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## Intestinal stem cells remain viable after prolonged tissue storage

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### Abstract

Intestinal stem cells (ISCs) are responsible for renewal of the epithelium both during normal homeostasis and following injury. As such they have significant therapeutic potential. However, it is unknown whether ISCs can survive tissue storage. We hypothesized that, although the majority of epithelial cells may die, ISCs would remain viable for at least 24 h at 4°C. To explore this hypothesis, jejunum of C57Bl6/J or *Lgr5-LacZ* mice were removed and either processed immediately or placed in phosphate buffered saline (PBS) at 4°C. Delayed isolations of epithelia were performed after 24, 30, or 48 h storage. At the light microscope level, despite extensive apoptosis of villus epithelial cells, small intestinal crypts remained morphologically intact through 30 h and ISCs were identifiable via *Lgr5-LacZ* positivity. Electron microscopy showed that ISCs retain high integrity through 24 h. When assessed by flow cytometry, ISCs were more resistant to degeneration than the rest of the epithelium, including neighboring Paneth cells, with higher viability across all time points. Culture of isolated crypts showed no loss of capacity to form

complex enteroids after 24 h tissue storage, with efficiencies after 7 days of culture remaining above 80%. By 30 h storage, efficiencies declined but budding capability was retained. We conclude that, with delay in isolation, ISCs remain viable and retain their proliferative capacity. In contrast, the remainder of the epithelium, including the Paneth cells, exhibits degeneration and programmed cell death. If these findings are recapitulated with human tissue, storage at 4°C may offer a valuable temporal window for harvest of crypts or ISCs for therapeutic application.

## Keywords

Intestinal stem cells; light and electron microscopy; viability; resistance to storage; enteroid culture

## Introduction

The intestinal epithelium is the most rapidly proliferating tissue in the mammalian body, with complete turnover of the majority of cells occurring every 3–4 days. Pioneering work of Cheng and Leblond in the early 1970s suggested that this turnover arises from stem cells located deep in the intestinal crypts (Cheng and Leblond, 1974). The general concept that these stem cells give rise to all differentiated lineages within the epithelium has stood the test of time and has now been confirmed by numerous groups (see reviews by Garrison, et al., 2009, King and Dekaney, 2013, Montgomery and Breault, 2008, Shaker and Rubin, 2010, Wong, 2004). Additionally, as more sophisticated tools have become available, accumulated evidence indicates that there are in fact at least two relatively distinct populations of intestinal stem cells (ISCs). The most extensively studied population is one that is marked by the G-protein-coupled receptor *Lgr5*. This population is actively cycling and is normally responsible for the day-to-day homeostatic turnover of the epithelium (Barker, et al., 2012). In contrast, there is another population, marked by *Bmi1*, *m-Tert*, *Hopx*, *Lrig1* and *Dclk1*, which is relatively quiescent and appears to be responsible for epithelial regeneration after injury (Carlone and Breault, 2012, Lund, 2012, Potten, et al., 2009, Scoville, et al., 2008).

The use of preparations which maintain ISCs in their niche, such as isolated crypts or organoids (i.e. crypts with adherent myofibroblasts), has long been recognized as having valuable therapeutic applications for situations where a critical mass of the intestine is damaged. Several labs have reported functional engraftment of tissue-engineered small intestine grown from organoid units (Avansino, et al., 2005, Bitar and Raghavan, 2012, Levin and Grikscheit, 2012). Initially, a critical limiting factor in such applications was the inability to expand intestinal organoids *in vitro* (Gupta, et al., 2006). Recently however, culture conditions have been reported which allow net expansion of murine crypts (Fuller, et al., 2012, Sato, et al., 2009), thus bringing therapeutic transplantation significantly closer to reality. To date, however, all studies of ISC/crypt expansion and transplantation have utilized freshly isolated, healthy tissue (Lahar, et al., 2011, Sato, et al., 2011, Yui, et al., 2012). While such an approach is easy in the laboratory, in the clinic that may rarely be the case. Therapeutic sources would typically include tissue collected either postmortem or at surgery. In either case the ability to store tissue prior to crypt or ISC isolation would constitute a significant advantage.

Data from other organ systems suggest that therapeutically useful cells are present postmortem. Hematopoietic stem cells have long been known to survive with delayed bone marrow harvest (Liu, et al., 1978, Mugishima, et al., 1985, Yesus, et al., 1981). More recently, hepatocytes were found viable and capable of engraftment after harvest up to 27 h postmortem (Erker, et al., 2010). On the basis of these findings, we hypothesized that

following storage of intestinal tissue at 4°C, ISCs would remain viable even if other components of the epithelium degenerate. To investigate this hypothesis, viability was assessed by light and electron microscopy, flow cytometry, and crypt culture. The results indicated, that just as in other tissues, stem cells of the small intestine are remarkably resistant to apoptosis and necrosis during storage up to 30 h postmortem.

