ABSTRACT

GONZALEZ, LIARA MEG. Large Animal Models of Intestinal Disease. (Under the direction of Dr. Anthony T. Blikslager).

This project was conducted to develop large animal models for the study of intestinal disease, particularly ischemia/reperfusion (I/R) injury, with a focus on the epithelial stem cell zone. I/R is an abdominal emergency with a mortality rate that generally exceeds 50% in both veterinary and human patients. The limited availability of human-derived tissues requires that information gained from animal models be extrapolated to humans. However, findings obtained from the commonly used rodent models have often failed to translate into clinically useful therapeutic advancements. Recent exciting developments in the field of intestinal epithelial stem cell (IESC) biology have presented encouraging findings with regard to the possible utility of IESCs to aid in intestinal repair. However, these studies were performed in rodent models and none have studied the specific impact of I/R. Therefore, this project aimed to study I/R as it occurs in veterinary clinical cases as well as in porcine models of experimentally induced mesenteric ischemia. Chapter III describes a study that evaluated the impact of I/R injury in cases of large colon volvulus (LCV) in horses. The predictive value of a series of histomorphometric parameters on short-term outcome, as well as the impact of colonic resection on horses with LCV was evaluated. Interstitial: crypt ratio (I:C) >1 and hemorrhage score ≥3 were significantly associated with poor outcome in cases of LCV. A digitally quantified hemorrhage area measurement strengthened the association of hemorrhage with non-survival. However, resection did not significantly impact short-term outcome. Chapter IV presents the development of a porcine model for the study of intestinal epithelial regeneration. Techniques for cell lineage identification as well as stem cell culture are described. This work represents the foundation for translational studies of IESC-driven
regeneration of the intestinal epithelium in physiology and disease. In a subsequent project (Chapter V) the equine intestinal epithelium was similarly characterized as this had not been previously described. All lineages of epithelial cells were identified in normal tissues, including stem cells and post-mitotic cell types. Finally, the application of these techniques to clinical cases of LCV in horses is presented in Chapter VI. An early study determined that loss of greater than 50% of the epithelium that lines an intestinal crypt is associated with a poor outcome in cases of LCV. However, at the time of that study, the tools to distinguish unique cell populations did not exist. With the development of the tools described in this project, a correlation between loss of stem and progenitor cells and patient death has been established. There is likely a critical number of IESC that must remain in order for damaged tissue to repair and regain barrier function, which likely translates into patient survival. There was a significant reduction in the number of stem and progenitor cells in the crypts of horses with LCV. Additionally, among LCV cases, there were significantly fewer stem and progenitor cells in horses undergoing resection. Future work aims to evaluate the direct impact of ischemic injury on the IESC populations using the porcine model that was developed in this project. Preliminary results from this study suggest that the reserve or quiescent intestinal epithelial stem cell population is resistant to ischemic injury. Further work will determine the mechanism of resistance; however, we propose that the hypoxia inducible factor pathway mediates this resistance by inhibition of apoptosis.
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Large Animal Models of Intestinal Disease

by
Liara M. Gonzalez

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APPROVED BY:

________________________________  ______________________________
Dr. Anthony T. Blikslager    Dr. Jorge A. Piedrahita
Committee Chair

________________________________  ______________________________
Dr. Scott T. Magness     Dr. Callie A. Fogle
DEDICATION

I dedicate this project to my grandfather, Professor Henry Weiman (October 27, 1919- July 16, 2014), who taught me the value of hard work and dedication to one’s profession. Que en paz descanse.
BIOGRAPHY

Liara M. Gonzalez was born on January 25, 1981, in White Plains, New York. She is the youngest of five children with an oldest sister and three brothers. Liara attended Edwin O. Smith High School in Storrs, CT and graduated in 1998. She received a Bachelor of Science degree in biology from the University of Connecticut in 2002. Liara subsequently attended Cornell University College of Veterinary Medicine graduating in 2006. A one year internship at New England Equine Practice, a tertiary care equine hospital, was then followed by a three year large animal surgery residency at North Carolina State University. After completion of the residency, Liara began her first year in the graduate program in Comparative Biomedical Sciences under the guidance of Dr. Anthony T. Blikslager. That same year she became board certified as a large animal surgeon. Liara looks forward to a long career as a clinician-scientist.
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CHAPTER I

PORCINE MODELS OF DIGESTIVE DISEASE: THE FUTURE OF LARGE ANIMAL TRANSLATIONAL RESEARCH
Abstract

The pig serves as a useful animal model for the study of pathophysiological conditions relevant to the human intestine. The pig has proven its utility for the study of fundamental disease conditions such as ischemia/reperfusion injury, stress-induced intestinal dysfunction, and short bowel syndrome. Pigs have also shown great promise for the study of intestinal barrier function, surgical tissue manipulation and intervention, as well as biomaterial implantation and tissue transplantation. Advantages of pig models highlighted by these studies include the physiological similarity to human intestine and similarities of mechanisms of disease. Emerging future directions for porcine models of human disease include the fields of transgenic pigs and stem cell biology, with exciting implications for regenerative medicine. There is increasing interest in non-rodent models. This review assesses currently used porcine models of gastrointestinal physiology and disease and provides a rationale for the use of these models for future translational studies.
**Introduction**

Digestive disease results in more than 230,000 deaths annually in the United States, with colorectal cancer as the leading cause of mortality in adults (119). Animal models are imperative for translational research targeted at improving human health. Because of the limitations of directly studying human disease in a clinical setting, animal models have been used extensively to expand basic science knowledge. Rodents, particularly mice, have been commonly used animal models of disease because of their relatively low cost, ease of maintenance, and rapid reproduction rate. Furthermore, they are highly amenable to genetic manipulation (32, 62). The utilization of transgenic and knockout mice has provided invaluable insight into the impact of genetic mutations and specific genes on disease etiology and progression (41, 83, 101). However, murine models often lack key clinical signs or pathological changes representative of human gastrointestinal disease which are essential to improve translational studies and drug discovery (Table 1) (5, 6, 8, 11, 20, 21, 43, 45, 62, 118). Therefore, there is renewed interest in large animal models that more closely resemble human disease processes (107, 108) and provide a non-rodent model for drug recovery. Aside from physiological considerations, the larger size of pigs is advantageous for models requiring surgical manipulation, such as Thiry-Vella loops in which an isolated cannulated segment of intestine is studied in vivo (18), or where research involves tissue transplantation (174). Of other large animals used in biomedical research, dogs have been used extensively, particularly for the study of ischemia/reperfusion injury. However, with increasing social
pressures to limit use of drugs as experimental animals and the high mortality rate associated with some disease models, the use of dogs is declining (21, 174).

The pig has a number of distinct advantages that has made this species a useful translational research animal model (Table 1). In particular, there are important anatomical and physiological similarities to human beings (42, 118). The pig has a comparably sized genome with extensive homology to humans. The pig genome has a 60% sequence homology to humans as compared to rodents with only 40% homology (68, 158). Additionally, the pig chromosomal structure is more similar to humans than mouse, rat, dog, cat or horse (91, 102). Pigs, like humans, are omnivores and share similar metabolic and intestinal physiological processes (36, 74, 118). For example, a comparison of the recommended daily allowances of vitamins and minerals in the human diet and the daily nutrient requirement of pigs reveal striking similarities between the two species in infancy, growth, reproduction, and lactation (92, 118). This likely contributes to their comparable mucosal barrier physiology and enteric microbiota, as well as susceptibility to select enteric pathogens (112, 118). The role of the intestinal microbiota in maintaining intestinal health has been highlighted in recent years, and disturbances in microbial composition have been associated with important human diseases such as diarrhea, neonatal necrotizing enterocolitis and obesity. Conversely, studies focusing on the relationship between the composition of the gut microbiota and disease have shown widely diverging results when comparing mice to humans (112, 147, 163). Similarities in the intestinal microbial ecology between pigs and humans have made the pig a useful non-primate animal model for studies of dietary modulation of microbiota (65).
Specifically, both human and porcine gut microbiota consist mainly of Firmicutes and Bacteroidetes phyla and both share similarities in gastrointestinal microbial diversity (65, 80, 87). Additionally, pharmaceutical bioavailability and nutrient digestibility in pigs closely resemble that of people (15, 42, 118). These characteristics have led to the use of pigs for the development of pig models of a number of gastrointestinal diseases including necrotizing enterocolitis (8, 139, 141, 148, 160, 165), acute mesenteric ischemia (21, 26, 134, 146, 157), short bowel syndrome (15, 105, 114, 120-122), AIDS-associated Cryptosporidium infection (6, 47, 50), stress-induced intestinal dysfunction (94, 97, 98, 150), cystic fibrosis (132, 170) and familial adenomatous polyposis (45). This review will highlight strengths and limitations of pig models of intestinal ischemia/reperfusion injury, stress-induced intestinal dysfunction and short bowel syndrome. In addition, we have reviewed information that will extend the discussion on animal models in the fields of transplantation, bioengineering, and transgenics.

**Comparative gastrointestinal anatomy: Similarities and differences between humans and pigs**

Pigs have significant anatomical and physiological similarity with human beings, with some key comparisons noted in Table 2 and Figure 1. The structure of the small intestine is very similar in humans and pigs, including macroscopic features such as the ratio of intestinal length per kilogram bodyweight (Table 2) (118). Other gross similarities include the presence of sacculations and taenia (bands of longitudinal muscle) extending along the majority of the colonic length in both human and porcine colons (74, 153). These anatomical similarities contribute to the comparable transit time and analogous digestive and absorptive processes
reported for these species (54). Shared microscopic features also exist, including the structure of the villi and the types of cells that constitute the intestinal epithelium (Figure 1). For example, the villous projections within the small intestine of both species are finger shaped, whereas in rats they are flat leaf-like structures (74). Additionally, the cell lineages that constitute the intestinal epithelium, their phenotypic appearance, and their expression of unique protein biomarkers used for cellular identification are similar in pigs and people (49). These include the ultrastructural appearance of stem, goblet, and enteroendocrine cells as well as absorptive enterocytes (49). A comparative analysis of sub-cellular features of porcine intestinal epithelial cells located within the crypt base, using transmission electron microscopy, revealed multiple irregularly shaped, small, columnar cells with basally located nuclei and scarce cytoplasm (49). This morphologic appearance is consistent with the description of crypt base columnar stem cells in humans (9, 49). Taken together, these features contribute to fundamental digestive processes such as mucosal transport and motility that are similar between pigs and humans (118). Additionally, pigs, like humans, are true omnivores, whereas other potential mammalian models such as dogs, cats, ruminants, rabbits and rodents have evolutionarily developed alternative digestive strategies (74, 155). Furthermore, pigs and humans have similar neonatal gut development and gastrointestinal immunologic responses to insult (37, 74, 155). As far as differences in gastrointestinal tract anatomy, the porcine cecum is relatively large and clearly delineated as compared to the human cecum, and the porcine colon is orientated in a spiral fashion (Figure 1) (74). Additionally, the pig lacks an appendix (74, 155). Despite these differences, both pigs and
humans are colon fermenters, unlike rodents that are cecal fermenters, and have similar colonic microbiota composition (92, 112).

On a cellular level, there is controversy within the literature of the presence or absence of the Paneth cell within the pig (103, 104, 161); a fully differentiated cell type that secretes antibacterial substances and has a proposed role in supporting the adjacent crypt base columnar cells within the small intestinal crypt base of other species, including humans (44, 143, 144). Based on recent work, a cell sharing ultrastructural qualities attributed to the Paneth cell, including large size, elongated flattened nucleus and located adjacent to the crypt base columnar cells has been identified within porcine small intestine (49). However, these cells lack electron dense apically situated granules and antibody cross reactivity to lysozyme, a well-accepted and commonly used biomarker for Paneth cells (49). However, these differences appear to have relatively little effect on overall physiological function, which is critical when selecting an animal model for human digestive disease. In addition, there are important similarities between other cell populations thought to be critical to maintenance of the stem cell niche. For example, antibodies raised against human-derived proteins utilized as specific biomarkers for the identification of stem, progenitor, absorptive enterocyte, enteroendocrine and goblet cells cross react with porcine intestinal tissue, and demonstrate the same expression pattern observed in humans (49). These include antibodies against both SOX9 and HOPX that identify stem and progenitor cells. It should be noted, that antibody based tests are limited by available functional antibodies and none have specifically identified the crypt based columnar population. However, it has been shown that pigs do
express crypt base columnar cell gene biomarkers (49). Work is needed to further develop the pig as a model to study intestinal stem cells however, considering what is established, the pig serves as a valuable model for studies of human intestinal injury, repair and efficacy of novel therapeutics (Table 3).

**Stress-induced intestinal dysfunction**

Stress plays a central role in the onset and exacerbation of clinical symptoms in several gastrointestinal diseases of humans. Progress in the field of stress-related gastrointestinal disease has been hampered by the lack of relevant animal models of stress that recapitulate the chronic and complex biology of stress and the clinically relevant outcomes (e.g., diarrhea and weight loss) that are observed in people. Much of the work in this field has utilized rodent models of short term/acute stress (53, 142). These models have yielded important insight into the mechanisms by which stress initiates gastrointestinal disease; however, there are obvious developmental and biological differences between rodents and humans that limit their translational value. Porcine models are ideal for studying the early life origins of gastrointestinal disease because: (1) compared with rodents, the porcine gastrointestinal tract is most similar to humans with regard to development, anatomy, and function (156); (2) pigs possess a highly developed central and peripheral nervous system in order to perceive and integrate complex stress signaling processes in the brain and peripheral nervous systems (25); and (3) pigs, similar to humans, exhibit pathophysiological hallmarks of disease (intestinal inflammation) and relevant clinical signs (e.g. diarrhea and reduced weight gain) when subjected to stress.
Altered function of the enteric nervous system (ENS) plays a central, pathophysiological role in stress-related gastrointestinal diseases as it modulates stress-induced changes in motility, inflammation, intestinal permeability, and visceral hypersensitivity; therefore, the ENS is currently, and will continue to be, a therapeutic target. The porcine ENS has been shown to have similarities to human ENS that may offer advantages over commonly used rodent models (25). For example, intrinsic primary afferents in the human and porcine enteric nervous systems differ significant from rodents and other small laboratory animals in their distribution electrophysiological behavior, and synaptic properties (24, 25). These interspecies differences are important when considering animal models of human disease because it is imperative that therapeutic treatments for stress associated intestinal disorders such as IBS are based on results from animals with extensive similarity in their gastrointestinal tracts (25).

A novel porcine model of stress-induced gastrointestinal dysfunction that exhibits remarkable similarity to the pathophysiology of stress-related gastrointestinal disease in humans is the porcine early weaning stress model. In the porcine early weaning stress model, pigs are weaned from the sow at an early age (prior to 21 days of age), as compared to the natural weaning process which occurs gradually over months. The early weaning model has been valuable in studying the mechanisms of early life stress-induced gastrointestinal disease (94, 97, 98, 150). Early weaning stress in pigs involves an initial early life stress event followed by consistent, heterotypic stressors associated with changes in management and social conditions until they have reached market weight. This may model aspects of
heterotypic chronic stress in humans. Early weaning in pigs induces a stress response characterized by elevated serum and intestinal corticotropin releasing factor that has been shown to play a critical role in intestinal permeability and diarrhea (94, 98, 111, 150).

Ischemia/ reperfusion injury

Intestinal ischemic injury occurs when the blood supply to a segment of intestine is compromised. Paradoxically, tissue damage may continue when blood flow is re-established and this is referred to as reperfusion injury (Figure 2). Injury attributed to ischemia/reperfusion injury is associated with many disease states including: intestinal volvulus (128), acute mesenteric ischemia (175), intestinal transplantation (85) and systemic disease that lowers tissue perfusion such as hemorrhagic shock or cardiopulmonary disease (90, 162). In general, an excess of 50% of patients suffering from intestinal ischemia/reperfusion succumb to sepsis associated with a breakdown in intestinal barrier function (129). Mortality depends on the duration, location, and length of bowel affected by impaired perfusion. The type and degree of injury to the intestinal mucosa resulting from a pathologic decrease in intestinal blood flow is influenced by the microvascular architecture within the villi. Experimental models of ischemia have shown that the morphological signs of injury begin at the villus tip and progress toward the base of the villus (20). The villus circulation comprises blood entering the villus via a centrally-located arteriole, which arborizes at the tip of the villus and converges into venules located beside the arteriole and on the periphery of the villus (28). This sets up a counter-current exchange whereby oxygen diffuses from the arterioles toward low oxygen tension venous blood flowing in the opposite
direction within the venules. Diffusion is facilitated by the relatively low rate of blood flow within villi. This in turn makes the tip of the villous relatively hypoxic even under normal conditions, which is exacerbated when blood flow is reduced or obstructed. However, the degree to which ischemia promotes mucosal injury is dependent upon the anatomy of the villous microvasculature, which affects the efficiency of counter-current exchange. Importantly, this microvascular architecture varies between species. The pig and human villus microvascular architecture are very similar, arborizing at the tip of the villous into a fountain-like pattern and converging into one or two venules located beside the arteriole (10, 28). In contrast, in the rat, the flattened leaf-shaped villi are supplied by a single arteriole that passes unbranched from the base to the tip of the villus where it bifurcates into a capillary network, a “netted bag” pattern (31). The arterioles begin to converge and form two venules as the vasculature approaches the base of the villus (28, 31). In the mouse, two arterioles supply oxygenated blood to the villus, and then divide into a capillary network that rejoins to form a single venule at the tip of the villus. The venule then travels down the center toward the base of the villus (29). These differences are thought to impact the physiological function of the intestinal mucosa between species and potentially their susceptibility or response to injury. To this end, multiple in vivo and in vitro intestinal permeability studies have demonstrated significant differences between man and rodents and greater correlation between humans and pigs (14, 39, 109). In particular, Bijlsma et. al. hypothesize that the differences in permeability between rodents and humans is due to the interspecies variation in villus blood vessel architecture resulting in varying countercurrent exchange efficiency
that directly impacts villus epithelial cell function (14). The specific microscopic
architectural features of pigs and humans that are shared including villus structure and
vascular anatomy likely contribute to interspecies similarities in ischemic damage (14, 21).
Additionally, since many forms of intestinal ischemic injury involve decreased tissue
perfusion and the villi are the first and most dramatically damaged in ischemic injury, the pig
may more accurately model this disease process in humans (20, 164).

