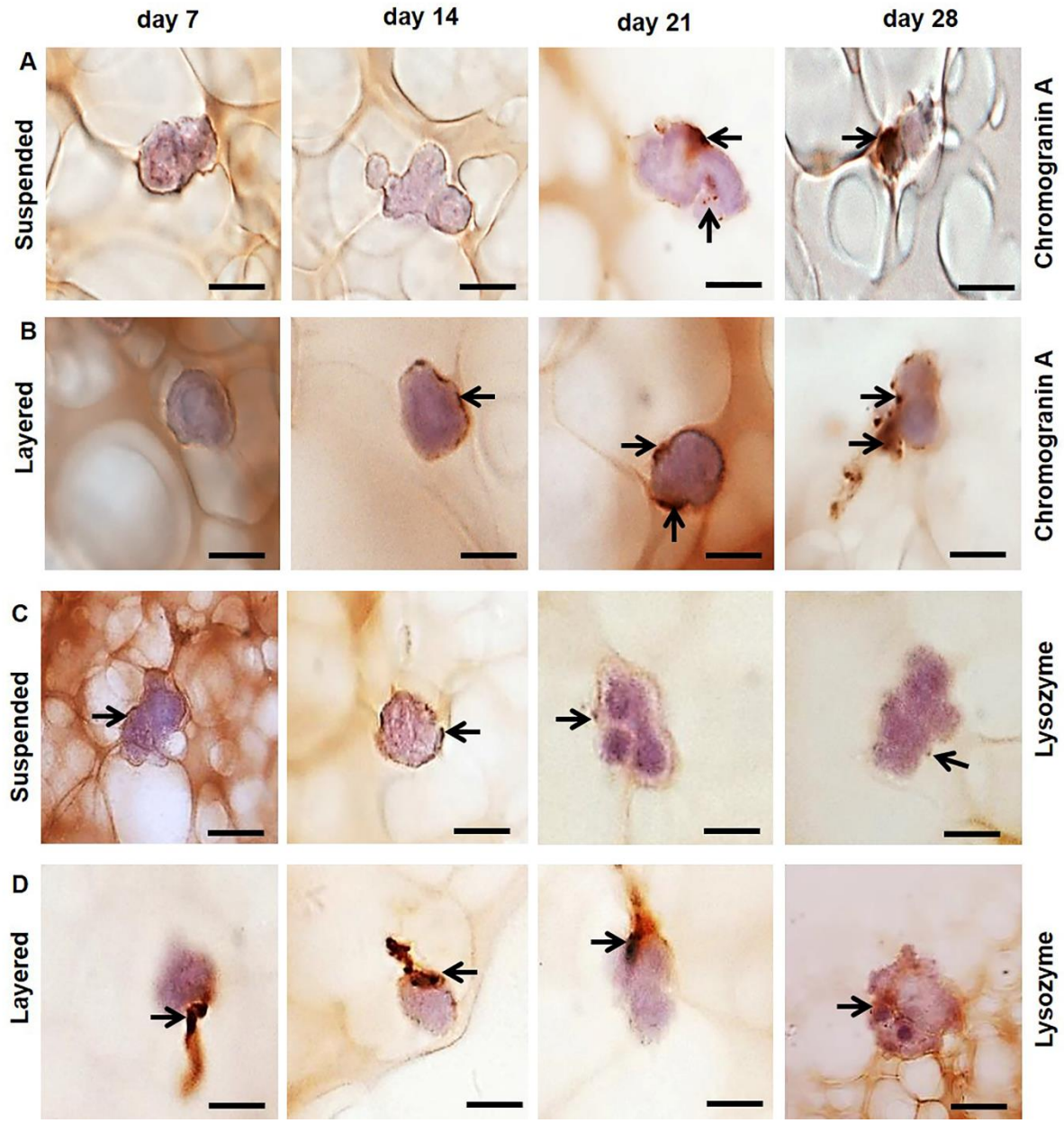

Figure 7 Immunohistochemistry staining (black arrows) for chromogranin A (A&B) and lysozyme (C&D) of the isolated Lgr5^{+} intestinal stem cells suspended within Matrigel for 7 days then the organoids were released from Matrigel and dissociated into single cells and suspended within L-pNIPAM hydrogel or layered on L-pNIPAM hydrogel under dynamic culture conditions for 7 to 28 days. Cell nuclei were stained with haematoxylin (blue). Scale bar = 20 μm.