## Materials and methods

### Animals and Reagents

All animal procedures were approved by the Institutional Animal Care and Use Committee at the respective two institutions. Male, C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Heterozygote breeder pairs of *Lgr5-LacZ* mice (Barker, et al., 2007) were generously provided by Lexicon Pharmaceuticals (The Woodlands, TX). All mice were housed 4–5 per cage on a 12 h light dark cycle in American Association for Accreditation of Laboratory Animal Care-approved facilities and were 6–8 weeks of age at the time of usage. Mice were killed by overdose of isoflurane (until breathing stopped) followed by cervical dislocation, then the jejunum was removed and used as described in Experimental Design below.

Reagents used for histology were: anti-cleaved caspase-3 [Asp 175] (Cell signaling #9661, Danvers, MA), and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside [X-gal] (SIGMA #7240-90-6). Antibodies used for flow cytometric analysis included: Alexa Fluor 647 (Alexa 647) conjugated anti-CD31 (Biolegend #102415, San Diego, CA), Alexa 647 conjugated anti-CD45 (Biolegend #103124), Pacific Blue conjugated anti-CD24 (Biolegend #101820), Fluorescein isothiocyanate (FITC) conjugated bovine anti-rabbit IgG (Santa Cruz Biotechnology #SC-2365, Santa Cruz, CA), and FITC conjugated lectin from *Ulex europaeus* [UEA] (SIGMA #L9006, St. Louis, MO).

### Experimental Design

In the first experiment, viability of the entire jejunal epithelium was assessed following immediate dissociation, as compared with 24 h storage under various conditions: a) storage in phosphate buffered saline (PBS) at 4°C after initial flushing of the intestine; b) storage in PBS at 4°C *without* flushing of the intestinal contents; and c) placing the entire mouse at 4°C. Jejunal epithelial cells were isolated via the EDTA/dispase method as previously described, except that dithiothreitol was omitted (Formeister, et al., 2009). Each epithelial preparation was stained with trypan blue, then trypan-positive (dead) and trypan-negative (alive) cells were counted in a hemocytometer. Viability was expressed as the number of alive cells as a percentage of total (alive plus dead) cells. While this approach gives useful information regarding the overall pattern of epithelial viability, it should be recognized that absolute values for percent viable may be an over estimation if some proportion of the dead cells (i.e. part of the denominator) become degraded to the point of being unrecognizable as cells assessed for overall viability by trypan blue.

For all subsequent experiments, jejunum of C57BL6/J or *Lgr5-LacZ* mice was dissected, flushed with ice cold PBS and split into four aliquots for immediate processing (0 h) and delayed processing (24, 30, and 48 h). For delayed processing, flushed jejunum was kept in a 15mL conical of PBS at 4°C for 24, 30, or 48 h. When splitting the intestine of each mouse jejunum, sections were taken either with diagonal sectioning or small transverse sections, which were systematically split between time points, to prevent regional differences between time points.

## Histology/ Microscopy

Tissue was pinned flat and fixed with 4% paraformaldehyde (PFA) for 3 h at room temperature (RT) or processed for whole-mount  $\beta$ -galactosidase (X-gal) staining. After whole-mount staining and/or fixation, tissue was immersed in 70% ethanol for at least 24 h and then paraffin embedded into a single block per time point. Longitudinal sections of 6  $\mu$ m were utilized for morphology and immunohistochemistry.

Slides were evaluated using an Axio Imager A1 microscope and pictures taken with an AxioCam MRC 5 high-resolution camera (Carl Zeiss, Microimaging, Thornwood, NY). Overall morphology was examined with hematoxylin and eosin (H&E). Apoptosis was assessed with cleaved caspase-3. For caspase-3, heat induced epitope retrieval was performed in a decloaking chamber at 120°C for 30 s followed by 90°C for 10 s (DAKO #S1699, Glostrup, Denmark). Sections were treated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min at RT, then incubated for 1 h in blocking buffer (DAKO X0909, Glostrup, Denmark) or 10% normal goat serum at RT. Subsequently, sections were incubated with primary antibody at 1:800 (cleaved caspase-3) in antibody diluent (Thermo #TA125-ADQ, Waltham, MA) at 4°C overnight. Sections were rinsed in Tris buffer between all applications of antibodies, reagents and endogenous peroxidase blocking step. Secondary antibody, biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch, #111-065-144, West Grove, PA), was applied at 1:500 in antibody diluent (Thermo #TA125-ADQ, Waltham, MA) for 1 h at RT. Vectastain Elite avidin-biotin horseradish peroxidase complex in Tris, was applied at 1:50 (Vector Labs, #PK6100, Burlingame, CA) for 30 min at RT. Quatro chromogen, 3,3'-diaminobenzidine (Thermo #TA125-QHDX, Waltham, MA) was then applied for 1 min. Finally, sections were counterstained with either nuclear fast red, or hematoxylin, dehydrated, cleared in xylene and DPX coverslipped.