Disease entities causing intestinal ischemia include volvulus, acute and chronic
mesenteric ischemia, cardiopulmonary disease, and hemorrhagic shock. In addition, ischemia
is thought to contribute to necrotizing enterocolitis (NEC), the most common life-threatening
gastrointestinal emergency in neonatal patients (58, 106, 110). Since the degree of damage
varies according to the type of ischemic injury, it is not possible to fully understand all of the
features of ischemia/ reperfusion injury with a single model. In light of this, porcine studies
elucidating mechanisms of ischemia/ reperfusion injury relevant to human disease have
assessed mesenteric ischemia, volvulus, and hemorrhagic shock to understand the relative
contribution of ischemia and reperfusion to mucosal injury (3, 18, 21, 26, 70, 76, 134, 146,
157, 174). Mesenteric ischemia was found to cause the most severe time-dependent injury,
which was marginally exacerbated by reperfusion, and only at select time points (2-hours of
ischemia, 1-hour reperfusion) (20). Alternatively, low-flow ischemia induced by hemorrhagic
shock in pigs caused minimal ischemic injury, with no evidence of reperfusion injury (20).
This is in marked contrast to rodent and feline studies exploring a range of low-flow states,
all of which induced minimal ischemic injury, but were accompanied by marked reperfusion injury (23, 56, 63, 117, 151).

A great deal of the research on ischemia/ reperfusion injury has focused on the reperfusion phase, with the hope that basic findings would translate to the clinical ability of blocking injury by treating prior to reperfusion. Mechanisms of reperfusion injury have largely been documented in rodent and feline models, with the seminal discovery of xanthine oxidase as a central enzyme in the development of reactive oxygen metabolites (20, 56, 57, 59, 116, 151). Studies in these laboratory animals have shown that small intestinal ischemia is followed by a robust reperfusion injury as a result of mucosal xanthine oxidase-induced oxidant release. However, there is doubt as to the clinical relevance of these findings in humans, which lack mucosal xanthine oxidase at birth, and intestinal enzyme expression remains relatively low throughout development to adulthood (20, 35). This pattern of xanthine oxidase expression is very similar in pigs, whereas rodents maintain levels 4-5 fold higher (20). Therefore information gleaned from rodent and feline models that attribute reperfusion injury to activation of xanthine oxidase not be readily extrapolated to humans or may be relevant only to particular types of injury (41, 56, 82, 117, 154, 178). Another critical element shown to induce and perpetuate reperfusion injury is the neutrophil. Neutrophils are activated by chemoattractants induced by reactive oxygen metabolites, after which they greatly amplify reperfusion injury (48). The key neutrophil population involved in reperfusion injury in rodents is the resident mucosal population, as opposed to neutrophils recruited from the circulation (79). However, pigs have a relatively small population of
mucosal neutrophils, as do people, in contrast to the large population of resident mucosal neutrophils in rodents and cats (20). Collectively, these findings may explain the relative lack of reperfusion injury in the pig under a variety of ischemic conditions, including mesenteric ischemia, volvulus, and shock. These considerations are relevant to effective translation of basic science research to the treatment of human ischemia/ reperfusion injury (39, 40, 84).

The physical size of the pig as a model of human disease also enhances the feasibility of creating surgical models that recreate clinically relevant disease, including acute mesenteric ischemia (AMI) (3, 21, 157). Approximating the human size is also crucial in the development and testing of imaging modalities for early diagnosis of AMI (2, 175). This is likely why research focused on improving early diagnosis of AMI commonly utilizes porcine models to evaluate the efficacy of imaging modalities such as radiography, angiography, computed tomography and magnetic resonance imaging (26, 76, 113, 134, 146).

**Mucosal repair**

Progress has been very slow to translate basic science findings on reperfusion injury to clinical trials (40, 84). More information on mechanisms of mucosal repair is critical. Three important stages of intestinal epithelial repair have been identified (Figure 2). First, the immediate post injury or acute stage of repair is when barrier function is re-established by villous contraction and cellular restitution. The process of epithelial restitution has been re-examined in porcine models of ischemic injury, and found to include a novel component that had received little attention in cell models: tight junction repair (17, 19, 51). Specifically, initial studies on post-ischemic epithelial repair in pigs revealed a lack of barrier function
recovery until tight junctions within restituting epithelium had closed (19). Thus, the term restitution now integrates the concepts of villus contraction, epithelial crawling, and tight junction re-organization. This proliferation independent process ensures that the basement membrane is covered, epithelial continuity is established and inter-cellular tight junctions are re-assembled (17, 115). During the subsequent or sub-acute period, cellular proliferation, differentiation and the onset of inflammation predominate (18). Finally, during the chronic stage of mucosal repair the normal mucosal architecture is re-established, the small intestinal villi for example, in addition to varying degrees of fibrosis that depend on the initial degree of injury and inflammation (127). Each of these processes has been dissected in depth, particularly the latter which continues to be a strong thrust of porcine studies (89, 93, 96). In terms of translation of such studies, the potential for pharmacologically hastening tight junction repair has been demonstrated in porcine post-ischemic bowel and awaits human trials (95).

Evaluation of mucosal repair following ischemic injury in pigs has also revealed a different time course of neutrophil infiltration as compared to murine and feline studies. In particular, ischemia in rodents and cats showed peak infiltration of neutrophils within 1-hour of reperfusion injury (55, 59, 66), whereas in porcine studies, peak neutrophil infiltration occurred 6-hours following ischemic injury (48). The importance of this finding was that rather than exacerbating injury, this delayed neutrophil infiltration may hamper restitution, particularly tight junction repair, as neutrophils coursed through the paracellular spaces of restituting epithelium. Of translational significance, inhibiting neutrophil adhesion, or
treating with superoxide dismutase could restore optimal repair (48). Porcine studies have also shown that neutrophils induce physical damage to the epithelial tight junctions as they transmigrate across ischemic-injured mucosa (48). Other porcine studies have focused on understanding mechanisms of tight junction repair following ischemic injury, including clinically relevant pharmacological interventions (4, 18, 95, 130).

**Necrotizing enterocolitis**

NEC is an abdominal emergency that afflicts approximately 10% of infants born with low birth weight, and has a mortality rate of approximately 30% (8, 58). Severe, irreversible damage to the intestine often necessitates extensive surgical resection with resultant short bowel syndrome (SBS). This syndrome is associated with debilitating malnutrition, diarrhea, abdominal pain and fatigue (52, 58). The piglet is the only animal model that develops NEC under the same conditions that predispose human infants, namely prematurity, bacterial colonization and enteral feeding (8, 34, 139). Pigs also demonstrate pathognomonic signs of human disease including abdominal distension, food intolerance and regurgitation as well as mucosal necrosis in the distal small intestine and colon and pneumatosis intestinalis (65, 86, 138, 139). One of the most important features of NEC shared by neonates and piglet models is that disease is only seen in premature individuals (141). Importantly, ‘total parenteral nutrition’ (TPN), which is associated with onset of NEC in preterm infants, can be administered to piglets. Piglets not only share predisposing factors and clinical signs of NEC but also the histologic indicators of disease. This has led to extensive work utilizing the pre-term piglet model for treatment and drug discovery to improve the care of affected infants.
For example, the severity and degree of mucosal atrophy were reduced in a piglet model when probiotics consisting of *Bifidobacterium animalis* and four *Lactobacillus* species were administered post parturition (148). Notably, the positive findings in porcine studies have been applied to human subjects and have confirmed the decreased incidence of NEC resulting from probiotic therapy (65, 67, 88).

**Short bowel syndrome**

Although the preterm piglet is an ideal model of SBS in NEC patients, variations between infant, juvenile and adult growth rates as well as developmental physiology preclude extrapolation of knowledge based on neonatal models of SBS to older patients. Here again, pigs provide a translational advantage because developmental and age-related changes in the gut are similar between pigs and humans (15, 46, 99, 177). Short bowel syndrome as a result of extensive resection of ischemic-injured or Crohn’s disease-affected bowel is the most common cause of intestinal failure in adults (52). Following extensive intestinal resection, the mucosal surface area is decreased, impairing nutrient and water absorption. This may result in diarrhea, fluid and electrolyte abnormalities, and malabsorption and weight loss (52). To offset SBS, the intestinal remnant undergoes an adaptive response that includes villus hyperplasia, increased crypt depth, and increased brush border enzyme activity that results in increased mucosal surface area with improved fluid and nutrient absorption.

Although, morphological and functional changes characteristic of intestinal adaptation have been reported in rodents, the pig provides a more suitable model to study specific nutritional requirements critical to the treatment of SBS patients (12, 13, 15, 36, 92, 100, 105, 114, 159,
Total parenteral nutrition is currently the treatment of choice in humans and as was previously noted, can be administered to pigs (52). However, treatment with TPN is fraught with complications in SBS patients. These include venous thrombosis, infection and organ failure as well as sleep disruption, anxiety and depression (52). These clinical obstacles have driven extensive research into ways of minimizing TPN use by identifying effective mitogenic agents that hasten intestinal adaptation and improve the absorptive capacity of the remaining intestine. Murine models have successfully demonstrated the mitogenic effects of Glucagon-like peptide 2 (GLP-2) therapy; however, the FDA required the use of non-rodent models for pharmaceutical testing prior to human clinical trials. Porcine studies effectively demonstrated the rapid intestinal growth and maturation, increased intestinal brush border enzyme expression, decreased apoptosis and proteolysis, increased nutrient absorption and intestinal blood flow following treatment with GLP-2 (27, 60, 61, 123, 124, 140, 167). GLP-2 has now emerged as a potential therapeutic option for some patients and is currently used in humans (71-73). Further work has focused on the appropriate time frame for administration of therapies aimed at enhancing the adaptive response.\(^{(122)}\) For example, a porcine model was utilized to specifically identify changes in the absolute numbers of epithelial cells within distinct mucosal populations and the time course of those changes in remnant bowel following extensive resection to give insight into potential targeted therapies (122). That study found that despite an early increase in proliferative and enterocyte cell number, following bowel resection, the cells were immature and incapable of normal absorptive function. It was therefore concluded that therapeutic interventions should be aimed at
increasing cellular differentiation into mature cell types, not just general cellular proliferation to hasten return of normal absorptive function (122).

**Transplantation studies**

Ultimately, when extensive and irreversible injury to the intestine occurs and when TPN therapy fails to effectively maintain SBS patients, intestinal transplantation is indicated (52). Unfortunately, there is an unacceptably high mortality following intestinal transplantation. For example, death occurs in 40% of patients within five years of receiving an allograft (1). Acute rejection, chronic allograft dysfunction and ischemia/reperfusion injury within the transplanted bowel are the most severe and common complications associated with bowel transplantation (85, 174). The limited options for replacing resected intestine make allotransplantation, xenotransplantation and tissue engineering research integral to improving available therapies for SBS patients. The pig represents an ideal model for each of these types of studies because of their shared size and specific physiologic, immunologic and organ developmental similarities with humans that translate into answering questions of practicality and generalized efficacy (69, 75, 135, 169, 174). For instance, the current reference standard for detection of acute cellular rejection of allografts is a histological grading system (136) similar to the grading system used to identify allograft rejection in pigs used in transplantation research (174). The grading scale ranges from mild rejection, as determined by the presence of greater than 6 apoptotic cells out of 10 crypts and a mild to moderate inflammatory infiltrate of predominantly mononuclear cells, to severe rejection characterized by crypt destruction, mucosal erosions and severe inflammatory infiltration (174).
Although rodent models of allotransplantation have contributed to improvements within the field, the increased gross size of the pig permits investigation of alternative surgical therapies that mimic those possible in people, combined with low mortality in experimental animals as compared to rodents (7, 78, 174). Examples of porcine models of transplantations include: orthotopic (graft in gastrointestinal continuity), heterotopic (graft not in gastrointestinal continuity), segmental (Thiry-Vella loop), whole small bowel or combined small bowel with colon (169, 174). Therefore, porcine experimental work directly facilitates the development of procedures for use in clinical patients (78, 169, 174).

Despite improvements in the success of alloengraftment surgery in recent years, particularly in specialized centers, recipients must cope with lifelong immunosuppressive therapies and the co-morbidities and mortality associated with this therapy (52, 75). The limitation of rodent models for these translational studies is associated with the major immunological differences that exist between species. In recent years, significant advances have been made in the development of ‘humanized’ mice to study human biological processes. However, these developments do not preclude the need for a large, more biologically complex system, to verify findings (145). Although there are immunological differences between humans and pigs, specific similarities and recent advances in the development of genetically modified animals has made the pig the most likely potential source for xenografts for humans (42, 69, 137). The demand for human organ donations significantly overwhelms the supply with over 100,000 recipient candidates awaiting organ transplants in the U.S. (171). The first transgenic pig was generated in 1985 and the first
swine model derived specifically for the betterment of human health was in the field of xenotransplantation (64, 171). Pigs are considered the preferred donor species because their organ size is compatible with humans and because using non-human primate species poses difficult ethical concerns and infectious diseases (69, 77, 126). The development of genetically modified pigs expressing human complement pathway regulatory proteins (hCPRPs) or that have the α1,3-galactosyltransferase gene “knocked-out” (GalT-KO), has overcome the initial barriers posed by acute graft rejection (77, 81, 126). With these genetic modifications to inhibit recipient immunologic responses, porcine to non-human primate heart and kidney transplants have been successful (168).

Bioengineering offers an alternative source of tissue for intestinal replacement and avoids the complications associated with ischemia/ reperfusion injury of the donor tissue and life-long immunosuppressive therapy for the transplant recipient. Creating bioengineered tissues is an exciting prospect for regenerative medicine in the future, but advancements have been hindered by the anatomic and physiologic complexity of the intestine. Compared to other areas of intestinal research, studies on intestinal tissue engineering to replace segments of native intestine are limited (16, 137, 152). A porcine model was successfully used to generate tissue-engineered small intestine from autologous tissue, thereby re-creating conditions required for successful clinical use (137). Organoid units (isolated intestinal crypt cultures with epithelial stem cells and intact surrounding mesenchymal elements) were derived from resected small intestine, placed on biodegradable polyglycolic acid tubes, and implanted into the omentum or mesentery of the autologous animal. Intestinal tissue
composed of all anatomic layers, including mucosa adjacent to an innervated muscularis, was shown to have developed (137). The far-reaching therapeutic potential of this research could serve as the basis for tissue replacement for a multitude of diseases. Ultimately, large animals and particularly the pig are biologically complex systems that serve a critical role in addressing the safety of a therapeutic regimen and verifying mechanistic findings from rodent models.

**Transgenic pigs**

The impetus for advancement in the field of transgenic pig development has been due to limitations in rodent models when their small size, short life span or inadequate representation of a disease phenotype prevents successful translational research (5). The majority of transgenic pig work has been in the field of xenotransplantation, as previously discussed. However, in recent years there have been significant improvements in the technology used to create genetically modified pigs to model human diseases (5, 43, 171). Comprehensive tables listing the available genetically modified porcine models for use in biomedicine and of human disease can be found in Whyte et. al. and Fan et. al. (43, 171).

One of the best examples with regard to intestinal disease is the development of the porcine model of cystic fibrosis (CF) (170). Mouse models lack typical disease manifestations, including the intestinal obstruction phenotype that is observed in human patients (168, 171). Transgenically modified $CFTR^{-/-}$ and $CFTR^{AF508}$ pigs have proven superior by displaying the same clinical signs as seen in people, including intestinal, bile duct, and pancreatic duct
obstructions, as well as liver lesions, low levels of IGF-1 and the hallmark phenotype of lung
disease (132, 168).

