AGE-DEPENDENT DEFECT IN SUBACUTE INTESTINAL RESTITUTION ASSOCIATED WITH UNDERDEVELOPED GLIAL NETWORK IS RESCUED BY JUVENILE MUCOSAL HOMOGENATE IN A NEONATAL PIG MODEL OF INTESTINAL ISCHEMIA

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Gastroenterology

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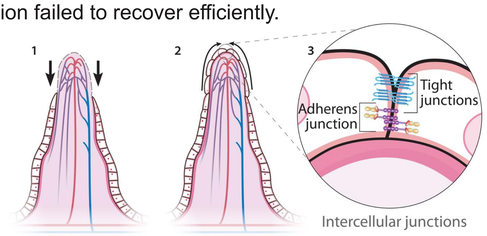
Introduction

- Necrotizing enterocolitis (NEC) is the leading cause of death in neonatal intensive care units. (Neu et al, N Engl J Med, 2011)
- In NEC and intestinal accidents such as volvulus, ischemia damages the intestinal epithelium leading to sepsis and death unless the mucosal barrier is rapidly restored, or injured intestine is resected.
- Using a highly translatable porcine model, we have shown juvenile (6-8-week-old) pigs completely and efficiently restore barrier function through villus contraction, rapid epithelial restitution, and tight junction re-assembly (below).
- In prior studies with younger neonatal (2-week-old) pigs, barrier function failed to recover efficiently.



Hypotheses

1. **There is a defect in mucosal repair in neonates associated with incomplete epithelial restitution.**
2. **This defect is associated with insufficient pro-reparative signals from the subepithelial compartment and exogenous application of injured juvenile mucosal homogenate containing pro-reparative factors will rescue neonatal repair.**

Methods

- Neonatal (2-week-old) and juvenile (6-8-week-old) Yorkshire cross pigs (NC State Swine Educational Unit)
- Laparotomy was performed under general anesthesia
- 8-10 cm loops of jejunum were isolated and 30-minutes ischemia was induced with ligatures (top image at left)
- Mucosa was stripped from serosa and mounted on Ussing chambers for 120-minutes *ex vivo* recovery
- Injured neonatal or juvenile mucosal homogenate was added to the Ringer solution of recovering neonatal tissues (bottom image at left)
- Transepithelial electrical resistance (TEER) and mannitol fluxes were used to measure barrier repair
- Recovered tissues were collected for histomorphometry, immunohistochemisty, immunolabeled 3D imaging of solvent cleared organs (iDisco), electron microscopy, and western blot.

Results

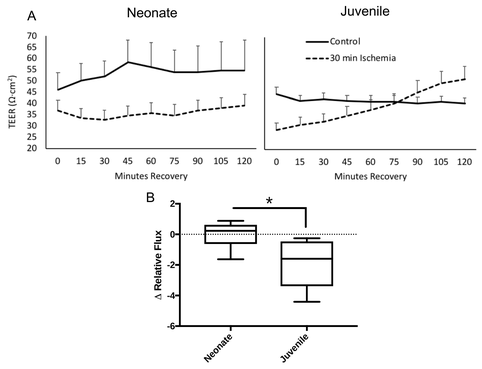


Figure 1. Neonates fail to recover barrier function *ex vivo* following 30-minutes ischemia. (A) Change in TEER over the *ex vivo* recovery period. Note the return of TEER to control levels (dotted line) in injured juvenile mucosa (solid line) within 75 minutes of *ex vivo* recovery, while neonates fail to recover control TEER levels within 120 minutes (n=5-17, significant interaction between recovery and age on two-way repeated measures ANOVA, P<0.001). (B) Change in ^{3}H-Mannitol flux over the *ex vivo* recovery period. Note the trend toward increasing small molecular flux over the recovery period in neonates, while juvenile tissue trends toward decreasing small molecular flux (n=6, Student's t-test, *P<0.01).