### $\beta$ -galactosidase staining

To determine whether the *Lgr5* positive crypt base stem cells remain over time, whole mount staining was performed with intestinal tissue from male heterozygote *Lgr5-LacZ* mice as per published protocol with slight modifications (Takeda, et al., 2011). All segments were pinned flat and flushed with 3mM dithiothreitol to remove mucus. Briefly, jejunum was fixed with glutaraldehyde and PFA, incubated with X-gal solution for 16 h overnight at RT, and then further fixed with 4% PFA. Tissue was then transferred to cassettes in 70% ethanol and embedded in paraffin.

### Electron microscopy (EM)

For assessment of the structural integrity of the intestine at 0 and 24 h time points, 5mm pieces of jejunum were immersion-fixed in 2% PFA/2.5% glutaraldehyde in 0.15M sodium phosphate buffer, pH 7.4, and stored at 4°C for several days before processing. Following several washes in 0.15M sodium phosphate buffer, the samples were post-fixed in 1% osmium tetroxide/1.25% potassium ferrocyanide in 0.15M sodium phosphate buffer, pH 7.4, for 1 h (Russel and Burguet, 1977). Samples were dehydrated in a graded series of ethanols, followed by propylene oxide, and infiltrated and embedded in Polybed 812 resin (Polysciences, Inc., Warrington, PA). 1 $\mu$ m semithin transverse sections were cut with a glass knife, mounted on slides, stained with 1% toluidine blue and viewed using a light microscope to select the region of interest. Ultrathin sections (70nm) were cut using a diamond knife, mounted on 200 mesh copper grids and stained with 4% aqueous uranyl acetate and Reynold's lead citrate (Reynolds, 1963). Samples were observed using a LEO EM910 transmission electron microscope operating at 80kV (Carl Zeiss Microscopy, Peabody, MA) and digital images were acquired using a Gatan Orius SC1000 CCD Digital Camera with Digital Micrograph 3.11.0 (Gatan, Inc., Pleasanton, CA). The integrity of cells

at the crypt base was assessed by a pathologist who was blinded to the experimental conditions.

### Flow Cytometry

Jejunal epithelial cells were isolated as described under Experimental Design and 1 million cells were then incubated in 100  $\mu$ L of PBS for 45 min on ice with either: no antibody, isotype-FITC Bovine IgG (0.5  $\mu$ g); CD31-Alexa674 (0.5  $\mu$ g) and CD24-PB (0.25  $\mu$ g); or CD45-Alexa647 (0.5  $\mu$ g), CD31-Alexa647, CD24-PB, and UEA-FITC (1:250). Samples were then rinsed and resuspended in 500  $\mu$ L of PBS with addition of 1  $\mu$ g of propidium iodide (PI). Cells were analyzed using a Beckman-Coulter (Dako) CyAn ADP. Debris was first excluded based on size via bivariate plot of forward scatter (FSC) versus side scatter. Doublets were excluded using successive gating on both a bivariate plot of pulse-width versus FSC and a bivariate plot of FSC area versus FSC linear. CD45<sup>+</sup> and CD31<sup>+</sup> events, representing leukocytes and endothelial cells respectively (von Furstenberg, et al., 2011), were then excluded based on positivity for Alexa-647. To determine which crypt base cell populations survive over the course of time, the remaining cells were analyzed for PI, CD24 and UEA, to assess viability of ISC and Paneth cell fractions as previously described (von Furstenberg, et al., 2011, Wong, et al., 2012). A separate aliquot was also analyzed for overall viability by trypan blue.