Previously, CF was the only intestinal disease with a representative porcine model
(133, 170). However, gene-targeted pigs with mutations in the *adenomatous polyposis coli*
(*APC*) gene have recently been established (45). Colorectal cancer is one of the most
frequent causes of cancer worldwide and is the leading cause of gastrointestinal related
mortality (119, 131). For this reason, much emphasis has been placed on the study of familial
adenomatous polyposis (FAP) in humans. Murine *Apc* mutants have been used in this
research but have significant limitations including differing location and histologic
appearance of lesions as compared to humans (22, 45). In particular, in humans, colonic
adenomas develop during childhood, whereas gastric polyps and duodenal adenomas occur
during adulthood (45). In contrast, murine models develop polyps in the duodenum and small
bowel, and these polyps lack dysplastic cells in the superficial mucosa (45). The porcine
model of FAP, on the other hand, shares the anatomical location and histologic appearance of
human disease (45). With regard to the diagnosis and treatment of FAP, the porcine model
illustrates the advantage of large animals to facilitate advances in pharmaceutical,
endoscopic, and surgical interventions. The completed pig genome project will provide the
platform for further transgenic work to create pertinent pig models of human disease,
particularly where rodent models fail to recapitulate clinical signs of disease (168).
**Future promise for translational research**

The need for large animal models to improve translational research is well accepted (107, 108). The limited availability of human-derived tissues requires that information gained from animal models is extrapolated to humans. The fact that mice and other rodents are the most commonly used preclinical models has been implicated in the “pipeline problem” (108, 125). One of the reasons for this problem is that rodent injury models fail to recapitulate human disease. For instance, murine injury models used in the study of intestinal epithelial stem cells (IESCs) have utilized chemical or mechanically induced mucosal injury or whole body irradiation to create damage (7, 30, 38, 166, 176). None of these are common sources of mucosal injury in human clinical cases. Therefore, a great deal of additional research is needed before stem cell therapy can become a clinical reality. For example, a study examining the engraftment potential of IESCs following mucosal injury in Thiry-Vella loops was hampered by high mortality rates and the authors indicated the need for a large animal model in which Thiry-Vella loops are well tolerated (7). Until recently, a large animal model to study intestinal stem cell biology remained elusive. Currently, the cross reactivity of commercially available antibodies to identify specific cell types in pig intestinal tissue has been demonstrated (45, 47, 49, 122, 137, 172). Validation of tools for histologic, protein and mRNA based analysis of porcine IESCs have also been completed (49). Additionally, this study describes a method for successful long-term culture of porcine crypts into fully differentiated enteroids (isolated intestinal crypt cultures with epithelial stem cells without intact surrounding mesenchymal elements). Collectively, the work provides a platform for
translational work, including for stem cell engraftment studies that are currently limited by the small size and high mortality rates of rodents (7). The pig as a model to study IESCs in disease affords the opportunity to bridge the gap between lab bench discovery and bedside application.
<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Porcine</td>
<td>• Models exhibit pathophysiology and clinical signs of disease</td>
<td>• Cost</td>
</tr>
<tr>
<td></td>
<td>• Approximate human size</td>
<td>• More difficult to manage</td>
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<tr>
<td></td>
<td>• Xenotransplantation potential</td>
<td>• Special housing facilities</td>
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<td></td>
<td>• TPN administration possible</td>
<td>• Few transgenic models available</td>
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<tr>
<td></td>
<td>• Share nutritional requirements</td>
<td>• Time consuming</td>
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<tr>
<td></td>
<td>• Fulfill FDA requirement for pharmaceutical testing</td>
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<tr>
<td></td>
<td>• Comparable microbiome</td>
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<tr>
<td></td>
<td>• Possess highly developed central and peripheral nervous system</td>
<td></td>
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<tr>
<td></td>
<td>• Genome is only ~7% smaller than humans</td>
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<tr>
<td>Rodent</td>
<td>• Low cost</td>
<td>• Pathophysiological differences</td>
</tr>
<tr>
<td></td>
<td>• Ease of maintenance</td>
<td>• Absence of clinical signs of human disease in some models</td>
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<tr>
<td></td>
<td>• Ease of genetic manipulation</td>
<td>• Technically difficult to create surgery models due to size</td>
</tr>
<tr>
<td></td>
<td>• Rapid reproduction rate</td>
<td>• Mortality rate</td>
</tr>
<tr>
<td></td>
<td>• Number of available and well established models</td>
<td>• Genome is ~14% smaller than humans</td>
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## Table 2. Anatomical Comparison between Pigs and Humans (Adapted from Patterson et al. (118))

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average birth weight (kg)</td>
<td>3.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Average mature weight (kg)</td>
<td>60-100</td>
<td>200-300</td>
</tr>
<tr>
<td>Water composition at birth (%)</td>
<td>82</td>
<td>82</td>
</tr>
<tr>
<td>Water composition as adult (%)</td>
<td>69</td>
<td>72</td>
</tr>
<tr>
<td>Average lifespan (years)</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>Small Intestinal length as adult (m)</td>
<td>5.5-7</td>
<td>15-22</td>
</tr>
<tr>
<td>Large Intestinal length as adult (m)</td>
<td>1.5</td>
<td>4-6</td>
</tr>
<tr>
<td>Length of intestine per kilogram bodyweight (ratio)</td>
<td>~0.1</td>
<td>~0.1</td>
</tr>
<tr>
<td>Small Intestinal weight as adult (g)</td>
<td>1040</td>
<td>2310</td>
</tr>
<tr>
<td>Large Intestinal weight as adult (g)</td>
<td>590</td>
<td>1970</td>
</tr>
</tbody>
</table>
Table 3. Direct Clinical Application of Porcine Models

<table>
<thead>
<tr>
<th>Clinical Impact</th>
<th>Research Support Using Porcine Models</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Understanding role of early life stress on onset and exacerbation of clinical symptoms of human gastrointestinal diseases</td>
<td>Early life stress induces a marked elevation of serum and intestinal corticotropin releasing factor (98) that plays a critical role in intestinal permeability and diarrhea. Administration of CRF receptor antagonists and mast cell stabilizers to pigs prevented stress-induced increases in intestinal permeability (94, 98, 111, 150).</td>
</tr>
<tr>
<td>• Treatment of reperfusion Injury</td>
<td>Activation of xanthine oxidase plays less important role than initially postulated in inciting injury. Injury is mediated by neutrophil release of oxygen metabolites and transmigration through epithelial tight junctions creating physical damage (48).</td>
</tr>
<tr>
<td>• Treatment of NEC patients</td>
<td>Oral probiotics reduce the incidence and severity of NEC in very low birth weight infants (88).</td>
</tr>
<tr>
<td>• Dietary considerations following massive bowel resection</td>
<td>GLP-2 hastens intestinal adaptation; FDA drug approval (15, 71-73, 105, 114).</td>
</tr>
<tr>
<td>• Successful intestinal transplantation</td>
<td>Simultaneous liver transplant does not decrease the risk of bowel graft rejection and is no longer recommended in patients with SBS with normal liver function (175).</td>
</tr>
<tr>
<td>• Cause and treatment of gastrointestinal dysfunction associated with cystic fibrosis</td>
<td>Pig model of CF accurately displays clinical manifestations of human disease permitting study of pathogenesis of intestinal obstruction (5, 43, 154, 172).</td>
</tr>
<tr>
<td>• Diagnosis of Familial Adenomatous Polyposis</td>
<td>Porcine transgenic model accurately represents disease manifestation, including location and histologic appearance of lesions (45).</td>
</tr>
</tbody>
</table>
Figure Legend

**Figure 1.** Comparative Gastrointestinal Anatomy

The porcine gastrointestinal anatomy (top) shares many similarities and some distinct differences to that of humans (bottom). The porcine intestinal length is greater than humans but the ratio of total length per kilogram of bodyweight for both pigs and humans is similar. The comparable portions of porcine and human colon are demonstrated with color. Images of histologic sections of porcine and human duodenum and colon are included for comparison.
Figure 2. Ischemia-Reperfusion Injury and Recovery.
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CHAPTER II

ANIMAL MODELS OF ISCHEMIA-REPERFUSION INDUCED INJURY:
PROGRESS AND PROMISE FOR TRANSLATIONAL RESEARCH
Abstract

Research in the field of ischemia-reperfusion injury continues to be plagued by the inability to translate research findings to clinically useful therapies. This may in part relate to the complexity of disease processes that result in intestinal ischemia, but may also result from inappropriate research model selection. Research animal models have been integral to the study of ischemia-reperfusion induced intestinal injury. However, the clinical conditions that compromise intestinal blood flow in clinical patients ranges widely from primary intestinal disease to processes secondary to distant organ failure and generalized systemic disease. Thus, models that closely resemble human pathology in clinical conditions as disparate as volvulus, shock, and necrotizing enterocolitis are likely to give the greatest opportunity to understand mechanisms of ischemia that may ultimately translate to patient care. Furthermore, conditions that result in varying levels of ischemia may be further complicated by the reperfusion of blood to tissues that, in some cases, further exacerbates injury. This review assesses animal models of ischemia-reperfusion injury as well as the knowledge that has been derived from each in order to aid selection of appropriate research models. In addition, a discussion of the future of intestinal ischemia-reperfusion research is provided to place some context on the areas likely to provide the greatest benefit from continued research of ischemia-reperfusion injury.

Introduction

The survival rate for human and veterinary patients with ischemic intestinal injury is generally less than 50% (16, 68, 169, 180) because tissue hypoxia, inflammation and cell
infiltration result in loss of the mucosal barrier. This barrier is primarily composed of a single layer of columnar epithelial cells bound together by interepithelial junctions, which collectively prevents the translocation of bacteria and associated toxins into the systemic circulation (137). Intestinal ischemia is associated with a broad range of clinical conditions including neonatal necrotizing enterocolitis (123), acute mesenteric ischemia (AMI) (180), volvulus (152), trauma (40), cardiopulmonary disease (148), hemorrhagic shock (40, 52) and intestinal transplant rejection (110) (Figure 1). Ischemic injury may be coupled with subsequent reperfusion of tissue that is known to exacerbate injury in some of these disease processes (110). Since intestinal ischemia is rarely preventable, most research in the field has focused on advancing techniques for early detection of ischemia and the development of novel therapeutic approaches that target the post-ischemic insult (the reperfusion period). Injury attributed to reperfusion is thought to be primarily attributable to reactive oxygen metabolites, associated with activation of oxidant-producing mucosal enzymes, release of lipid chemoattractants from injured cellular membranes, and subsequent infiltration of neutrophils (Figure 2). Therefore, proposed therapeutic interventions to protect against reperfusion injury have targeted inhibition of oxidant injury and of neutrophil activation (141, 154). However, due to the relative failure of clinical therapy directed at reperfusion injury following early studies, the importance of targeting reperfusion injury in cases of intestinal ischemic injury has been brought into question (103). The most recent clinical perspectives on the efficacy of these treatments are derived from studies of tissues more commonly affected by reperfusion, such as the myocardium following infarction (7). These
too have failed to demonstrate the clinical utility of these therapies. Encouragingly, recent studies have recognized the modulation of cell death pathways as a novel target for therapeutic intervention (85, 178). Mechanisms of cell death mediated by necrosis and apoptosis have been shown to further exacerbate injury induced by ischemia and reperfusion. In fact, insufficient clearance of these dying cells has been shown to lead to increased inflammation and impaired tissue repair (178). As a result, there has been a shift of emphasis toward modulation of pathways of cell death (85, 178) and regenerative processes (111, 158). However, initial consideration of the animal model to select for study is of paramount importance, given the highly variable clinical presentation of diseases that involve ischemia. Animal models have been indispensable to the study of mechanisms of ischemia and reperfusion injury (21, 38, 132-135), but each model has distinct advantages and disadvantages so that none of these models can perfectly recapitulate the natural onset and progression of human disease. The aim of this review is to provide an in depth assessment of key animal models used for the study of ischemia-reperfusion injury, with the goal of allowing investigators to understand and select from available models for translational research. In addition, the future of intestinal regenerative medicine in light of recent advances will be discussed.

**Mechanisms of Ischemia**

Ischemia is the reduction or complete occlusion of blood flow to a target organ that results in a state of tissue oxygen deprivation. In the intestine, overt obstruction of the blood supply can result from mesenteric vascular occlusion derived from thrombus formation or emboli
secondary to cardiopulmonary disease (140, 163). Compromise of normal blood flow to the intestine can also be associated with severe intestinal distension and mechanical obstruction as occurs in cases of intestinal strangulation associated with hernia, volvulus and intussusception (1, 180). Additionally, ischemic injury is thought to contribute to the pathogenesis of neonatal necrotizing enterocolitis (NEC), the most common life-threatening gastrointestinal emergency in neonatal patients (68, 123, 125). Other major sources of intestinal ischemic injury result from diseases that reduce systemic blood flow such as cardiopulmonary diseases and shock states (52, 148). Finally, the interruption of blood flow is an important component of the process of intestinal transplantation that inevitably results in a period of ischemia (104, 110) (Figure 1).

Decreases in blood flow compromise the oxygen supply required for normal cellular function and results in both cellular damage and death. The intestinal epithelium has a particularly high energy demand, largely as a result of basolateral Na⁺-K⁺ATPase used to drive secretion and absorption, and is therefore particularly sensitive to reductions in blood flow. The process of epithelial cell loss that results from ischemic injury is well documented in many research animal species (38, 133, 135, 144). Cells closest to the intestinal lumen on the villus tips in the small intestine and inter-crypt surface epithelium in the colon initially lose their attachment to the basement membrane with progressive loss of cells extending toward the crypt base as the duration of ischemia increases (Figure 3) (38, 133). Interestingly, the cells closest to the luminal surface normally function in a state of ‘physiologic hypoxia’ due to an oxygen gradient that is derived from the mucosal vascular
architecture (143, 168). Although there are species differences in the anatomy of the mucosal vasculature (Figure 4), a general construct of counter current exchange exists whereby oxygen diffuses from the arterial blood supply that ascends the mucosa toward the lumen and venous drainage flowing in the opposite direction, progressively decreasing the arterial concentration of oxygen as the vessels near the luminal surface (3, 9). This creates a steep oxygen gradient that makes the villus tips hypoxic (32, 156). A similarly decreasing gradient of oxygen from the crypt base to the luminal surface exists within the colon (168). Therefore, further decreases in oxygen concentrations, as occurs with intestinal ischemia, impact the cells at the villus tips and colonic surface epithelium first. This initially creates a distinct lesion identified histopathologically, called Gruenhagen’s space (Figure 3) (15, 38). Originally, this space was solely attributed to the accumulation of cytoplasmic fluid from ischemically damaged cells (38). More recent studies using in vitro techniques and a novel in vivo human study have shown that contraction of the myofibroblasts within the lamina propria also contribute to the creation of this initial defect (45, 138). In fact, the creation of the space was shown to occur within 30 minutes of complete ischemia and reseal by 60 minutes if the blood supply was subsequently returned (45). This demonstrates the remarkable capacity of the intestine to repair rapidly in cases where vascular compromise can be reversed. However, in most cases the duration of ischemia is likely to be lengthier and the tight junctions that serve to anchor the cells together break down, resulting in cell loss into the intestinal lumen. Epithelial cell separation from the basement membrane and complete cell loss then continue and progress toward the crypt base in a time-dependent manner (38).
It has been specifically demonstrated in a porcine model of jejunal mesenteric vascular occlusion that by 60 minutes, epithelium is lost from the upper third of the villus and by 120 minutes there is near complete loss of villus epithelium (21). Any exposure of the basement membrane causes marked compromise of barrier function, allowing luminal bacteria and toxins to gain access to the underlying vasculature within the lamina propria (74, 109, 137). The sequelae are septicemia and multiple organ dysfunction, which are responsible for the high morbidity and mortality rates associated with severe intestinal ischemic injury (84, 140, 163, 180).

Models of Ischemia

Complete Vascular Occlusion

Complete ischemia by temporary vascular occlusion (using atraumatic vascular clamps) or permanent vascular occlusion (using ligation) of the superior mesenteric artery (SMA – technically the cranial mesenteric artery in animals) in rodent models is currently the most commonly used method of inducing intestinal ischemic injury. The SMA is approached through a midline incision and occluded for varying lengths of time (85). In most of these studies, heparin is injected intravenously to prevent thrombus formation within the SMA which enables the circulation to be re-established once the atraumatic vascular clamps are removed for measures of reperfusion injury. However, an early study by Megison et. al. noted a highly variable degree of injury and often high mortality of rats in studies in which occlusion of the SMA was performed (114). That study noted that SMA occlusion alone was
unreliable in creating consistent and reproducible injury, whereas SMA occlusion combined with ligation of collateral arcades created a greater degree of injury and a higher but consistent level of mortality in the animals. These findings lent support to a similar study performed in cats in which SMA occlusion reduced blood flow to the mucosa an average of 35% in the proximal duodenum, 61% in the distal duodenum, 71% in the jejunum and ileum, and 63% in the proximal colon. No reduction of blood flow was identified in the mid and distal colon (139). Thus, the effect of SMA occlusion is highly dependent on the intestinal segment being evaluated. These findings may be complicated further by the fact that different segments of the intestine may be more or less sensitive to ischemic injury. For example, differences in resistance to ischemic injury have been identified between the jejunum, ileum and large colon with studies indicating a progressive increase in resistance to ischemic injury in aboral segments (36, 105, 144). Furthermore, a porcine study of small bowel transplantation demonstrated microscopic injury in the ileum only after 5 hours of ischemic injury compared to 1.5 hours in the jejunum (34). Little reperfusion injury was noted in either segment. It should be noted, however, that this study only evaluated injury histologically. Unfortunately, many studies of ischemic injury either assess injury in a single segment of intestine, many times the ileum, or do not specify which segment is being evaluated (Table 1). Additionally, few studies have focused on the colon despite the fact that colonic ischemia is a more common clinical entity in people than small intestinal ischemia (1, 50, 72, 120). The colon does, in fact, respond differently to insult. For example, a study compared regional
differences in susceptibility to injury between the small intestine and colon and demonstrated that the colon was more resistant to ischemia in rats (105).

A more recent technique used to achieve complete irreversible ischemia was established in a porcine model by embolization of the SMA. The SMA was accessed either percutaneously (94) or via endovascular catheterization (2, 24, 145) and a solution of either butyl-2-cyanoacrylate (94) or polyvinyl alcohol particles and gel foam (2, 24, 145) were injected intravascularly. These models are particularly useful for imaging studies aimed at improving diagnosis in patients suspected of having AMI. Using this approach, the abdominal contents are not exposed or manipulated in creating the vascular obstruction. Since bowel manipulation alone creates inflammation that otherwise would not be present in clinical cases of AMI, minimally invasive approaches to the SMA may be optimal for studies modeling AMI.