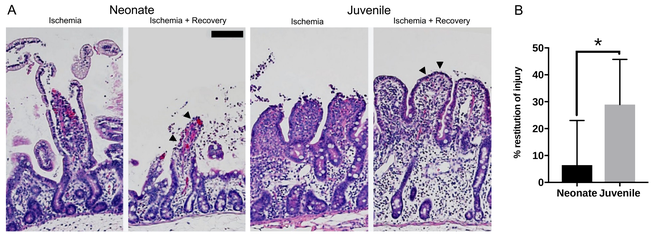


Figure 2. Defect in barrier repair e*x vivo* corresponds to failure of restitution in ischemia-injured neonatal jejunum. (A) Representative histology shows the remaining defect in the mucosal epithelium at the villus tips in neonates after recovery as compared to the restituted epithelium (arrowheads) of the juvenile villi (scale bar 100 μm) (B) Histomorphometry quantified a 30% increase in epithelialization of injured juvenile villi over the *ex vivo* recovery period while neonatal epithelium only restituted 5% of the wounded area (n=6, *P=0.026, student's t-test).

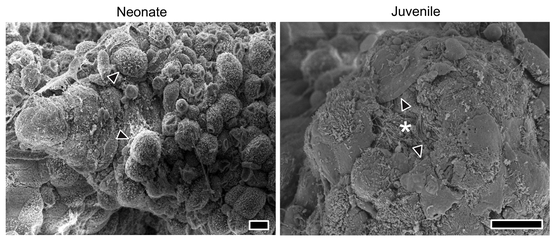


Figure 3. Scanning electron microscopy shows neonatal wound-associated enterocytes fail to assume migratory phenotype seen in juveniles. Note the sphering and persistence of microvilli (arrowheads) in the neonatal wound-adjacent cells (left) versus the smoothened leading edges of the lamellapodia (arrowheads) extending into the remaining defect (white asterisk) in the juveniles (right). (n=3, neonate at 2000x, juvenile at 5000x. Scale bars 10μm.)

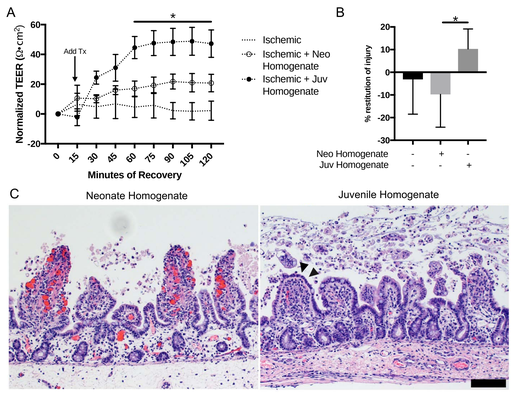


Figure 4. Exogenous application of injured juvenile mucosal homogenate partially rescues barrier repair in injured neonate jejunum. (A) Application of juvenile (juv), but not neonatal (neo), injured mucosal homogenate rescues the TEER of ischemia-injured neonatal jejunum during *ex vivo* recovery. Data presented is normalized relative to each individual's own initial TEER. (n=6, *P<0.0001, two-way ANOVA) (B) Histomorphometry quantified a 10% increase in epithelialization with juvenile homogenate versus a decrease with neonate homogenate or Ringers control (RM one-way ANOVA, P=0.041). (C) Representative histology showing remaining defects in neonatal homogenate-treated tissues as compared to evidence of partially restituted epithelium (arrowheads) in juvenile homogenate-treated tissues (scale bar 100μm)

Discussion & Future Directions

- Neonates have impaired epithelial restitution following 30-minutes ischemic injury in our *ex vivo* recovery model, whereas juveniles repair rapidly.
- Exogenous application of injured juvenile, but not neonatal, mucosal homogenate partially rescues barrier repair in injured neonate jejunum.
- The component of the injured juvenile homogenate which is driving repair is yet to be identified.