### Tissue Harvest for Intestinal Crypt Culture

Crypts were isolated, cultured, and quantified as previously published. (Fuller, et al., 2012) Proximal jejunum of male C57B16/J mice was removed and processed (0 h) or placed in PBS at 4°C for 24, 30, or 48 h (n= 6, 8, 8, 4 respectively). Tissue was manually shaken for crypt removal after incubation with 3mM EDTA. Pelleted crypts were resuspended in growth factor reduced Matrigel (BD Bioscience) supplemented with EGF (50ng/mL), Noggin (100ng/mL), R-spondin (500ng/mL) and wnt3a (5ng/mL). All concentrations are for total well volume. With the exception of wnt3a, these conditions are as described by Sato *et al.* (Sato, et al., 2009) Crypts in 10 $\mu$ L of Matrigel were then plated in 6 experimental replicates per time point on 48 well plates. Each was then overlaid with Advanced DMEM/F12 (GIBCO, Carlsbad, CA) supplemented with Hepes (10mM), N2 (1:100), B27 (1:50), L-glutamine (1:100), and penicillin/streptomycin (1:100) (Invitrogen). The final number of crypts plated was counted per well and followed over the course of 7 days for enterosphere and enteroid formation. Definition of these structures was as per recent nomenclature recommendations (Stelzner, et al., 2012). Enteroid efficiencies were defined as a percentage of enteroids formed divided by the number of crypts initially plated. Among formed enteroids, lateral crypt buds were counted across a minimum of two wells and ten enteroids per well per mouse.

### Statistics and Quantification

To assess the effect of storage conditions on epithelial viability, 1-way analysis of variance with Bonferroni post-test corrections for multiple comparisons was performed in GraphPad Prism 5 (LaJolla, CA). For analysis of viability over time by cell type (ISC v Paneth) a 2-way analysis of variance was performed in GraphPad Prism 5. To assess the maintenance of culture efficiency and crypt budding over time of tissue storage at 4°C, 1-way analysis of variance with Bonferroni post-test corrections for multiple comparisons was performed in GraphPad Prism 5. P-values less than 0.05 were considered statistically significant.



## Results

### Viable intestinal cells are present at least up to 24 hours post mortem

Based on the report that viable hepatocytes can be harvested from postmortem mice that have been stored as intact animals at 4°C (Erker et al. 2010), we initially assessed whether viable epithelial cells could be isolated from jejunal tissue stored for 24 h at 4°C under three different conditions: a) flushed intestine; b) removal of intestine with no flushing; and c) an entire carcass with intestine left *in situ*. As can be seen in Fig. 1, viability of the entire epithelium (as assessed by trypan blue staining after isolation) remained above 50% after 24 h when the intestine was flushed prior to storage, but decreased significantly when the tissue was left unflushed or *in situ*. Therefore, for all further experiments the jejunum was flushed prior to storage in PBS at 4°C.

### Morphology of the small intestinal crypts remains intact through 30 hours at 4°C

Tissue architecture, was initially assessed by hematoxylin and eosin (H&E) staining performed after 0, 24, 30, and 48 h after storage. The results (Fig. 2 a–e) demonstrate overall maintenance of crypt architecture through 30 h postmortem. Meanwhile, by 30 h the villi begin to slough from their laminal cores and are nearly completely absent by 48 h. At 48 h some tissue sections exhibit conservation of crypts (Fig. 2 d). However, in other areas there are crypts detaching from the submucosa with demonstrable cellular shrinkage (Fig. 2 e).

To further assess the viability of the crypt compartments, we used tissue from *Lgr5-LacZ* mice to simultaneously identify apoptotic cells by staining with antibodies to cleaved caspase-3 and *Lgr5* positive ISCs by LacZ staining (Fig. 2 f–j). Consistent with the H&E staining of wild-type mice, the crypts of *Lgr5-LacZ* mice retain good architecture through 30 h but show signs of degeneration by 48 h. Moreover, through 24 h crypts show no signs of apoptosis, despite extensive activated caspase-3 staining on the villi. By 48 h postmortem, there is near complete loss of the apoptotic villi, and at this time, the crypts begin to display caspase positivity (Fig. 2 i and j). As the ISCs reside within the crypt base and are responsible for maintenance of the small intestinal epithelium, we further characterized our tissue sections for the presence of crypt base columnar ISCs marked by *Lgr5*. *Lgr5-LacZ* expressing stem cells (Barker, et al., 2007) are clearly evident through 30 h but cannot be identified by 48 h delayed isolation (Fig. 2 f–j).

### Small intestinal stem cells are resistant to degeneration

The EM images in Fig. 3 show that at the ultrastructural level the ISCs of the crypt base appear to retain high integrity after 24 h storage of tissue. In contrast, some of the adjacent Paneth cells have become necrotic at this time, although others still appear to be relatively healthy with intact electron dense secretory vesicles.

Based on the report that the CD24<sup>lo</sup>UEA<sup>-</sup> population represents a relatively pure fraction of ISC by flow cytometry (Wong, et al., 2012), we examined viability of that fraction using PI-positivity to identify dead cells. In the same epithelial preparations, the viability of Paneth cells was assessed utilizing the CD24<sup>lo</sup>UEA<sup>+</sup> fraction. As can be seen in Fig. 4a, there was a decline in the percent of viable stem cells and Paneth cells over time postmortem (p<0.001). However, ISCs are significantly more viable (Fig. 4c) than their neighboring Paneth cells (p<0.001) (Fig. 4d). This difference is most pronounced by 30h, (Fig. 4a).