The critical assessment of the type of ischemia model with regard to studies of AMI cannot be overstated. Acute mesenteric ischemia is a complex of diseases that can be classified based on whether the mechanism of obstruction is occlusive or nonocclusive. Occlusive intestinal ischemia is further subdivided into acute or chronic and whether arteries or veins are affected. Nonocclusive mesenteric ischemia is a disease process that is more poorly understood but occurs with patent mesenteric arteries and is associated with disease processes such as congestive heart failure, aortic insufficiency, renal or hepatic disease and patients following cardiac surgery (169). Therefore, other approaches to create ischemic injury such as the low-flow model likely better recapitulate this form of injury.
**Low-Flow Ischemia**

Most of the original low-flow ischemia studies were performed in cats (Table 1). This model likely best recreates the type of intestinal injury that would be expected following hemorrhagic shock or other events that dramatically decreases the volume of blood delivered to an intestinal segment. In the low-flow ischemia model, blood flow is reduced to 20% of baseline levels (~25-35 mmHg). Using this method, the abdomen is approached through a midline incision and a segment of ileum is typically isolated. An arterial circuit is then established between the superior mesenteric and femoral arteries and pressure cannulas are used within the vessels in order to measure vascular pressure. Reduced blood pressure within the superior mesenteric artery (25-30 mm Hg) is achieved using an adjustable vascular clamp. The majority of injury associated with this model has been attributed to reperfusion as a result of increases in xanthine oxidase and neutrophil activation, making the ischemic event more of a priming mechanism for subsequent injury. This may or may not adequately represent clinical scenarios, in which injury caused by ischemia may be so severe that reperfusion injury is of limited significance. Again, careful selection of models depending upon the human condition being studied is critical.

**Segmental Mesenteric Vascular Occlusion**

An additional model of ischemia is segmental mesenteric vascular occlusion, in which the blood flow within a discrete loop of intestine is interrupted by clamping the local mesenteric vascular supply and cross-clamping the bowel. Within a single animal, multiple loops can be
subjected to varying lengths of ischemia with or without reperfusion, thereby titrating the
degree of injury within each loop. This model can be readily performed in large animals,
such as the pig, and rodents (72, 73), and has the advantage of providing multiple treatment
groups and controls within a single animal. However, this model of strangulating obstruction
relies on complete occlusion of the arterial and venous blood supply, whereas clinically the
venous circulation is commonly compromised prior to the arterial blood supply due to
differences in vessel wall thickness and compliance (133). This results in continued arterial
supply of oxygenated blood for a variable period of time, until the tissue becomes so
congested in the absence of venous drainage that the arterial vascular supply ultimately
collapses. This complex form of ischemic injury includes elements of ischemia as well as
excessive interstitial pressure, which together likely affect the level of epithelial sloughing.

Perhaps the most interesting and significant application of the mesenteric vascular occlusion
model of intestinal ischemia has been its application in human subjects (45, 46). In these
studies, a normal segment of jejunum from patients undergoing Roux-en-Y or similar
intestinal reconstruction were isolated and subjected to 30-45 minutes of mesenteric vascular
occlusion, with varying lengths of reperfusion, prior to removal from the patient (45, 46, 74,
113). The limitation of these studies is that severe ischemic damage as applies to most
patients suffering from ischemia-reperfusion injury cannot be induced in these clinical
studies.

With regard to each of these models of ischemia, the research animal species used
may contribute significantly to the outcome. As previously noted, variations of the mucosal
vascular supply, particularly in the small intestine, exist between species (Figure 4). The pig
and human villus microvascular architecture are very similar, arborizing at the tip of the
villus into a fountain-like pattern and converging into one or two venules located beside the
centrally located arteriole (9, 32). In contrast, in the rat, the flattened leaf-shaped villi are
supplied by a single arteriole that passes unbranched from the base to the tip of the villus
where it bifurcates into a capillary network, a so-called “netted bag” pattern (37). The
arterioles begin to converge and form two venules as the vasculature approaches the base of
the villus (32, 37). In the mouse, two arterioles supply oxygenated blood to the villus, and
then divide into a capillary network that rejoins to form a single venule at the tip of the villus.
The venule then travels down the center toward the base of the villus (33). These differences
may impact the physiological function of the intestinal mucosa between species and
potentially their susceptibility or response to injury. Multiple in vivo and in vitro intestinal
permeability studies have demonstrated significant differences between man and rodents and
greater correlation between humans and pigs (14, 44, 124). In particular, Bijlsma et. al.
hypothesize that the differences in permeability between rodents and humans is due to the
interspecies variation in villus blood vessel architecture resulting in varying countercurrent
exchange efficiency that directly impacts villus epithelial cell function (14).

**Mechanisms of Reperfusion Injury**

The concept of progressive injury that occurs following the restoration of normal vascular
circulation to an ischemically compromised region seems counter-intuitive. After all, re-
establishing blood flow is ultimately critical to rescue and maintain cell function. However,
ischemia creates an environment primed for the production of injurious metabolites, the influx of inflammatory cells and epithelial cell apoptosis and necrosis (Figure 2). Early studies of reperfusion in cats and rodents attributed the exacerbation of injury during this period to the production of reactive oxygen metabolites that directly contribute to injury as well generation of neutrophils chemoattractants (25, 47, 64, 65, 67, 69, 76, 77, 80, 96, 97, 99, 126-128, 134-136, 150, 161). During ischemic conditions, as tissues continue to utilize adenosine triphosphate as a source of energy, the metabolic byproduct, hypoxanthine, accumulates. In addition, in this oxygen deprived environment, xanthine dehydrogenase which would normally function to metabolize hypoxanthine is converted to xanthine oxidase by locally produced tissue proteases (128). When tissue perfusion is re-established, xanthine oxidase utilizes the now freely available and abundant oxygen as an electron acceptor as it metabolizes hypoxanthine, producing superoxide (Figure 2) (65-67, 69). The direct injury induced by oxygen free radicals combined with their role in the activation and chemoattraction of neutrophils creates the severe tissue injury attributed to reperfusion.

Neutrophils inflict tissue injury by a multitude of proposed mechanisms that include occlusion and increased permeability of the microvascularature (43, 81), release of reactive oxygen metabolites (47, 128, 162), cytotoxic enzyme release (183) and mechanical injury induced by migration (57). The vast majority of the studies used to determine these findings used a feline low-flow ischemia model. Upon activation, neutrophils release the contents of their granules that include potent proteases such as elastase, myeloperoxidase, protease-3 and metalloproteinases as well as reactive oxygen species including H₂O₂ and hypochlorous acid
(106, 150, 183). These compounds are bactericidal and aid in neutrophil migration as connective tissues are lysed, but concomitantly cause extensive collateral damage to surrounding matrix proteins and nearby cells. Additional injury is created by the mechanical damage induced by their migration across the epithelial monolayer which has been shown to increase paracellular permeability (57). The detrimental role of neutrophil accumulation on epithelial barrier function post ischemia was supported by findings in a porcine model of complete mesenteric vascular occlusion (57) in which pre-treatment of animals with anti CD11/CD18 monoclonal antibody, targeting neutrophil adhesion, significantly reduced neutrophil infiltration and improved measures of epithelial barrier function (57).

**Model Dependent Reperfusion Injury**

The significance of reperfusion to intestinal injury following an ischemic event has been and continues to be controversial. This is partly due to the fact that the degree of reperfusion injury appears to depend on the type of ischemia (complete versus incomplete vascular occlusion) and duration of ischemic injury as well as the research animal species and segment of affected intestine.

*Complete Vascular Occlusion*

Currently, the complete vascular occlusion method for induction of ischemic injury in rodents is the most commonly used approach for the study of ischemia-reperfusion injury (Table 3 and 4). Rodents are advantageous to use because of their relatively low cost, ease of maintenance, and rapid reproduction rate. In recent ischemia-reperfusion research, mouse
models have been used to determine genes associated with oxidative stress (12) as well as to provide evidence of increased epithelial proliferation and barrier function in response to endogenous factors such as keratinocyte growth factor administration (29). Furthermore, mice are highly amenable to genetic manipulation. Attenuation of reperfusion injury in mice genetically modified to overexpress superoxide dismutase has provided further proof of the deleterious role of reactive oxygen metabolites in reperfusion (47). Additionally, other transgenic mouse models have been used to demonstrate the protective role of mu opioid receptor signaling (60), and the detrimental effects of cytokines such as IL-17A (102) in ischemia-reperfusion injury. A large number of studies have also utilized rat models. Aside from transgenic murine models, the role of a host of factors has been successfully investigated in rodents, highlighting the beneficial effects of epidermal growth factor (11, 58), heparin-binding epidermal growth factor (35, 49, 112), anti-apoptotic signaling (84) and xanthine oxidase blockade (154) to prevent injury or improve healing. Other studies using rats have shown that atenolol (30), a beta-blocker and L-arginine (61), a substrate of nitric oxide biosynthesis both attenuate enteric nervous system dysfunction that occurs as a result of ischemia-reperfusion injury. However, multiple studies have demonstrated that the degree of injury attributed to reperfusion following mesenteric vascular occlusion is variable. The degree of reperfusion injury appears to depend on the duration of preceding ischemia and the segment of intestine (105, 133). Evidence of reperfusion injury has been shown to occur following intervals between 30 to 60 minutes of ischemia but not when the duration of ischemia is shorter or longer (105, 133). Additionally, the colon appears to be more resistant
to reperfusion injury compared to the small intestine and susceptibility to injury along the length of the small intestine is variable with the ileum appearing to be more resistant to reperfusion injury than the jejunum. The reason for these differences remains poorly understood (34, 36, 105), although the lack of oxidant-producing enzymes in the colon may be one reason that injury differs from the small intestine (75, 118, 119).

Low-Flow Ischemia

As noted above, in models utilizing the low-flow method of ischemia, the majority of tissue damage occurs during reperfusion. In vivo feline studies have shown a direct correlation between elevated xanthine oxidase levels and tissue damage as well as microvascular injury (64, 67, 161, 181). However, important differences exist between species that influence their susceptibility to reperfusion injury. The robust reperfusion injury identified in cats and rodents as a result of mucosal xanthine oxidase-induced oxidant release is not found in other species (21). In fact, humans and pigs lack mucosal xanthine oxidase at birth, and levels remain low even into adulthood making the clinical relevance of the feline and rodent studies questionable (13). Furthermore, no definitive clinical utility has been identified for xanthine oxidase inhibitors in patients with intestinal ischemia-reperfusion injury (103, 130). Perhaps with the development of the novel human model of ischemia-reperfusion injury (46) the controversy regarding the contribution of xanthine oxidase to reperfusion injury will be resolved.
Another important species difference is the role of neutrophils in the induction and perpetuation of reperfusion injury. The resident mucosal population of neutrophils, as opposed to neutrophils recruited from circulation during onset of injury, appears to play a significant role in reperfusion injury in rodents and cats (47, 99, 128, 150). In a low-flow cat model, acute versus chronic pre-treatment with a CD18-specific monoclonal antibody enabled the investigators to assess the role of resident mucosal neutrophils, with chronic administration of the antibody depleting mucosal neutrophil reserves. These studies indicated that it was the resident neutrophils, rather than those infiltrating from the microvasculature, that were responsible for the majority of the reperfusion injury documented (99, 128, 150). However, humans have a relatively small population of resident neutrophils (21), a finding that was also found to be the case in horses. Interestingly, equine ischemia studies have shown very limited evidence of reperfusion injury (20, 118). Together, differences in oxidant enzyme expression and neutrophil populations likely affect the reliability of basic studies to translate findings to the treatment of humans with ischemia-reperfusion injury.

*Segmental Mesenteric Vascular Occlusion*

Most of the segmental mesenteric vascular occlusion studies have been performed in pigs (Table 5) although other species have been used (Tables 1, 2 and 4). This is likely because the larger size of the pig facilitates temporary occlusion of smaller vessels, such as those found within the mesentery, without compromising the integrity of the vessels, thereby allowing reperfusion. There are other advantages to using the porcine model for the study of reperfusion injury. Pigs share a similar pattern of xanthine oxidase expression as humans.
(21), whereas in rodents, enzyme expression is 4-5 fold higher (13, 21). Additionally, pigs like humans have a relatively small population of resident neutrophils unlike rodents and cats (21).

The larger size of pigs is also advantageous for models requiring surgical manipulation, such as Thiry-Vella loops in which an isolated cannulated segment of intestine can be subject to ischemic injury (18), or where research involves tissue transplantation (179) or testing advanced diagnostic imaging (27). Similar surgical manipulations in rodents have been fraught with complications (6).

Although evidence supports the use of pigs to more closely recapitulate human intestine, obvious morphological differences exist which may influence the use of pigs for the study of reperfusion injury (10, 44, 90, 146). In many mammalian species, including humans, the small intestinal epithelium is composed of stem cells that reside within the crypt base, and four post-mitotic cell types: absorptive enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. However, the existence of Paneth cells in pigs remains controversial and there is currently no definitive evidence to prove or disprove the existence of a similarly functioning cell type (62, 121, 122). Paneth cells are unique in that they reside adjacent to the intestinal stem cells within the small intestinal crypt base, and are thought to migrate toward the crypt base following complete maturation as compared to other maturing cell lineages that migrate toward the lumen. Paneth cells are thought to play an integral role in modulating stem cell self-renewal or differentiation especially during times of insult (39, 56, 149), making the study of regenerative events following ischemic injury potentially more complex.
in porcine models. However, there is recent evidence that Paneth cells may actually contribute to the injury induced by ischemia-reperfusion injury (71, 91, 102). Work in rodent models attribute injury within the gut, systemic inflammation and remote organ injury following intestinal ischemia-reperfusion injury to Paneth cell degranulation. In fact, it appears that Paneth cell depletion may attenuate ischemia-reperfusion injury (102).

Knowledge regarding the advantages and disadvantages to each model is critical to appropriate experimental design and data interpretation.

**Future Directions**

*New Insight into Mechanisms of Cell Death*

Recent insight into the process of cell death by apoptosis and necrosis in both rodent and human models of intestinal ischemia-reperfusion injury have begun to shed light into the role of these pathways in the process of injury and subsequent repair (31, 45, 113, 153, 178). An important component to the combined injury attributed to ischemia and reperfusion is the direct impact on the intestinal epithelial cells. Controlled cell death is critical to the maintenance of normal barrier function and serves to maintain epithelial continuity along the luminal surface while expelling dying cells. Under homeostatic conditions, cells mature as they gradually migrate along the crypt-villus axis. As they near the luminal surface a normal process of desquamation occurs that results in epithelial cell shedding. Briefly, this process is thought to be driven by an interaction between the extracellular matrix and integrins and cadherins, the transmembrane proteins that anchor cells to the basement membrane, and
which signal cell survival through pathways including focal adhesion kinase (p125fak), phosphatidylinositol 3’-kinase (PI-K)/Akt and mitogen-activated protein kinases (ERK, JNK, p38 MAPK) (48, 53, 54, 82, 171). Changes in these surface proteins and signaling pathways occur as cells approach the villus tip that signal a normal process of cell shedding into the intestinal lumen (28). During ischemia and reperfusion, cell injury results in death by both apoptotic and necrotic processes (74, 85, 107). Sustained tissue injury appears to be associated with inflammation that is perpetuated by the presence of excessive apoptotic and necrotic cells mediated by the complement system (78, 159, 175, 182). However, the exact pathophysiologic mechanisms that occur are complex and incompletely understood (178). Nonetheless, inhibition of apoptosis as well as the facilitated clearance of apoptotic cells appears to reduce bacterial translocation and promote repair by minimizing prolonged inflammation (45, 84, 113, 178). Based on research dedicated to understanding ischemia-reperfusion injury, it seems that the attenuation or modulation of the inflammatory response would be critical to future therapeutic approaches aimed at reducing tissue destruction. However, thus far, little progress has been made in improving clinical outcomes. Therefore, a better understanding of reparative processes may provide an alternative means to improve care.