- A growing body of evidence indicates that enteric glial cells (EGC) play a pivotal role in promoting intestinal epithelial repair and barrier function through the release of paracrine factors. (Van Landeghem et al, Am J Physiol Gastrointest Liver Physiol, 2011; Savidge et al, Gastroenterology, 2007; Bach-Ngohou et al, J Physiol, 2010; Coquenlorge et al, Sci Rep, 2007; Xiao et al, Mol Neurobiol, 2014)
- EGC form a dense network in the lamina propria in close proximity to intestinal epithelial cells, and this network has been shown to continue development into the postnatal period. (Kabouridis et al 2015, Cossais et al 2016)

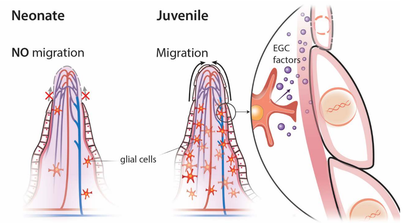


Figure 5. Illustration depicting proposed mechanism of neonatal restitution defect. We propose that neonates have an underdeveloped EGC network which leads to reduced EGC-mediated barrier repair signaling and impaired epithelial restitution, and that this is rescuable with EGC factors.

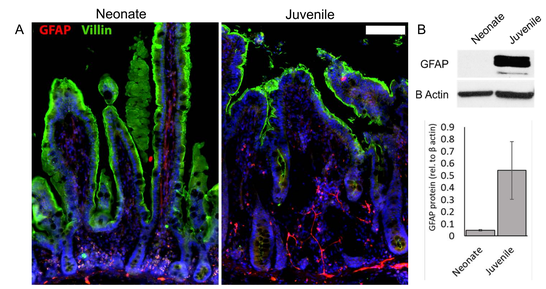


Figure 5. There is lessened expression and distribution of EGC marker GFAP in the mucosa of neonates as compared to juveniles. (A) Immunostaining for GFAP (red) and villin (green), an enterocyte marker, in jejunal mucosa.. Note lower distribution of GFAP-marked cellular processes in the neonatal lamina propria as compared to juvenile (n=3 , scale bar 100μm) (B) Representative western blot of whole mucosal homogenates for GFAP and β Actin loading control and densitometry of GFAP expression relative to β Actin (n=2)

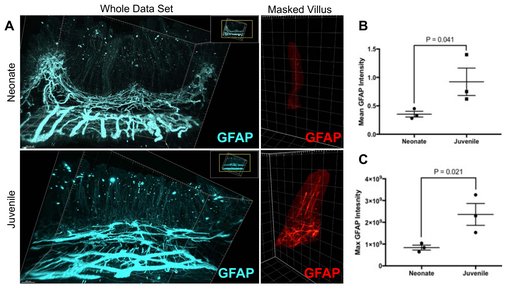


Figure 6. Light sheet imaging of GFAP-marked EGC networks in whole solvent-cleared jejunum revealed lower signal intensity in the lamina propria of neonatal jejunum as compared to juveniles. (A) 2D representations of 3D EGC networks labeled against GFAP in neonatal (top panels) and juvenile (bottom panels) jejunum. Panels in blue represent the entire data set while panels in red represent a single villus masked for intensity quantification. (B) Quantification of mean intensity of GFAP revealed higher levels in juvenile villi as compared to neonatal villi. (C) Quantification of maximum intensity of GFAP revealed higher levels in juvenile villi as compared to neonatal villi. (n=3, P-values derived by student's t-test)

- Our preliminary data shows lower EGC markers in the lamina propria of neonatal jejunum (figures 5,6) supporting the proposed postnatal development of the EGC network, and a smaller and/or less mature EGC population in this age group.
- Ongoing RNA sequencing and mass spectrometry studies will differentiate EGC-mediated restitution pathways in our neonatal and juvenile models.
- Identifying rescuable mechanisms of defects in neonatal mucosal repair could elucidate novel methods to enhance intestinal recovery in affected infants for future clinical use.

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