### Small intestinal stem cells retain proliferative capacity after storage at 4°C

As ISCs appeared viable postmortem, we further assessed their functionality via *in vitro* crypt culture. Freshly isolated crypts showed culture efficiencies over 80% and remarkably there was no decline in culture efficiency after a 24 h delay in isolation (Fig. 5). Efficiency

dropped to 36% when crypts were isolated from tissue that had been stored for 30 h and no crypts formed enteroids when storage was extended to 48 h. However, if enteroids form, they retain their proliferative capacity as evidenced by no statistically significant differences in their ability to form buds (Fig. 6a,  $p > 0.3$ ). Multilobulated structures that equally progressed from enterospheres to enteroids were observed in tissue that had been stored for 0, 24, and 30 h. Moreover, the observation of approximately 6 buds per enteroid from tissue that had been stored for 30 h (Fig. 6b) is not only within the range of that observed for fresh tissue in the current study, but is also consistent with the extent of budding reported for crypts isolated from fresh tissue in a previous study (Fuller, et al., 2012).

## Discussion

Current advances in tissue engineering and cellular transplantation models require analysis of potential tissue sources, both autologous and heterologous. Some tissue types have proven viable after delayed isolation and useful for therapy (Erker, et al., 2010, Mugishima, et al., 1985). For the intestine, numerous studies have shown that this tissue is remarkably rejuvenative after various types of *in vivo* damage, due to ISC subpopulations being relatively resistant to cell death (Barker, et al., 2012, Potten and Hendry, 1975, Wright, 2000). However, survival of intestinal tissue postmortem has not been systematically investigated. In this study, we sought to determine if viable and therapeutically useful ISCs were present and functional after delayed isolation.

Our initial experiment explored various storage conditions with the rationale that depending on the nature of the harvest (e.g. heterologous tissue postmortem versus autologous tissue via surgery) there may be limited opportunity to prepare the tissue for storage. In this experiment our assessment was based on viability of the entire epithelium. The data (Fig. 1) made a convincing case, that to ensure reasonable viability after 24 h storage at 4°C, the intestinal tissue needs to not only be removed from the body, but also flushed prior to storage. Whether loss of viability in the tissue stored *in situ* or without flushing could have reflected slower cooling or adverse effects of intestinal contents remains to be investigated. Likewise, the possibility that the ISC fraction may be resistant even under adverse storage conditions remains open to question. Nevertheless on the basis on these initial observations we chose to use flushed tissue for all subsequent experiments.

Our images of tissue architecture after storage for various times revealed dramatic differences between villi and crypts. While the villi showed extensive apoptosis at 24 h and almost complete disintegration by 48 h, the crypts remained essentially intact. Moreover healthy ISCs at the crypt bases were observed both at the light microscopic (by *Lgr5-LacZ*) and the EM level. Similar morphology of apoptotic villus loss with crypt preservation has also been noted in models of intestinal ischemia and reperfusion (Itoh, et al., 2002, Park and Haglund, 1992, Udassin, et al., 1994). Although no studies have looked specifically at the ISCs following ischemia and reperfusion, thymidine incorporation was noted at the base of the crypt in one study at the time of regeneration (Park and Haglund, 1992). Given the common findings of villus degeneration and crypt preservation, the same protective mechanisms may be in place both *ex vivo* following storage at 4°C and *in vivo* following ischemia at body temperature. As many of the pathologic diseases resulting in intestinal failure are secondary to ischemia, our data may suggest that ISCs remain viable in these disorders.

Beyond the morphologic evidence of ISC viability, we found they are capable of supporting crypt growth *in vitro* after delayed isolation up to 30 h. Furthermore, once growth is established *in vitro*, cells maintain their proliferative capacity as evidenced by the lack of significant difference in the number of buds observed in enteroids derived from 30 h tissue

as compared with fresh tissue. Successful expansion of crypts *in vitro* is consistent with our finding that the ISCs are more resistant to degeneration than their neighboring Paneth cells over time as demonstrated via flow cytometry and EM. However, we cannot say if these dying Paneth cells still play a role in protecting their neighboring stem cells as has been demonstrated *in vivo* for live Paneth cells (Parry, et al., 2012). Protection by compromised Paneth cells would be consistent with a study which explored bone marrow transplant of both apoptotic support cells and live hematopoietic stem cells and found improved engraftment and declined rates and severity of graft v host disease (Pessach, et al., 2012). Interestingly, despite lack of nutrients the majority of cell death appears to occur via apoptosis in a controlled fashion.