_Advances in Understanding Epithelial Renewal_ 

Recent advances in the field of intestinal stem cell biology and regenerative medicine have improved our understanding of epithelial renewal (142). The recent discovery of biomarkers to clearly distinguish intestinal stem cell populations has contributed to the rapid
advancement of the field (8, 63, 164, 172, 173). Interestingly, rodent models of intestinal
ischemia have shown architectural preservation of the intestinal epithelial stem cells (55, 86,
132, 170). Additionally, the ability of the intestinal epithelial stem cell compartment to
expand after intestinal resection, radiation injury, and doxorubicin treatment has been
demonstrated in rodent models (41, 42, 83, 174). Together this makes the intestinal epithelial
stem cells a promising therapeutic target to hasten mucosal epithelial regeneration following
ischemia-reperfusion injury. It is our hope that this new knowledge and advanced technology
will facilitate targeted therapies to improve treatment and outcome of this devastating
disease.
### Table 1. Experimental details of in vivo ischemia-reperfusion models in cats

<table>
<thead>
<tr>
<th>Type of Experimental IR Injury</th>
<th>Segment of Intestine</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMA Complete Occlusion</td>
<td>Ileum</td>
<td>(91)</td>
</tr>
<tr>
<td>SMA Complete Occlusion</td>
<td>Jejunum + Ileum</td>
<td>(50)</td>
</tr>
<tr>
<td>Mesenteric vascular occlusion</td>
<td>Small intestine segment not specific</td>
<td>(77)</td>
</tr>
<tr>
<td>Low-flow SMA occlusion</td>
<td>Ileum</td>
<td>(65, 68, 78, 79, 86, 93, 96, 97, 123, 124, 131-133, 147, 158, 159, 174, 180, 181)</td>
</tr>
<tr>
<td>Low-flow SMA occlusion</td>
<td>Jejunum + Ileum</td>
<td>(94)</td>
</tr>
<tr>
<td>Low-flow SMA occlusion</td>
<td>Jejunum</td>
<td>(125)</td>
</tr>
<tr>
<td>Low-flow SMA occlusion</td>
<td>Small intestine does not specify segment</td>
<td>(87, 95, 178)</td>
</tr>
</tbody>
</table>

SMA, Superior Mesenteric Artery
Table 2. Experimental details of in vivo ischemia-reperfusion models in dogs

<table>
<thead>
<tr>
<th>Type of Experimental IR Injury</th>
<th>Segment of Intestine</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMA occlusion</td>
<td>Ileum</td>
<td>(5, 90, 157)</td>
</tr>
<tr>
<td>Mesenteric vascular occlusion</td>
<td>Jejunum</td>
<td>(25, 69)</td>
</tr>
</tbody>
</table>

SMA, superior mesenteric artery
## Table 3. Experimental details of in vivo ischemia-reperfusion models in mice

<table>
<thead>
<tr>
<th>Type of Experimental IR Injury</th>
<th>Segment of Intestine</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMA occlusion</td>
<td>Jejunum</td>
<td>(11, 175)</td>
</tr>
<tr>
<td>SMA occlusion</td>
<td>Jejunum + Ileum</td>
<td>(179)</td>
</tr>
<tr>
<td>SMA occlusion</td>
<td>Colon</td>
<td>(138)</td>
</tr>
<tr>
<td>SMA occlusion*</td>
<td>Jejunum + Ileum</td>
<td>(100)</td>
</tr>
<tr>
<td>SMA occlusion*</td>
<td>Jejunum</td>
<td>(99)</td>
</tr>
<tr>
<td>SMA occlusion*</td>
<td>Small intestine segment not specified</td>
<td>(46, 156)</td>
</tr>
<tr>
<td>SMA occlusion + collateral vessel ligation*</td>
<td>Ileum</td>
<td>(59)</td>
</tr>
</tbody>
</table>

SMA, superior mesenteric artery; Asterisk indicates transgenic animal use.
Table 4. Experimental details of in vivo ischemia-reperfusion models in rats

<table>
<thead>
<tr>
<th>Type of Experimental IR Injury</th>
<th>Segment of Intestine</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenteric Vascular Occlusion</td>
<td>Proximal colon</td>
<td>(71, 72)</td>
</tr>
<tr>
<td>SMA occlusion</td>
<td>Proximal colon</td>
<td>(103, 117, 173)</td>
</tr>
<tr>
<td>SMA occlusion</td>
<td>Jejunum + Ileum</td>
<td>(10, 103, 110, 151)</td>
</tr>
<tr>
<td>SMA occlusion</td>
<td>Ileum</td>
<td>(129, 130, 144)</td>
</tr>
<tr>
<td>SMA occlusion</td>
<td>Jejunum</td>
<td>(28, 29, 58, 60, 82, 84, 126)</td>
</tr>
<tr>
<td>SMA occlusion</td>
<td>Ileum</td>
<td>(152, 162, 163)</td>
</tr>
<tr>
<td>SMA occlusion</td>
<td>Small intestine does not specify segment</td>
<td>(57, 175)</td>
</tr>
<tr>
<td>SMA + jejunal and colic artery occlusion</td>
<td>Jejunum</td>
<td>(83, 112)</td>
</tr>
<tr>
<td>SMA + jejunal and colic artery occlusion</td>
<td>Ileum</td>
<td>(167)</td>
</tr>
<tr>
<td>SMA, superior mesenteric artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of Experimental IR Injury</td>
<td>Segment of Intestine</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SMV Occlusion</td>
<td>Jejunum, ileum, colon</td>
<td>(164)</td>
</tr>
<tr>
<td>Mesenteric Vascular Occlusion</td>
<td>Jejunum + Ileum</td>
<td>(33)</td>
</tr>
<tr>
<td>SMA Embolism</td>
<td>Jejunum + Ileum</td>
<td>(142)</td>
</tr>
<tr>
<td>SMA Embolism</td>
<td>Small intestine + colon</td>
<td>(92)</td>
</tr>
<tr>
<td>SMA Embolism</td>
<td>Small Intestine segment not specified</td>
<td>(2, 23)</td>
</tr>
<tr>
<td>Mesenteric Vascular Occlusion</td>
<td>Jejunum</td>
<td>(44, 45, 73, 111)</td>
</tr>
<tr>
<td>Mesenteric Vascular Occlusion</td>
<td>Proximal Colon</td>
<td>(71, 72)</td>
</tr>
</tbody>
</table>

SMV, superior mesenteric vein; SMA, superior mesenteric artery
Table 6. Experimental details of in vivo ischemia-reperfusion models in humans

<table>
<thead>
<tr>
<th>Type of Experimental IR Injury</th>
<th>Segment of Intestine</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenteric Vascular Occlusion</td>
<td>Jejunum</td>
<td>(44, 45, 73, 111)</td>
</tr>
<tr>
<td>Mesenteric Vascular Occlusion</td>
<td>Proximal Colon</td>
<td>(71, 72)</td>
</tr>
</tbody>
</table>
Figure 1. Clinical conditions that cause ischemic intestinal injury. Clinical conditions that cause ischemic intestinal injury. Intestinal ischemia is associated with a broad range of clinical conditions including neonatal necrotizing enterocolitis, acute mesenteric ischemia (AMI), volvulus, trauma, cardiopulmonary disease, hemorrhagic shock and intestinal transplant rejection.
Figure 2. Mechanism of ischemia-reperfusion injury. During ischemic conditions, as tissues continue to utilize adenosine triphosphate as a source of energy, the metabolic byproduct, hypoxanthine, accumulates. In addition, in this oxygen deprived environment, xanthine dehydrogenase which would normally function to metabolize hypoxanthine is converted to xanthine oxidase by locally produced tissue proteases. When tissue perfusion is re-established, xanthine oxidase utilizes the now freely available and abundant oxygen as an electron acceptor as it metabolizes hypoxanthine, producing superoxide. The direct injury induced by oxygen free radicals combined with their role in the activation and chemoattraction of neutrophils creates the severe tissue injury attributed to reperfusion.
Figure 3. Epithelial loss associated with ischemic intestinal injury in porcine jejunum. As the duration of ischemia increases there is progressive loss of epithelium that begins at the villus tip and continues toward the crypt base. Asterisk indicates Gruenhagen’s space. Scale Bar 50 μm.
Figure 4. Species differences in villus microvascular architecture. A general construct of counter current exchange exists along the length of the villus whereby oxygen diffuses from the arterial blood supply that ascends toward the lumen and venous drainage flowing in the opposite direction. This results in the progressive decrease in the arterial concentration of oxygen as the vessels near the luminal surface. However, species differences exist. The pig, cat and human villus microvascular architecture are very similar, arborizing at the tip of the villus into a fountain-like pattern and converging into one or two venules located beside the centrally located arteriole. In contrast, in the rat, the flattened leaf-shaped villi are supplied by a single arteriole that passes unbranched from the base to the tip of the villus where it bifurcates into a capillary network, a so-called “netted bag” pattern. The arterioles begin to converge and form two venules as the vasculature approaches the base of the villus. The dog consistently has two arterioles and two venules although the villus shape is cylindrical. These differences may impact the physiological function of the intestinal mucosa between species and potentially their susceptibility or response to injury. Note: Villus size is not to scale.
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CHAPTER III

OPERATIVE FACTORS ASSOCIATED WITH SHORT-TERM OUTCOME IN HORSES WITH LARGE COLON VOLVULUS: 47 CASES FROM 2006 TO 2013

Publication:

Summary

**Reasons for performing the study:** There is an important need for objective parameters that accurately predict the outcome of horses with large colon volvulus.

**Objectives:** To evaluate the predictive value of a series of histomorphometric parameters on short-term outcome, as well as the impact of colonic resection on horses with large colon volvulus.

**Study Design: Retrospective case-series study**

**Methods:** Adult horses admitted to the Equine and Farm Animal Veterinary Center at North Carolina State University as well as Peterson & Smith and Chino Valley Equine Hospitals between 2006-2013 undergoing an exploratory celiotomy, diagnosed with large colon volvulus of ≥360 degrees, where a pelvic flexure biopsy was obtained, and that recovered from general anesthesia, were selected for inclusion in the study. Logistic regression was used to determine associations between signalment, histomorphometric measurements of interstitial: crypt ratio (I:C), degree of haemorrhage, percentage loss of luminal and glandular epithelium, as well as colonic resection with short-term outcome (discharge from the hospital).

**Results:** Pelvic flexure biopsies from 47 horses with large colon volvulus were evaluated. Factors that were significantly associated with short-term outcome on univariate logistic regression were Thoroughbred breed (P=0.04), I:C >1 (P=0.02) and haemorrhage score ≥3 (P=0.005). Resection (P=0.92) was not found to significantly impact short-term outcome. No combined factors increased the likelihood of death in forward stepwise logistic regression.
modeling. A digitally quantified haemorrhage area measurement strengthened the association of haemorrhage with non-survival in cases of large colon volvulus.

**Conclusions:** Histomorphometric measurements of I:C ratio and degree of haemorrhage predict short-term outcome in cases of large colon volvulus. Resection was not associated with short-term outcome in horses selected for this study.

**Potential relevance:** Accurate quantification of mucosal haemorrhage at the time of surgery may improve veterinary surgeons’ prognostic capabilities in horses with large colon volvulus.

**Introduction**

Large colon volvulus (LCV) is an acute, severe abdominal crisis in the horse. LCV accounts for 10-20% of horses presented for colic that undergo exploratory laparotomy. Reported short term survival rates for horses with LCV vary markedly, with reported ranges of 35-86% [1-9]. Long-term survival of horses with LCV is the lowest amongst surgical causes of colic, with a recent report of 48.3% and 33.7% survival following one and two years, respectively [7]. The accurate prediction of survival of horses with LCV has been an ongoing area of interest for many years but remains elusive. Indicators used for prognosis have included preoperative parameters such as plasma lactate levels and abdominal ultrasound findings, operative findings including colonic luminal pressure and surface oximetry, and histomorphometric measurements obtained from pelvic flexure biopsies [4, 6, 8, 10-13]. However, the accuracy of pelvic flexure biopsies to predict short-term survival in cases of LCV has recently been called into question [4]. Consequently, the most common
basis for prognosis continues to be the surgeon’s clinical impression of colonic viability based on the appearance of the bowel and the mucosa at the time of surgery [8, 10]. Therefore, there is an important need for objective parameters that accurately predict survival of horses with LCV. Resection and anastomosis of the large colon is one available treatment option recommended by some surgeons to improve survival outcomes, but recent studies suggest resection has a limited impact on survival [11]. For example, reported short-term survival for horses treated by resecting the large colon are not significantly different than for horses treated by manual replacement of the large colon alone [1, 2, 11]. It is not possible to resect the entire large colon because of its attachment to the base of the cecum and a short area of the dorsal body wall, leading proponents of resection to propose that ‘de-bulking’ severely injured colon may enhance survival [14]. However, resection of the large colon is time-consuming and expensive. Therefore, in addition to overall evaluation of prognostic indicators, there is a need to determine if resection and anastomosis improves survival in horses with LCV so that more informed operative decisions can be made. The aim of this study was to evaluate the potential predictive value of a series of histomorphometric parameters on short-term outcome (discharge from the hospital), as well as the impact of colonic resection on horses with LCV.

Materials and methods

Study population and inclusion criteria

Adult horses admitted to the Equine and Farm Animal Veterinary Center at North Carolina State University, as well as Peterson & Smith and Chino Valley Equine Hospitals between
February 2006 and March 2013 undergoing an exploratory celiotomy, diagnosed with LCV of $\geq 360$ degrees where a pelvic flexure mucosal biopsy was obtained, and that recovered from general anesthesia were selected for inclusion in the study. Pelvic flexure biopsy samples from cases of LCV were banked for future studies at these institutions during this time. Medical records from these cases were retrospectively reviewed. Perioperative data and survival information were obtained for each horse included in the study, including survival from surgery, and survival until discharge (short-term outcome). However, statistical analyses to identify factors associated with short-term outcome were only performed on cases that fully recovered from general anesthesia following surgical correction of volvulus. This was done to avoid analysis of horses euthanized at surgery for economic reasons. Mucosal biopsies were obtained from a control group of horses that had pelvic flexure enterotomies for non-strangulating lesions, and used to establish baseline histomorphometric parameters. Mucosal biopsies were obtained with the consent of the owners, and biopsy procedures were approved by the NC State University Institutional Animal Care and Use Committee.

**Surgical and Perioperative Management**

The recommendation for manual correction of the volvulus alone or correction with large colon resection and anastomosis was based on clinical assessment of large colon viability by the surgeon. The wishes of the owner, based on discussion of surgical assessment, were honoured in all cases, including the decision to perform euthanasia on economic grounds. Factors used to assess colon viability have been previously published and included serosal color, wall thickness, friability, motility and a palpable pulse [2]. In all cases of LCV,
complete evacuation of colonic contents was based on the degree of colonic distension but was performed in the majority of cases. Pre-operative antibiotics and analgesics therapy for all cases included potassium penicillin G (22,000 U/kg intravenously (IV) every 6 hours), gentamicin (6.6 mg/kg IV every 24 hours) and flunixin meglumine (1.1 mg/kg IV every 12 hours). Post-operative antibiotics and analgesics were continued for at least five days and further administration was determined on a per case basis as indicated by evaluation of the patient and results of laboratory tests. Abdominal drains were routinely placed at surgery if a resection and anastomosis was performed and postoperative abdominal lavage was performed. The administration of lidocaine (bolus 1.3 mg/kg IV followed by constant rate infusion of 0.05 mg/kg/min IV) was based on clinical assessment and presence of ileus. IV fluids were administered as needed postoperatively. Polymyxin B (6000 U/kg IV every 12 hours) and plasma were administered as needed for treatment of clinical evidence of endotoxemia and hypoproteinemia. The decision to euthanize select horses in the postoperative period was made on the basis of clinical assessment and further discussion with the owner.

**Specimen preparation**

All mucosal biopsies were collected at the time of enterotomy and immediately placed in 10% neutral buffered formalin solution. Tissues were routinely processed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin by the same histopathology laboratory (NC State University, College of Veterinary Medicine).
Histomorphometric Measurements

Morphologic changes were evaluated and graded (in the case of epithelial loss) or scored (in the case of haemorrhage) based on established criteria [15] by two of the authors (LMG, CAF). The evaluators performed histological evaluations independently and were blinded to horse and short-term outcome data. Evaluation by light microscopy (10X and 40X objective lenses) of at least 4 visual fields was performed. The interstitial: crypt ratio (I:C) was defined as the ratio of the measured lamina propria space occupied by the interstitium and the space occupied by the crypts (Figure 1A). A normal value for the I:C ratio was considered to be ≤1 [15]. The percentage loss of luminal epithelium (LEL) was based on visual estimation of the percentage of luminal epithelium that was separated from the basement membrane [15]. A grade of 1 to 4 was assigned to estimated ranges of epithelial loss (grade 1, 0-25%; grade 2, 25-50%; grade 3, 50-75%; grade 4, 75-100%). In addition, the percentage of glandular epithelial loss (GEL) was calculated by measuring the distance from the crypt base to the point of glandular epithelial loss or separation from the basement membrane divided by the total length of the crypt. A haemorrhage score within the lamina propria of the mucosal biopsy was assigned a score of 0 to 4 (score 0, no haemorrhage; score 1, few individual red blood cells (RBCs) within the lamina propria; score 2, increased number of individual RBCs; score 3, appearance of clumps of RBCs; score 4, confluent RBCs obscuring the demarcation of the lamina propria) (Figure 1B).
Digital quantification of haemorrhage

Degree of haemorrhage was digitally quantified by measuring the area taken up by RBCs within a discrete space in an image using a software program\textsuperscript{1}. More specifically, each digital image was imported into a graphic software package,\textsuperscript{1} and the resolution was set to 118 pixels/cm. A 6.4cm x 6.4cm square, abutting the muscularis mucosa and centered on the image was selected and transferred into a new canvas. The processed image was then loaded into an image analysis package,\textsuperscript{2} and the settings were adjusted in order to accurately identify the red blood cells (hue 232/255, saturation 0/255 and brightness 178/255 using the Huang thresholding method [16]. The haemorrhage area (HA) was subsequently acquired (Figure 1C). Three images obtained from each evaluating author (LMG, CAF) were assessed using the software.

Statistical Analysis

Short-term outcome was defined as horses that were discharged from the hospital. Chi square analysis was used to determine if an association existed between institution (NCSU vs. other) and short-term outcome as well as between breed distribution and institution. Age, breed, gender, I:C ratio, crypt length, % luminal epithelial loss, % glandular epithelial loss, haemorrhage score, haemorrhage area and whether or not a resection was performed were evaluated for association with short-term outcome. The cut off points for dichotomizing the quantitative variables entered in the logistic regression model were determined from measurements obtained from control cases and from previous studies [8, 15]. The normal

\textsuperscript{1} Adobe Photoshop v. 10.0
\textsuperscript{2} ImageJ is a public domain, java-based image processing program developed at the National Institutes of Health (NIH, Bethesda, MD). Available as freeware from http://rsbweb.nih.gov/ij
value for crypt length (392µm) was determined by averaging the measurements obtained from the control cases as a normal value has not been previously published. The I:C ratio cut off point of ≤1 as normal and >1 abnormal, % LEL <50% as normal and ≥50% abnormal, and %GEL <50% as normal and ≥50% as abnormal have been previously described as indicators of severity of injury [8, 15]. The haemorrhage scoring system has also been previously described and a score associated with moderate to severe haemorrhage (≥3) was selected as the cutoff for logistic regression analyses [8, 15]. A raw P<0.05 was considered statistically significant. To test for potential multivariable models that might be significant, forward selection, using a Bayesian Information Criteria (BIC) was performed to build a potential multivariable association model. Using this BIC selection criteria, a null model was built. Modeling was performed in Stata V11\(^3\) (www.stata.com). To test for concerns of potential confounding variables for the one statistically significant variable after multiple comparisons, multiple logistic regression modeling was performed, where these variables were entered to test for the significance of each of these variables after correcting for the other. While true estimates of predictive values are not possible in the current retrospective study, contingency table measures of model fit (including sensitivity, specificity, and a contingency table estimate of negative and positive predictive value) were calculated.

The sensitivity (the probability that a truly affected individual would test positive), specificity (the probability of a surviving horse having a negative test result)) and accuracy (the sum of the number of true positives and true negatives divided by the sum of the number of true positives, false positives, false negatives and true negatives) of haemorrhage score

\(^3\) StataCorpLP, College Station, TX
and area for prediction of death and short-term outcome in horses with LCV were calculated. Haemorrhage area calculations were used to generate a probability of death curve. The probability of death was calculated by counting the number of deaths as HA incrementally increased and dividing by the total number of individuals with HA measurements equal to or less than each measurement. A standard error of mean was calculated and represented graphically for each measured haemorrhage area by averaging all of the measured areas for a single case (6 measurements per case; 3 images per case from each author; LMG, CAF).

A Fisher’s exact test was used to test for an association between surgical resection and haemorrhage score.

Results

Pelvic flexure biopsies from seventy-one cases of LCV were obtained. Twenty-seven cases with biopsies from NCSU, forty-three from Peterson and Smith Equine Hospital and one from Chino Valley Equine Hospital were evaluated. Of all horses admitted for surgery, 36 of 71 (51%) survived until discharge. Of the seventy-one LCV cases taken to surgery, 24 horses were not recovered from anesthesia, 5 having intra-operative colon rupture, 5 due to owner declining further intervention despite the surgeon’s recommendation and 14 because the surgeon’s prognosis was grave and the owner elected euthanasia. Forty-seven horses with LCV met the inclusion criteria for further analyses. Of cases with LCV, the breeds were Thoroughbred (n=22), Quarter Horse (n=10), Warmblood (n=9), Arabian (n=2), Paso Fino (n=2), Tennessee Walking Horse (n=1), and American Paint Horse (n=1). The breed distribution for each of the hospitals was Warmbreads (23%), Quarter Horses (59%),
and other breeds (10%) from NCSU and Thoroughbreds (73%), Warmbloods (17%), Quarter Horses (3%) and other breeds (7%) from the two other contributing hospitals. The mean age was 9 years (range, 2-23 years), with three horses of unknown age. There were 35 mares, 10 geldings and 2 stallions. Thirty six of 47 (76.6%) survived until discharge from the hospital. Nineteen of 25 (76%) horses that had manual reduction alone survived, whereas 17 of 22 (77%) that had a resection and anastomosis following correction of LCV survived. All horses in which a large colon resection and anastomosis was performed that recovered from general anesthesia were from Peterson and Smith Equine Hospital except for one performed at Chino Valley Equine Hospital. Two large colon resections were performed at NCSU, one was ultimately euthanized intra-operatively due to systemic deterioration under general anesthesia and another was unable to recover and was humanely euthanized upon the owner’s request. All anastomoses were performed as end-to-end as described [2].

Fourteen horses that underwent exploratory celiotomy necessitating pelvic flexure enterotomy for reasons other than a strangulating lesion were used as controls. Of the group of control horses, the breeds were Thoroughbreds (N=4), Quarter Horses (N=3), Morgans (N=2), warmbloods (N=2), draft horse (N=1), Tennessee Walking Horse (N=1) and a pony (N=1) The mean age was 11 years (range, 1-27 years). There were 6 mares and 8 geldings. The average crypt length in control horses was 392 µm. The mean I:C was 0.9 (standard deviation ±0.3). The median percent glandular epithelial loss and grade of luminal epithelial loss were 0 (inter-quartile range, 0-0.478),and 1 (IQR 1-2.5) respectively. The average haemorrhage score was a score of 1 and the average haemorrhage area was 0.2 ± 0.2ppi.
Factors that were nominally significantly associated with short-term outcome on univariate logistic regression were Thoroughbred breed (P=0.042), I:C >1 (P=0.022) and haemorrhage score ≥3 (P=0.005) (Table 1). 21 of 21 (100%) cases of LCV with LEL <50% survived, whereas 15 of 26 (58%) of horses with LEL ≥50% survived. Of cases of LCV with GEL <50%, 35 of 44 (80%) survived. 2 of 3 (67%) of LCV cases with ≥50% GEL survived. The I:C ratio >1 and haemorrhage score ≥3 were strongly associated with non-survival (Table 1). Haemorrhage score was accurate for prediction of death and survival in horses with LCV in that 7 horses were accurately predicted to die out of 13 horses with a haemorrhage score ≥3, (sensitivity, 64%; 95% CI, 0.354-0.848; positive predictive value (PPV), 54%), and 30 horses were accurately predicted to survive out of 34 with a haemorrhage score <3, (specificity, 83%; 95% CI, 0.681-0.921; negative predictive value (NPV), 88%). Thoroughbred breed was slightly protective (Table 1). In the univariate logistic regression model, resection was not found to significantly impact short-term outcome (Table 1). However, in cases with haemorrhage score <3, 18 of 22 (82%) without resection survived and all 12 horses (100%) with resection survived. When the haemorrhage score was ≥3, 1 of 3 (33%) horses without resection survived and 5 of 10 (50%) horses with resection survived. A Fisher’s exact test for association of haemorrhage score with survival was not found to be statistically significant (P=1.00). In a forward stepwise logistic regression model, no combined factors increased the likelihood of death.

In multivariable analysis testing for potential confounding and collinearity amongst the three nominally associated variables: Thoroughbred breed, I:C >1, and haemorrhage
score, results indicated that the haemorrhage score had the strongest association, and the only statistically significant association. When controlling for I:C and breed, haemorrhage score was still significant \((p<0.03)\), and the other two variables were no longer significant when haemorrhage score was included in the model \((p>0.05\) in all cases).

The statistical strength of the haemorrhage score and its strong association with death led us to pursue a more objective means to measure the amount of haemorrhage within the lamina propria. Haemorrhage score was 79% accurate for prediction of death and short-term outcome. Digital quantification of haemorrhage severity was therefore calculated to address the subjectivity of the haemorrhage grading system. A haemorrhage area \(\geq 0.84\) ppi was associated with a 26-fold increased likelihood of non-survival \((OR \, 26; \, CI \, 2.9 \, - \, 230.3; \, P=0.003)\). Haemorrhage area was 79% accurate for prediction of death and short-term outcome in horses with LCV with 10 horses predicted to die out of 19 with a haemorrhage area \(\geq 0.84\) (sensitivity, 91%; 95%CI, 0.623-0.984; PPV, 53%), and 27 horses predicted to survive out of 28 with a haemorrhage area <0.84 ppi, (specificity, 75%; 95%CI, 0.589-0.863; NPV, 96%). No horses died with a haemorrhage area measurement <0.2 ppi. Only one horse died with a haemorrhage area measurement <0.84 ppi (Figure 2). When the haemorrhage area measurement was \(\geq 0.84\) ppi, 10 of 19 (53%) animals died.

**Discussion**

There is a need for objective parameters that associate the severity of colonic injury with survival in horses with LCV. Since the information gained from histomorphometric assessment is limited to the post-operative period in most practices, the more readily
available pre and intraoperative parameters such as heart rate, packed cell volume, and plasma lactate have remained commonly utilized prognostic tools despite their indirect indication of colonic compromise. We therefore reasoned that more in depth histological evaluation, including objective measurement of mucosal haemorrhage, may provide a more accurate assessment of the likelihood of short-term outcome in the postoperative period. In addition, no study has performed risk analyses to evaluate if large colon resection improves short-term outcome in cases of LCV.

Histomorphometric analysis of colonic tissue has long been considered the gold standard for assessing tissue viability [8, 15]. The observation that the histologic changes observed at the pelvic flexure are uniformly distributed throughout all colonic tissue involved in the volvulus further contributed to the perceived value of histologic assessment in grading tissue damage [17]. Specific histomorphometric parameters found to be associated with loss of intestinal viability and death in cases of LCV are I:C ratio ≥3 and GEL >50% [8]. The basis for increased I:C ratio in cases of severe injury has been attributed to increased haemorrhage that causes architectural damage as the interstitium expands and glandular epithelium is compressed [10, 17]. The association of the degree of GEL with loss of tissue viability has been attributed to the integral role of the glandular epithelium in the renewal of the mucosal lining [8, 10]. Recently, though, the reliability of histomorphometric grading of tissues derived from pelvic flexure biopsies in cases of LCV has been questioned [4]. Levi et al. determined that short-term survival could not be accurately predicted by histopathologic evaluation, whereas heart rate at admission, as well as heart rate and packed cell volume 24
hours postoperatively were significantly associated with survival [4]. However, in that study, histopathologic measures were not assessed individually, but were grouped and then assigned a numerical score [4]. We found that LEL and GEL were difficult to measure accurately and seemed to be susceptible to processing artifact. Therefore our overall impression was that LEL and GEL were not consistent measures of tissue damage. Additionally, within our study population, a small number of cases had an I:C ratio of \( \geq 3 \). Since an I:C ratio measurement \( \leq 1 \) is accepted as normal, and the mean measurement of the control cases was \(< 1\), we used \( >1 \) as a cut-off value. This led to the finding that horses with an I:C ratio \( >1 \) were 12-fold more likely to be non-survivors during hospitalization. However, I:C ratio did not further increase the likelihood of non-survival when combined with other factors when performing multiple logistic regression.

Interestingly, the degree of haemorrhage (as assessed by hemorrhage score), a parameter not associated with death in any other study, was found to have a strong statistical association with non-survival. This was of particular interest since determining haemorrhage within tissue would seem to have a greater potential for use in clinical practice in the absence of a pathologist at the time of surgery. The accuracy of the hemorrhage score in predicting survival or non-survival was 79%. However, the haemorrhage score was most useful in predicting which horses would mostly likely survive. For example, those horses predicted to survive based on the haemorrhage score would likely survive but those predicted to die may actually survive (PPV 54%; NPV 88%; sensitivity 64%; specificity 83%). Because of the low sensitivity based on this technique for the measurement of haemorrhage and to further
determine the reliability and strength of the association of haemorrhage with death, we developed a more objective measure of this parameter using morphometric software. The strong association of poor short-term outcome with increasing area of haemorrhage may be due to the architectural damage that results from extravasation of red blood cells into the lamina propria. The resulting increase in lamina propria area may contribute to the detachment of the epithelial lining which results in a breakdown of barrier function, although this was difficult to appreciate in the histologic sections (Figure 1). An additional mechanism of injury caused by the presence of increased red blood cells within the lamina propria may be release of free radicals and subsequent lipid peroxidation that would be expected to contribute to tissue damage [18, 19]. Others have indicated that a return of normal mucosal colour following surgical correction of LCV intraoperatively is an indicator of viability, as opposed to mucosa that remains black after correction of volvulus that indicates a poor prognosis for survival [20-22]. We speculate that this gross black appearance is directly associated with haemorrhage within the lamina propria, suggesting that accurate quantification of mucosal haemorrhage at surgery may improve veterinary surgeons’ prognostic capabilities.

One limitation of the current study was the exclusion of cases that did not recover from general anesthesia. An owner’s decision to euthanize is based on many factors that may include economic concerns or the fact that a surgeon can rarely provide a definitive prognosis. Currently, the intraoperative decision to resect the large colon is based on surgeon preference according to the perceived level of colonic injury, and prior experience with the
techniques. In cases of LCV, there is rarely a clear demarcation between viable and nonviable intestine, which makes the site and utility of large colon resection unclear [23]. Therefore, data on the survival of horses with LCV that undergo resection as compared to those that do not is critically needed to guide intraoperative decision making. The overall short-term survival of horses that recovered from surgery in our study where resection and anastomosis was performed was 77%, similar to that found by Mathis et. al., whose case inclusion criteria similarly only evaluated horses that recovered from anesthesia [11]. These findings were substantially higher than the 47% reported by Driscoll et. al., however that study population included horses euthanized intra-operatively [1]. Despite an encouraging short-term outcome among the horses that recovered from general anesthesia in our study, resection was not found to be statistically associated with an increased likelihood of short-term outcome. Furthermore, no parameters combined with resection in the multivariable model strengthened its association with short-term outcome. However, the population size was small (n=47), reducing the statistical power necessary to properly interpret the negative finding of a lack of effect of resection or to build multivariable models. Additional studies using a larger sample size will be necessary to obtain more definitive evidence for or against large colon resection and anastomosis as a treatment for large colon volvulus. Additionally, this retrospective study could not control for the individual reasons surgeons selected cases for resection. Additional prospective studies are warranted to control for the many clinical decisions that are necessary when managing a horse with large colon volvulus. Future work on LCV will be directed towards further determining the impact of resection in these cases
and to provide a surgery table-side test that accurately measures haemorrhage as a tool to more accurately guide surgical decisions.
Table 1. Univariate logistic regression analysis of factors potentially associated with death in cases of large colon volvulus. Data represents information for forty-seven horses with LCV. The OR represents the odds that a horse with LCV will not survive. *Variable found to be statistically significant (p<0.05). I:C= Interstitial Crypt Ratio. OR= Odds ratio. CI= Confidence Interval. P=P value.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>No. (%) Horses</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crypt Length</td>
<td>≥392</td>
<td>17(36.2)</td>
<td>1.0</td>
<td>0.24-4.03</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>&lt;392</td>
<td>30(63.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>≥9</td>
<td>23(48.9)</td>
<td>0.9</td>
<td>0.22-3.64</td>
<td>0.870</td>
</tr>
<tr>
<td></td>
<td>&lt;9</td>
<td>21(44.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>3(6.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breed *</td>
<td>TB</td>
<td>22(46.8)</td>
<td>0.2</td>
<td>0.03-0.94</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>Non-TB</td>
<td>25(53.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>12(25.5)</td>
<td>0.5</td>
<td>0.12-2.15</td>
<td>0.352</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>35(74.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal Epithelial Loss</td>
<td>≥50%</td>
<td>26(55.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;50%</td>
<td>21(44.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glandular Epithelial Loss</td>
<td>≥50%</td>
<td>3(6.38)</td>
<td>1.1</td>
<td>0.10-11.8</td>
<td>0.937</td>
</tr>
<tr>
<td></td>
<td>&lt;50%</td>
<td>44(93.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I:C*</td>
<td>&gt;1</td>
<td>26(55.3)</td>
<td>13</td>
<td>1.44-108</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>≤1</td>
<td>21(44.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosal Haemorrhage *</td>
<td>≥3</td>
<td>13(27.7)</td>
<td>8.8</td>
<td>1.94-39.6</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>&lt;3</td>
<td>34(72.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgical Correction</td>
<td>Resection</td>
<td>22(46.8)</td>
<td>0.9</td>
<td>0.24-3.61</td>
<td>0.918</td>
</tr>
<tr>
<td></td>
<td>Manual</td>
<td>25(53.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospital</td>
<td>NCSU</td>
<td>17(36.2)</td>
<td>0.4</td>
<td>0.09-1.46</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>30(63.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. I:C ratio and haemorrhage grade and area measurements. Histomorphometric evaluation of colonic mucosal biopsies of horses with and without large colon volvulus. (A) The interstitial (I) crypt (C) ratio was defined as the ratio of the width of the inter-crypt lamina propria to the width of the crypts. The crypt length (CL) was also measured. (B) Haemorrhage score within the lamina propria of the mucosal biopsy was assigned a score from 0 to 4 (score 0, no haemorrhage; score 1, few individual red blood cells (RBCs) within the lamina propria; score 2, increased number of individual RBCs; score 3, appearance of clumps of RBCs; score 4, no clear demarcation between RBCs, obscuring the lamina propria). (C) Haemorrhage area was digitally quantified using ImageJ v.1.46.®
Figure 2. Box and whiskers plot of horses that were euthanized or survived based on haemorrhage area. Haemorrhage area measured from biopsies obtained from cases of LCV indicating that although most horses with a haemorrhage area less than 0.84 survived (n=27) there was overlap in horses with a measurement greater than 0.84 ppi in that approximately equal numbers of horses were euthanized as compared to those that survived. Boxes represent interquartile range, single dividing line is the median, error bars represent standard deviation, and dots represent outliers.
References


CHAPTER IV

CELL LINEAGE IDENTIFICATION AND STEM CELL CULTURE IN A PORCINE MODEL FOR THE STUDY OF INTESTINAL EPITHELIAL REGENERATION

Publication:

ABSTRACT

Significant advances in intestinal stem cell biology have been made in murine models; however, anatomical and physiological differences between mice and humans limit mice as a translational model for stem cell based research. The pig has been an effective translational model, and represents a candidate species to study intestinal epithelial stem cell (IESC) driven regeneration. The lack of validated reagents and epithelial culture methods is an obstacle to investigating IESC driven regeneration in a pig model. In this study, antibodies against Epithelial Adhesion Molecule 1 (EpCAM) and Villin marked cells of epithelial origin. Antibodies against Proliferative Cell Nuclear Antigen (PCNA), Minichromosome Maintenance Complex 2 (MCM2), Bromodeoxyuridine (BrdU) and phosphorylated Histone H3 (pH3) distinguished cells proliferating cells at various stages of the cell cycle. SOX9, localized to the stem/progenitor cells zone, while HOPX was restricted to the +4/’reserve’ stem cell zone. Immunostaining also identified major differentiated lineages. Goblet cells were identified by Mucin 2 (MUC2); enteroendocrine cells by Chromogranin A (CGA), Gastrin and Somatostatin; and absorptive enterocytes by carbonic anhydrase II (CAII) and sucrase isomaltase (SIM). Transmission electron microscopy demonstrated morphologic and sub-cellular characteristics of stem cell and differentiated intestinal epithelial cell types. Quantitative PCR gene expression analysis enabled identification of stem/progenitor cells, post mitotic cell lineages, and important growth and differentiation pathways. Additionally, a method for long-term culture of porcine crypts was developed. Biomarker characterization and development of IESC culture in the porcine model represents a foundation for
translational studies of IESC-driven regeneration of the intestinal epithelium in physiology and disease.