As viable crypts are present and expandable, they can provide a tissue source for cellular transplantation or tissue engineering small intestine. Furthermore, as culture techniques improve, single ISCs may provide therapeutic benefits. Proof of principle for cellular transplantation was recently reported by Yui, et al. who successfully transplanted colonoids into a mouse model of colitis. The aim of their study was to replace damaged mucosal surface area via colonoid delivery per anus; engraftment of colonoids and restoration of colonic architecture was achieved (Yui, et al., 2012). Although, as noted in a recent commentary (Shaker and Rubin, 2012), much work still needs to be done to prove functionality, this cellular approach may prove beneficial for some patients. But, for other patients a tissue engineering approach to provide additional absorptive surface area may be necessary. Tissue engineered small intestines are now well characterized and have shown abrogation of nutritional deficits in a small animal models and feasibility in a large animal model (Avansino, et al., 2005, Grikscheit, et al., 2004, Sala, et al., 2009). While transplantation may provide one therapy for short bowel syndrome and intestinal failure, the finding of viable ISCs after prolonged storage may also help us understand *in vivo* regeneration of bowel following damage.

An exciting area for future investigation would be to determine which cell is responsible for our observed renewal *in vitro*. Current evidence supports the existence of actively cycling 'workhorse' ISCs and slowly cycling 'quiescent' ISCs (Carlone and Breault, 2012, Li and Clevers, 2010, Potten, et al., 2009, Scoville, et al., 2008). Additionally, under stress these cells may interconvert to repopulate the crypt (Montgomery, et al., 2011, Takeda, et al., 2011, Tian, et al., 2011, Yan, et al., 2012). In other states of damage and adaptation like resection, doxorubicin, radiation, or development a marked expansion is noted within the stem cell compartment (Dehmer, et al., 2011, Dekaney, et al., 2007, Dekaney, et al., 2009, Van Landeghem, et al., 2012). We hypothesize that the same mechanisms may be in play as cells survive delayed isolation and then are able to proliferate *in vitro*. From the irradiation model, an expansion is noted in both the active and quiescent stem pools, but it is unclear which cell population initiates the proliferative response to damage (Van Landeghem, et al., 2012). Likewise, although we detect ISCs marked by *Lgr5* after 30 h delayed isolation, we cannot say definitively that these cells are responsible for initiating growth *in vitro*. More research is needed to define the interrelationships among crypt cells during damage and repair. Nevertheless, without knowing which stem cell compartment is responsible - we do know that isolation in a delayed fashion provides viable ISCs which are capable of growth and proliferation *in vitro*, and may be therapeutically beneficial.