INTRODUCTION

Complete physiologic renewal of the intestinal epithelium occurs in approximately one week and is driven by a pool of IESCs at the crypt base [1]. This impressive rate of renewal is tightly controlled in homeostasis. Dysregulation of IESC renewal results in intestinal disorders such as small intestinal and colorectal cancer, which is the leading cause of digestive disease-related mortality [2,3]. Impaired epithelial renewal can lead to ulceration, chronic inflammatory responses and sepsis [4,5]. Since the description of IESCs in 1974 by Cheng and Lebond, investigators have attempted to understand the factors that control IESC-driven epithelial regeneration in physiology and disease [6].

In general, logistical and ethical issues minimize the use of humans or human-derived tissues for research and discovery pertaining to conditions of the intestinal epithelium. These obstacles highlight the need for a research model that closely mimics human intestinal anatomy, physiology, disease and injury processes. Currently, the vast majority of basic studies focused on intestinal epithelial diseases, injury and regeneration utilize rodent models. Rats and mice in particular represent an important, cost effective animal model for basic genetic, cellular and molecular biology of IESC-driven regeneration of the intestinal epithelium. Despite these advantages, significant differences between rodents and humans confound or prohibit translational studies [7].
Important anatomical, behavioral and environmental conditions that impact epithelial regeneration are more closely shared between pigs and humans than between mice and humans [8,9]. Pigs and humans share parallel mucosal barrier physiology, food intake, enteric microbiota composition, and pathogenicity of key disease causing microbes [7]. Pigs, like humans, are true omnivores and share similar metabolic and intestinal physiologic processes [7,9]. A mucosal in vitro permeability study demonstrated greater correlation between humans and pigs when compared to rats [8]. Importantly, it has been demonstrated that pigs represents a more physiologically relevant model of neonatal necrotizing enterocolitis, intestinal ischemia-reperfusion injury, acute mesenteric ischemia, short bowel syndrome, AIDS-associated opportunistic Cryptosporidium infection, and stress-induced intestinal dysfunction [10-22]. Additionally, a large animal model is likely to serve as a more physiological relevant model to study segmental assessment of radiation exposure, focally induced ischemia and reperfusion as well as transplantation and cell-based therapies.

Severe intestinal disease necessitates approximately 200 intestinal transplantations each year in the United States [2]. In a prospective cross-sectional study of patients, 40% of visceral allograft recipients died within 5 years of transplantation [23]. The impact of digestive disease on rates of mortality and morbidity as well as health care costs in the United States has created an urgent need for advances in transplantation and tissue replacement therapies [2]. A key factor to the success of many translational studies is the gross size of the animal model. The small size of the intestines of experimental rodent models often prohibits tissue manipulation or implementation of candidate surgical interventions such as tissue
engraftment or transplantation. These limitations further highlight the need for a large animal model to advance cell or tissue based therapies.

This study focuses on eliminating many of the obstacles that limit the pig as a translational model to study IESC-driven regeneration of the intestinal epithelium. This work thoroughly characterizes the porcine intestinal mucosa by identifying, developing and validating a comprehensive set of reagents to study porcine stem/progenitor cells and their principal post-mitotic cell descendants \textit{in situ} and in culture.

\textbf{MATERIALS AND METHODS}

\textit{Ethics Statement}

All animal studies were approved by the Institutional Animal Care and use Committee at North Carolina State University.

\textit{Animals and sample collection}

Tissues were obtained from healthy 6-8 week-old wild type Yorkshire cross pigs euthanized for reasons unrelated to this project. Sections from the gastrointestinal tract including the duodenum, jejunum, ileum, proximal and distal colon were sharply dissected.

\textit{Histological and Immunofluorescence Analyses}

Tissues were rinsed with 1X phosphate-buffered saline (PBS) and opened longitudinally along the anti-mesenteric boarder. For immunohistochemical analysis, tissue was fixed in 10% formalin, embedded in paraffin and sectioned (~5-8 \textmu m thickness). Slides were stained with hematoxylin and eosin to visualize crypt and villus morphology. For immunofluorescence, rinsed tissue was fixed in 4% paraformaldehyde (PFA) solution for 14-
18 hours at 4°C. The tissue was transferred to 30% sucrose solution for at least 24 hours at 4°C, embedded in optimal cutting temperature (OCT) media, frozen and sectioned at 5-8μm thickness using a cryotome and mounted on positively charged glass slides. Sections were washed three times with PBS to remove OCT. When necessary, heat induced epitope retrieval (HIER) was then performed by placing slides into reveal decloaker solution (Biocare Medical, Concord, CA) for 30 seconds at 120°C and then 90°C for 10 seconds, in a pressure cooker. The slides were allowed to cool at room temperature for 20 min prior to continuing. Tissue permeabilization was performed on all slides with PBS-0.3% Triton X-100 for 20 min, washed twice with PBS and incubated in blocking medium (Dako, Carpinteria, CA). Primary antibodies were applied to the tissue section in an antibody diluent (Dako) and incubated overnight at 4°C. Dilutions for functional antibodies were as follows: αSOX9 (rabbit, 1:1000, Chemicon/Millipore, Temecula, CA), αMucin2 (rabbit; 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), αLectin from Ulex europaeus-Atto 488 conjugate (1:500, Sigma-Aldrich, St. Louis, MO), αSucrase isomaltase (goat, 1:500, Santa Cruz Biotechnology), αCD326/EpCAM (rat, 1:500, BioLegend, San Diego, CA), αVillin (goat, 1:500, Santa Cruz Biotechnology), αCleaved Caspase 3 (rabbit, 1:400, Cell Signaling Technology, Inc., Danvers, MA), αGlucagon (rabbit, 1:250, Santa Cruz Biotechnology), αSomatostatin (goat, 1:500, Santa Cruz Biotechnology), αCarbonic Anhydrase (goat, 1:250, Santa Cruz Biotechnology), αProliferating cell nuclear antigen (mouse, 1:100, Chemicon/Millipore), αSP-1 Chromogranin A (Bovine) (rabbit, 1:1000, Immunostar, Hudson, WI), αSP-1 Chromogranin A (Porcine) (rabbit; 1:1000, Immunostar), αBeta-catenin
(mouse, 1:200, Cell signaling technology, Inc.), αMinichromosome Maintenance Complex 2 (Goat, 1:200, Santa Cruz Biotechnology) and αPhospho-histone H3 (rabbit, 1:200, Cell Signaling technology, Inc.). The BrdU staining protocol was performed on donated tissue from animals treated as described using a monoclonal antibody against BrdU (mouse; 1:100, Dako) [24]. All secondary antibodies (Jackson ImmunoResearch or Sigma, conjugated to Dylight 488, Cy 3 or Alexafluor 555) were diluted 1:500, and counter stained with bisBenzimide H 33258 nuclear stain (1:1000, Sigma). Background staining was negligible as determined by nonspecific IgG staining. Images were captured on an inverted fluorescence microscope (Olympus IX81, Tokyo, Japan) fitted with a digital camera (ORCA-flash 4.0 or -03G, Hamamatsu, Japan). The objective lenses used were X10, X20 and X40 with numerical apertures of 0.3, 0.45 and 0.6, respectively (LUC Plan FLN, Olympus, Tokyo, Japan). Immunohistochemically stained slides were imaged with an Olympus Bx45 microscope fitted with and Olympus DP72 camera. The objective lenses used were X10, X20 and X100 with numerical apertures of 0.3, 0.5 and 1.3 oil, respectively (UPlanFLN, Olympus).

Appropriate antibody specificity to cellular biomarker was supported by western blot analysis (Table 1), known localization from published immunofluorescence imaging of porcine [10,24-27] and mouse studies [28-32], as well as consistent repeatability of staining and appropriate localization along the crypt villus axis.

**qRT-PCR**

The jejunal mucosa was physically separated from seromuscular layers by scraping with a glass slide and was placed in RNAsse free microtubes and immediately placed in liquid
nitrogen. They were then stored at -80°C until use. Total RNA from jejunal tissue was extracted using the Qiagen RNeasy Minikit (Qiagen, Valencia, CA). Yield and quality of the extracts were determined by measuring absorbance at 260 and 280 nm (NanoDrop Technologies Thermo Fisher Scientific Wilmington, DE). The ratio of absorbance at 260:280 was between 2.03 and 2.07. 1 µg of RNA was converted to cDNA using the iScript cDNA synthesis kit (Biorad) and pooled. The cycle conditions were 5 min at 25°C, cDNA synthesis at 42°C for 30 min, denaturation at 85°C for 5 min and held at 4°C. Primers were designed based on published sequences of the pig target genes either manually or using the NCBI online primer design tool (Primer-BLAST, http://www.ncbi.nlm.nih.gov/tools/primer-blast/), Primer3 input (version 0.4.0, frodo.wi.mit.edu/). The specificity of the primers was checked using the NCBI online Blast tool (Primer-BLAST, http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Quantitative RT-PCR was performed utilizing the iTaq Universal SYBR green Supermix (BioRad). Standard curves were generated using serial dilutions of pooled cDNA from all three normal pigs tested in triplicate. The StepOnePlus Real time PCR system (Applied Biosystems by Life Technologies, Carlsbad, CA) was used. Cycle parameters included polymerase activation and DNA denaturation at 95°C for 30 sec. Forty cycles of amplification were performed with a 15 sec denaturation at 95°C and annealing/extension and plate read for 60 sec at 60°C. The melting curve analysis was performed at 65°C-95°C at 0.5°C increments, 5 sec per step. Melting curves were checked to ensure consistent amplification of a single PCR product.
Primer efficiency was calculated using the equation, Efficiency = 10^(-1/slope) – 1. All primer efficiencies were greater than 92%.

**Target validation by sequencing**

RT-PCR amplicons were analyzed on a 1.5% Agarose TE gel to assess size. The amplicons were purified using the USB ExoSAP-IT PCR product cleanup reagent (Affymetrix, Santa Clara, CA). Samples were sequenced (GENEWIZ, Research Triangle Park, NC), and the sequences were aligned to the gene target using the NCBI online Blast tool and Vector NTI alignment program (Life Technologies) for validation.

**Transmission Electron Microscopy**

TEM imaging was performed by the laboratory for advanced electron and light optic methods at North Carolina State University. The tissue fixation, preparation and image acquisition were performed as previously published [6,33,34].

**Crypt isolation, Enteroid Culture and Analysis**

Tissue was taken from 2-14 day-old wild type Yorkshire piglets euthanized for other research purposes. An 8-10cm segment of distal duodenum/proximal jejunum was surgically excised and opened longitudinally. The tissue was incubated for 20 min in a phosphate-buffered saline solution (PBS) containing 30mM Ethylenediaminetetraacetic acid (EDTA), 10 M Y-27632 (Selleck, Houston, TX), 1mM DTT (Sigma-Aldrich), and 100 g/mL penicillin/streptomycin at 4°C on an orbital shaking platform moving at 80 rpm. Tissue was transferred into a 37°C pre-warmed PBS solution containing 30mM EDTA, 10 M Y-27632, and 100ug/mL penicillin/streptomycin. The tissue was incubated in this solution at 37°C for
10 min and then shaken at 2.5 cycles sec⁻¹ to mobilize the crypt/villi units. If the desired yield was not achieved, the tissue was incubated in solution at 37°C for an additional 2 min and shaken for an additional 30 sec up to 5 times. Longer incubations caused extremely poor survival. Following the final shake, the remnant intestine was removed from the solution and the crypt/villi units were quantified and pelleted.

The pelleted epithelium was re-suspended directly into hESC Matrigel (BD Bioscience, San Jose, CA) supplemented with 100ngmL⁻¹ recombinant mouse Noggin (Peprotech, Rocky Hill, NJ), 500ngmL⁻¹ recombinant human R-Spondin (R&D Systems, Minneapolis, MN), 50ngmL⁻¹ recombinant mouse EGF (Life Technologies, Carlsbad, CA), 100ngmL⁻¹ recombinant human Wnt3a (R&D systems), 10 M Y-27632 (Selleck), 10 M SB202190 (Sigma), and 500nM LY2157299 (Selleck). Between 50 and 200 crypt/villi units were plated in 50uL of matrigel on a 48 well plate. After allowing the matrix to polymerize for 30 min at 37°C, each well was overlaid with 500uL of Advanced DMEM/F12 containing the supplements 1x N-2 supplement (Life technologies), 1x B-27 supplement minus vitamin A (Life technologies), 1x Glutamax (Life Technologies), 100ugmL⁻¹ penicillin/streptomycin, 1mM Hepes buffer (Life Technologies), and 1mM N-Acetylcysteine. Growth factors were added to the media 48 hr after plating and every 72 hr following that. The entire volume of media was changed 72 hr following plating and every 72 hr after that. Every 1-2 weeks organoids were passaged at a 1:5 ratio by mechanical dissociation, pelleting, and re-plating the pellet, into new Matrigel containing the growth supplements described above.
For histological studies, enteroids were fixed in a PBS solution containing 4% paraformaldehyde at room temperature for 20 min. Fixed enteroids were washed in a 30% sucrose solution and embedded in Optimal Cutting Temperature (OCT) media. Enteroids were cut into 5-8 μm sections and heat induced epitope retrieval was preformed when necessary by heating in reveal decloaker solution (Biocare Medical, Concord, CA) to 120°C for 30 sec and then 90°C for 10 sec inside a pressure cooker. Slides were allowed to cool to room temperature for 20 min prior to staining. Enteroids were permeabilized in a 0.3% Triton X-100 PBS solution for 20 min and then blocked in protein block solution (Dako, Carpinteria, CA) for 30 min. Primary antibodies, α-SOX9, α-Mucin2, α-sucrose isomaltase, and α-PCNA, at the same dilutions previously described, were applied to the slides in antibody diluent (Dako) and incubated for 2 hr at room temperature. All secondary staining was preformed with Cy3 conjugated antibodies (Jackson ImmunoResearch, West Grove, PA) diluted 1:500 in antibody diluent (Dako) incubated at room temperature for 45 min. Nuclei were marked with bisBenzimide H 33258 nuclear stain (Sigma/ Aldrich) diluted 1:1000 in PBS and applied for 5 min at room temperature. Confocal images were obtained using a Zeis LSM 710 laser scanning microscope. The objective lenses used were X40 water and X63 oil with numerical apertures 1.1 and 1.4, respectively (C-Apochromat, Plan-Apochromat, Zeiss, Jena, Germany).
RESULTS

Identification of cells of epithelial origin

To distinguish epithelial cells from those of mesenchymal and hematopoietic origin, we tested whether two candidate antibodies raised against mouse EpCAM (CD326) and human Villin would exclusively label pig epithelial cells. EpCAM is a pan-epithelial transmembrane protein that functions as a homotypic calcium-independent cell adhesion molecule [35]. EpCAM expression was observed in the basolateral membrane of all cells arranged along the luminal monolayer of the epithelial mucosa (Figure 1) [35]. In the small and large intestine, Villin expression is localized to the apical border of the intestinal epithelial cells due to its association with the microvillar actin filaments [36]. Villin expression demonstrated a gradient of increased staining intensity from the crypt toward the lumen as has been described in normal mammalian tissue (Figure 1) [36].

Markers of proliferation and apoptosis identify cells within each stage of the cell cycle

Assessing the proliferative capacity of IESCs and their progenitors is essential for monitoring regenerative responses in the small intestine and colon. Proliferating Cell Nuclear Antigen (PCNA) is accepted as a general proliferation marker and localized to the nucleus of the majority of cells constituting the crypt base in porcine tissue (Figure 2). Minichromosome Maintenance Complex 2 (MCM2) serves as a biomarker for cells that are peaking at G1-S phase [28]. The thymidine analogs BrdU or EdU are well established markers for cells in S-phase [29]. Both MCM2 and BrdU were localized within the nuclei of a subset of cells within the proliferative zone (Figure 2). Histone H3 is phosphorylated (pH3) at the end of
prophase and represents a suitable marker for cells in G2-M-phase of the cell cycle. pH3 positive cells marked a minority of cells within all crypt bases consistent with the limited number of cells at this point in the cell cycle [30]. Immunostaining jejunal and colonic tissues for each of these proliferation markers demonstrates robust cross-reactivity with cells located in the proliferative zone of the crypt as has been demonstrated in mice (Figure 2) [28, 37, 38]. These validated antibodies for porcine gut tissue represent a comprehensive set of reagents for detailed study of the proliferative response in physiology, disease and injury induced regeneration.

Interrogating apoptotic dynamics is equally important to understanding mechanisms underlying a regenerative response [39]. Caspase3 cleavage represents the execution phase of apoptosis [39]. The antibody against cleaved human caspase 3 (CASP3) marked few cells at the villus tip in porcine small intestine and colon where an apoptotic event known as ‘anoikis’ typically occurs (Figure 2) [26]. Rare cells at the base of the crypts were observed which is consistent with rare apoptotic incidences in physiologic renewal.