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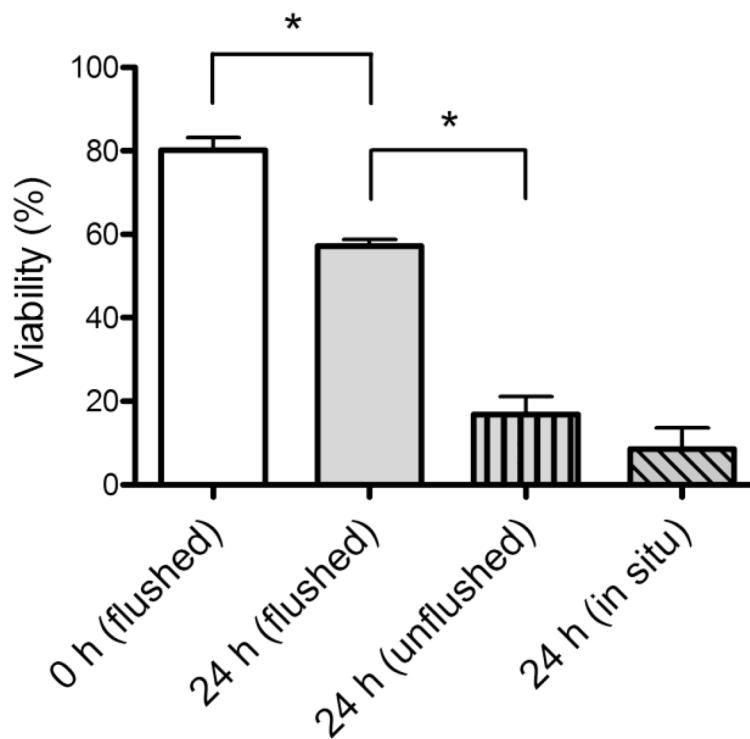
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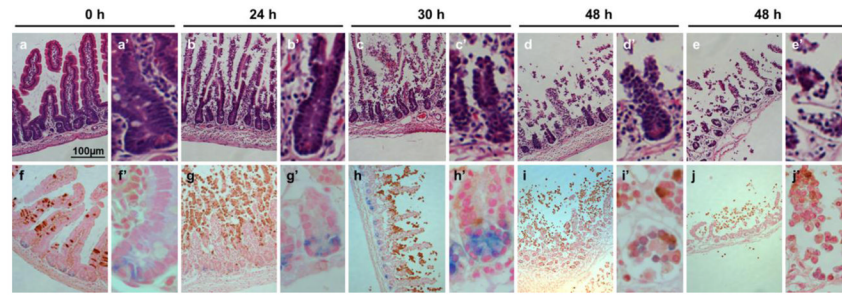
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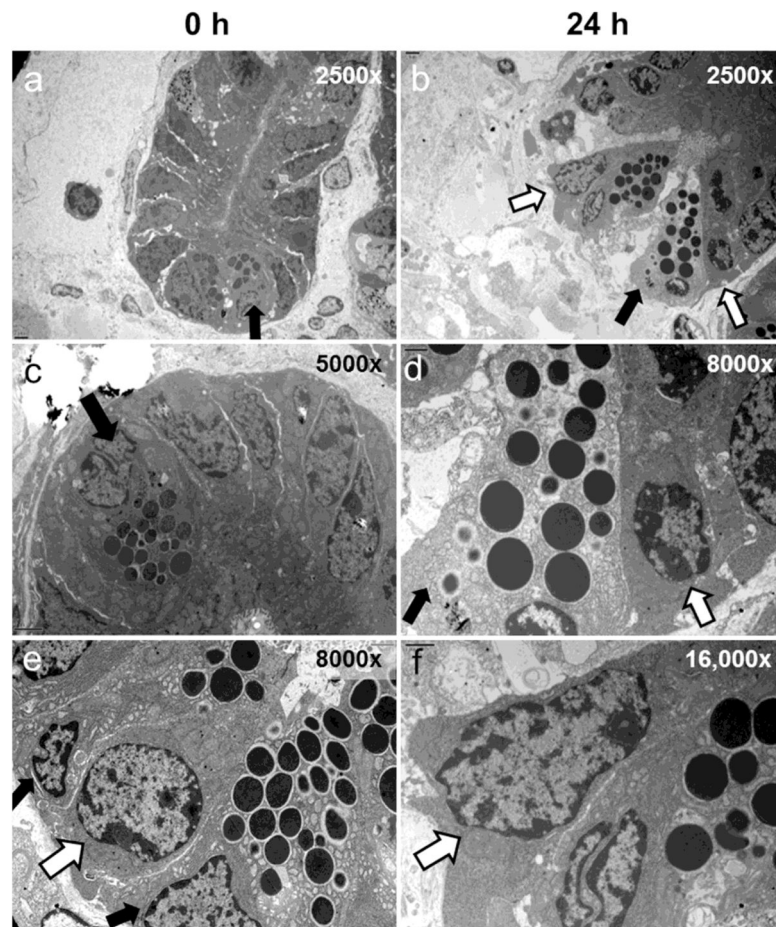
**Fig. 1.** Viability of jejunal epithelial cells following storage of tissue at 4°C. Cells were isolated either immediately (0 h flushed) or after 24 h storage of jejunum either: flushed with PBS (flushed); removed from carcass but not flushed (unflushed); or left within the whole mouse (*in situ*). Viability was measured by trypan blue staining. Data are shown as means  $\pm$  SEM (n=3) \*P < 0.05



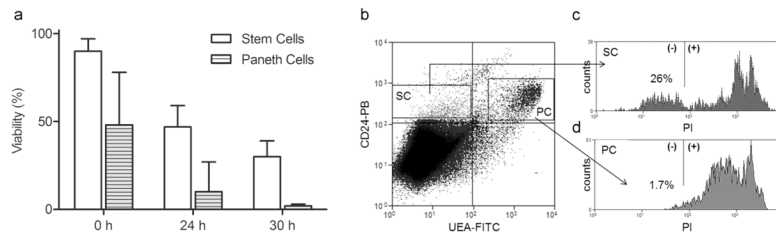
**Fig. 2.**

Architecture of flushed jejunal tissue stored for 4°C for 0, 24, 30, and 48 h. (a–e) H&E for demonstration of morphology. (f–j) cleaved caspase-3 staining (brown) for apoptosis and co-staining for Lgr5-LacZ (blue). Each time point is shown at low power (a–j) and high power (a–j) respectively. For the 48 h time point two different regions are shown to illustrate the variability observed. Because the tissue is very fragile at this stage it was very difficult to get good sections. Nevertheless: panels “d” and “i” show a region where the mucosa is nearly denuded, but the muscle layers have remained intact and negative for cleaved caspase-3; whereas panels “e” and “j” show a region where the muscle layers are also destroyed. Scale bar shown is 100µm (a).



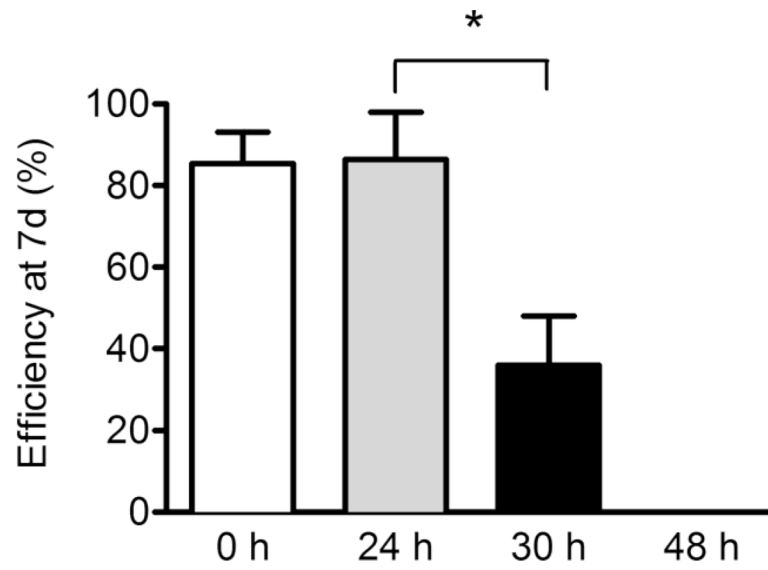


**Fig. 3.** EM images of jejunal tissue stored for 0 or 24 h at 4°C. Each vertical panel shows crypts of increasing magnification: left panel 0 h; right panel 24 h. Open arrows point to crypt base columnar ISCs compared to closed arrows which indicate Paneth cells (with electron dense granules).

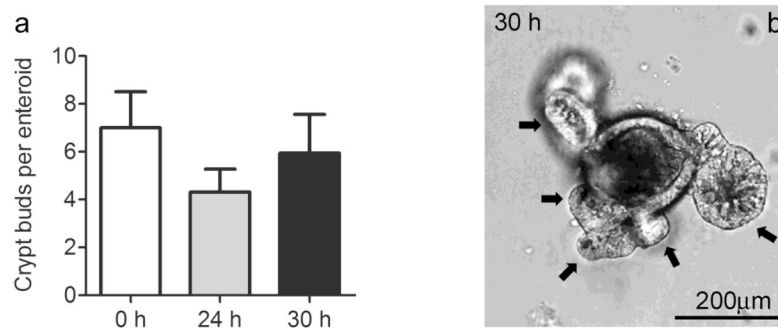


**Fig. 4.**

Viability of ISCs and Paneth cells isolated from jejunal tissue stored at 4°C for 0, 24, and 30 h. **(a)** ISC viability was computed as live (PI<sup>-</sup>) CD24<sup>lo</sup>UEA<sup>-</sup> cells as a % of total CD24<sup>lo</sup>UEA<sup>-</sup> cells and Paneth cell viability as live (PI<sup>-</sup>) CD24<sup>lo</sup>UEA<sup>+</sup> cells as % of total CD24<sup>lo</sup>UEA<sup>+</sup> cells. Data displayed as means ± SEM (n=4). **(b)** Example of gating strategy as illustrated by a 30 h sample, with gates termed (SC) for stem cell and (PC) for Paneth cell. The CD24<sup>lo</sup>UEA<sup>-</sup> (SC) and CD24<sup>lo</sup>UEA<sup>+</sup> (PC) subpopulations are gated forward onto single parameter histograms (panels “c” and “d” respectively) to determine the % of cells negative for PI (live).



**Fig. 5.** Survival and growth of murine crypts isolated from tissue stored at 4°C for 0, 24, 30, and 48 h. Data are shown as enteroid forming efficiency after 7 d of crypt culture, with means  $\pm$  SEM (n= 7, 7, 7, 4) \*P < 0.05.



**Fig. 6.** Proliferative capacity of murine ISC after storage at 4°C as measured by enteroid budding after 7d of crypt culture. **(a)** Composite data for 0, 24, 30 h displayed as means ± SEM (n=6,6,5)  $P > 0.3$ . **(b)** Representative enteroid from 30 h condition with black arrows marking crypt buds, scale bar of 200 μm shown.