**Identification of stem and progenitor cell populations**

Next, we aimed to distinguish stem/progenitor cells from fully differentiated lineages. Recent evidence supports the presence of IESCs that exist in different states of proliferative capacity [40, 41]. Crypt-based columnar ‘active’ stem cells (CBCs) are located intercalated between Paneth cells in mice, are constantly dividing, and primarily responsible for the burden of homeostatic epithelial regeneration. Unfortunately, the commercially available antibodies used to detect the CBC population, LGR5, OLFM4, and CD24 did not demonstrate cross
reactivity with active CBC stem cells in porcine intestinal tissue (data not shown; Table 2). However, antibodies raised against SOX9, a member of the SRY-family of transcription factors primarily expressed in CBCs and transit-amplifying progenitor cells, demonstrated the ability to detect proliferating cells in the base of the small intestine and colonic crypts with distinct localization to the nucleus of positive cells (Figure 3) [31,42,43].

Another putative stem cell population, termed ‘the +4 stem cells’, are a slower dividing ‘reserve’ or facultative stem cell that primarily reside above the Paneth cell compartment in mice and humans [41,44]. In order to identify a putative +4 stem cell population, an antibody against human HOPX, an atypical homeodomain containing protein, was tested. Immunostaining for HOPX showed cross reactivity to cells in pig intestinal tissue with expression pattern restricted to the ‘+4’ stem cell zone consistent with what has been observed in mice (Figure 3) [32].

**Identification of absorptive cell lineage**

Enterocytes function to absorb nutrients, electrolytes and water and are the predominant cell type within the intestinal mucosa [33]. The histologic identification of this cell lineage is used to assess whether appropriate cellular differentiation is occurring during a regenerative response [45,46]. Immunostaining for the digestive enzymes, sucrase isomaltase (SIM) and carbonic anhydrase II (CAII) clearly demonstrates localization to the apical brush-border of the absorptive enterocytes in the small and large intestine, respectively [47,48]. There was no positive immunostaining of SIM in the colon or CAII in the small intestine indicating
these antibodies are suitable to differentially distinguish between these two absorptive cell
types (Figure 4).

**Identification of secretory cell lineage**

In mice and humans three primary secretory lineages exist, enteroendocrine cells, goblet cells
and paneth cells. Enteroendocrine cells represent a minor population of cells that secrete
various hormones that regulate gut physiology and appetite control [49]. Chromogranin A
(CgA) is an acidic glycoprotein that localizes within secretory granules of nearly all
enteroendocrine cells and is considered a general marker for all enteroendocrine cell subtypes
[50]. Immunostaining for CgA localized to the cytoplasm of a minority of cells throughout
the length of the small intestine and colon, consistent in morphology with enteroendocrine
cells found in other animal species [31,51] (Figure 5). Subtypes of enteroendocrine cells
could also be identified in pig intestinal epithelium. Gastrin (GAST) is a hormone secreted
by G cells in the stomach and duodenum, and it functions as both a mucosal growth factor
and stimulator of mast and parietal cells [49]. An antibody against human Gastrin (GAST)
identified a minority of cells in the duodenum while no immunostaining was observed along
other segments of the small or large intestine, an observation consistent with the expression
pattern of GAST observed in humans [49] (Figure 5). Somatostatin (SST) is a hormone
secreted by delta cells throughout the length of the intestine and functions to block the
release of many gut hormones ultimately affecting epithelial transport and intestinal motility
[49,52]. SST-positive cells were observed intermittently in all segments of the porcine
intestine (Figure 5). The main role of glucagon-like peptide 1 (GLP-1) is to delay gastric
emptying and signal post prandial satiety and that of glucagon-like peptide 2 (GLP-2) is to stimulate mucosal enterocyte proliferation [53]. Reactivity to the glucagon antibody, with cytoplasmic localization, was observed within the enteroendocrine cells along the proximal distal axis of the small intestine (Figure 5) consistent with that observed in mice and humans [53,54].

Secretory goblet cells produce mucins that are integral to intestinal physiology by providing protection of the epithelial surface as well as aiding in absorption [55]. Immunostaining for Mucin 2 (MUC2) exclusively marked mucous-producing cells along the entire length of small and large intestine as well as the crypt-villus axis (Figure 6). Positive staining was localized to the cytoplasm of positive cells and within the luminal surface which is the expected location of mucinous secretions. *Ulex europaeus* agglutinin-1 (UEA-1) is a lectin that specifically binds to alpha-linked fructose receptors located on cell surface glycoproteins and glycolipids and is used to detect both goblet and Paneth cells in mice [56]. UEA-1 bound to the mucinous secretions of goblet cells in porcine small and large intestine (Figure 6). Besides marking goblet cells in fixed tissue sections, UAE-1 is likely suitable for fluorescence activated cell sorting of live cells [37].

The existence of the Paneth cell in the pig remains disputed [57,58]. No eosin or toluidine blue staining, which typically marks apically located granules in Paneth cells, was identified in the crypt base of porcine small intestine (data not shown). In mice lysozyme expression is a biomarker for Paneth cells [59,60]. Lysozyme staining was not observed in
the epithelium of porcine small intestine despite the use of multiple anti-lysozyme antibodies (data not shown; Table 2).

**TEM characterization of porcine crypt-based cells**

Transmission electron microscopy allows for the morphological identification of cell lineage by the presence or absence of sub-cellular features [6,33,61-63]. Comparative analysis of crypt-based cells from various organisms has enabled the characterization of cell types that are consistent with particular lineages [6,33]. A complete description of the pig small intestine and colon, to the best of our knowledge, has not been previously described. At least two distinct cell types were distinguishable in the crypt base of the pig small intestine and colon (Figure 7). Multiple irregularly shaped, small, columnar cells with basally located nuclei and scarce cytoplasm, consistent in appearance with CBC cells of mice and humans [33,64], were interspersed between large pyramidal shaped cells with large supranuclear clear mucoid vesicles and small electron dense bodies (Figure 7). The appearance of these large mucoid filled cells was not entirely consistent with the accepted morphological features of Paneth cells in other mammalian species [63,65]. The goblet cell within the small intestine and colon of the pig possessed small basally located nuclei that were notably distended apically with mucinous globules consistent with the accepted ultrastructural appearance in mammalian intestinal tissue (Figure 7) [61,66]. The enteroendocrine cells of the pig intestine demonstrated a narrow apex and wide base with many small, spheroidal, electron dense granules in the infranuclear region as is classically described in mammals (Figure 7) [62,66,67]. The mature absorptive enterocytes of the pig intestine were clearly
distinguishable as columnar shaped cells with centrally located nuclei. Other key features of these cells include multiple organelles and a lack of secretory granules within the cytoplasm and a position closer to the gut lumen in both the small intestine and colon, as has been described in other mammals (Figure 7) [33,66]. These electron micrographs of the porcine crypt base represent a foundation for morphologic description of cells in normal, injury and disease states.

**Assessment of gene expression in stem/progenitor and differentiated cell lineages**

Measuring gene expression is essential to understanding mechanisms of injury, disease and the stem cell-driven regeneration. Because qPCR primer sets to detect target gene expression in pig are limited, we designed and validated primers to genetic biomarkers of stem/progenitor cells, differentiated cell lineages, and important signal transduction pathways involved in regeneration [38,44,68,69]. Twenty one candidate PCR primers were designed and six were previously described [27,70,71] (Table 3). Amplification of cDNA generated from total intestinal RNA demonstrated expression of Wnt3a+ and Lgr5+, which are important regulators of IESC maintenance [59,59,72]. Genes important for Notch pathway regulation, Atoh1+, Dll4+, and Hes1+, were amplified from epithelial-derived cDNA. These genes are critical for appropriate cell differentiation and fate and important to interrogate in regeneration [38]. ‘Active’ stem cell markers, Olfm4+, Ascl2+, Sox9+, CD24+, were used to monitor stem cell renewal and maintenance. Hallmark genetic biomarkers for differentiated lineages were detected by amplification of Muc2+ and Itf+ (goblet cells), CgA+ and Cck+ (enteroendocrine cells), and Sglt1+ and L-Fabp+ (absorptive enterocytes) [43,73-75]. Single
amplicons of the appropriate size were verified by gel electrophoresis and DNA sequencing of all amplicons validated target gene amplification. Primers efficiencies were calculated using the equation, Efficiency = 10^(-1/slope) – 1 and demonstrated >92% efficiency (Table 3). Under the conditions described, no primer-dimers were observed.

**In vitro culture of porcine crypts**

Long-term culture of intestinal epithelial stem cells in mice and humans has only recently been accomplished and has revolutionized the ability to conduct detailed mechanistic studies in a highly controlled manner [38,76-78]. Pig crypts isolated from jejunal tissue were introduced into a modified 3-dimensional (3-D) culture environment similar to the culture conditions that support growth of mouse and human enteroids [42,76-79]. Within 24 hours of plating whole pig crypts in matrigel with defined medium, enterosheres formed (Figure 8, day 2) [80]. These structures persisted until day 4 and then began to convert into enteroids that possessed columnar epithelial cells and primitive crypt buds (Figure 8, day 4). Mature crypt buds developed by day 14 and by day 21 enteroids were fully formed (Figure 8, day 14 and 21). Enteroid cross sections demonstrated the presence of SOX9\(^+\) stem/progenitor cell populations, PCNA\(^+\) zones of proliferation, MUC2\(^+\) goblet cells, CgA\(^+\) enteroendocrine cells, and SIM\(^+\) absorptive enterocytes (Figure 9). Fully developed enteroids were allowed to persist in culture for two weeks at which point they were passaged. To date, enteroids have been passaged 8 times representing a total of 4.5 months in culture. There has been no apparent decrease in enteroid formation over this time.
Discussion

A recent NIH symposium entitled “Improving Animal Models for Regenerative Medicine” focused on the development of large animal models for the study of human disease [81]. The motivation for the symposium was the persistent failure in translating murine models to clinical treatments [81]. The utility of the pig as a large animal model has been well-documented for many body systems [15-21,82-88]; and the similarities between the pig and human gastrointestinal system position the pig as a promising species for animal models of gastrointestinal disease. Seminal advances made in murine intestinal stem cell biology now position investigators to answer clinically relevant problems from the perspective of stem cell-driven epithelial regeneration. Data presented in this study lay the foundation for developing the pig as a large and physiologically relevant animal model for these studies. This study identifies, develops and validates a range of genetic biomarkers and crypt culture strategies that will enable investigators to assess stem cell maintenance and potency in both the small and large intestine of pig models of physiology, injury and disease.

Few studies use commercially available antibodies on porcine intestinal tissue to assess cell lineage allocation, thus limiting the ability to effectively monitor and analyze epithelial regenerative responses [10,25-27,89,90]. To address this problem, we identified a comprehensive set of commercially available antibodies that would cross-react with target pig proteins to enable detection of stem/progenitor and post-mitotic lineages. The ability to specifically observe epithelial dynamics during physiology and disease is critical to understanding and quantifying regenerative processes and therapeutic interventions [10].
Appropriate designation of EpCAM expressing cells, for example, can offer insight into epithelial cell-cell adhesion, migration, signaling, differentiation and proliferation since it plays a key role in these cellular functions [35]. Additionally, assessing the proliferative capacity of IESCs and their progenitors is essential for monitoring regenerative responses in the small intestine and colon. Detailed analysis of cell cycle progression during injury and disease states could yield important information pertaining to repair mechanisms. The maintenance of epithelial barrier function depends on the tightly controlled balance between cellular proliferation, differentiation and apoptosis. Monitoring the phenotypic changes resulting from injury or in genetically modified animals can shed light into critical homeostatic pathways. These pathways can now be monitored in pig models utilizing the biomarkers for PCNA, MCM2, BrdU, pH3 and CASP3.

Identification of stem and progenitor cell populations has proven critical to deciphering important cellular pathways as well as the impact and response of these cells to injury. In mice, ‘active’ IESCs are marked by Lgr5, Olfm4, Ascl2, Sox9 [31,43,59,60,73,74,91,92]. Unfortunately, of the commercially available antibodies for CBCs tested, only SOX9 demonstrated positive staining. Sox9 is a member of the SRY-family of transcription factors that is primarily expressed in crypt-based columnar stem cells and transit-amplifying progenitor cells [31,42,43]. Enteroendocrine and Tuft cells also express very high levels of Sox9 and recent evidence suggests that the Sox9 high population has ‘reserve’ IESC capacity [31,42-44,75]. Slower dividing ‘reserve’ or facultative stem cells primarily reside above the Paneth cell compartment in mice and humans [41,44]. These have
been historically termed ‘the +4 stem cells’ which denotes the cell position in the crypt base where they most likely exist [93]. As the name suggests, facultative IESCs appear to respond to injury stimulus to re-enter an active state to regenerate the epithelium [44,69,94]. To some extent, the reserve IESC population is marked by *Bmi1, Tert, Hopx, and Lrig1* in mice [37,95-101]. Immunostaining for HOPX will enable future studies utilizing porcine models to evaluate the role of these putative ‘reserve’ IESCs during and following intestinal injury.

The ability to observe and quantify fully differentiated cells is critical to understanding the dynamics of epithelial regeneration in normal homeostasis, injury, and repair. CgA was used as a general marker of enteroendocrine cells but multiple sub-types were also characterized. The hormones produced by all of the enteroendocrine cells are integral to crypt cell physiology. Proglucagon, for example, is produced and cleaved within the L-type enteroendocrine cells into glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) [54]. Interestingly, glucagon-like peptides demonstrate immunoreactivity to antibodies against glucagon, the product of cleaved proglucagon in the pancreas [54]. The potential therapeutic benefits of both GLP-1 and GLP-2 are of interest for multiple important human diseases. GLP-1 is integral to both signaling satiety and in glucose homeostasis. GLP-1 based treatments are now well established in the management of type 2 diabetes and have been proposed for the treatment of obesity [102]. Research into the therapeutic benefits of GLP-2 administration to hasten epithelial proliferation with direct stimulatory effects on the stem cell population following intestinal resection have also been studied [12,103-105]. The
ability to clearly identify GLP-1 and GLP-2 producing cells may then prove integral in pig models that study weight management, diabetes and short bowel syndrome [12,102,103,106].

Electron microscopy supported the immunohistochemical findings on porcine small and large bowel showing clear ultrastructural characteristics of lineage states along the crypt villus axis. Electron microscopy provided morphological evidence suitable for identification of cells consistent with CBC stem cells, goblet cells, enteroendocrine cells and absorptive enterocytes. TEM studies have and will continue to contribute to understanding the ultrastructural cellular changes that occur during disease and repair processes as well as following signaling pathway manipulation that may not be possible with immunohistochemical studies [95,107,108]. TEM has been utilized, for example, to visualize invasion of intestinal epithelium by viral particles and detailed evidence of the impaired structural integrity of epithelial tight junctions in disease [108-110].

Quantitative gene expression analysis is highly sensitive and contributes to a more complete characterization of intestinal epithelium in the pig. In this study, the PCR primers were specifically designed to amplify target genes currently used as molecular signatures for each cell type and important signaling molecules known to regulate intestinal homeostasis. Interpreting gene expression dynamics during and following intestinal injury can give insight into the cell populations, CBCs (Lgr5⁺, Olfm4⁺, Sox9⁺, Ascl2⁺, CD24⁺) versus ‘reserve’ stem cells (Bmi1⁺), that are compromised or stimulated in the process [44,69,95]. Additionally, the impact on stem cell homeostasis (Wnt3a⁺), proliferative capacity (PCNA⁺), and cell fate determination (Atoh⁺, Hes1⁺) can be evaluated to interpret epithelial regenerative capacity
Ultimately, by evaluating the specific post-mitotic cell populations, absorptive enterocyte (Sglt1+, L-Fabp+) or specific secretory cells (Muc2+, Itf+, CgA+, Cck+) that are sensitive to or upregulated in response to injury, potential therapeutics aimed at signal pathway manipulation can be pursued [38,75,112,113]. Having defined qPCR primers allows for thorough and reliable interpretation of cellular pathway dynamics, and therefore, insight into mechanistic processes controlling epithelial regeneration.

An intriguing observation in cross species comparison of the small intestine epithelium is the presence of the Paneth cell lineage in some species and the absence in others. Paneth cells are long-lived post-mitotic cells that reside in the base of the small intestinal crypts in some species. In mice, Paneth cells have been implicated in serving as a ‘nurse’ cell for the CBC stem cells by secreting WNTs and presenting NOTCH-ligands [38,59,60]. In the colon, evidence indicates that a c-KIT+ MUC2+ cell may serve as an analogous counterpart to the Paneth cell in the colon [114]. The existence of Paneth cells in pigs is still debated [57,58]. Morphologically, Paneth cells can be identified by the ultrastructural presence of an elongated flattened nucleus, large cytoplasm and secretory granules [63,65]. In our porcine studies, TEM and immunostaining do not support the presence of a bona fide Paneth cell; however, our study presents data that is consistent with the interpretation that a Paneth cell equivalent, similar to that of the mouse colon, may be present in pig small intestinal crypts.

TEM and morphometric analyses presented in our study indicate that at least two different cell types reside within the crypt base. A small columnar shaped cell with a basally
located nucleus and sparse cytoplasm seems consistent with the accepted ultrastructural appearance of the CBCs in other species. Interspersed between these cells is a larger cell type with electron dense supranuclear secretory vesicles. Many of these cells also contain clear, mucoid in appearance, apically located vesicles. Interestingly, the ultrastructural appearance of the secretory granules vary in size and number as well as electron density between those species accepted as having Paneth cells [65]. Post-mitotic cells (PCNA and MCM2 negative) are also present at the crypt base in the pig. These cells appear to be Sox9+ and Muc2+, and UEA-1+, which is consistent with the Paneth-like cells identified in mouse colonic crypts and marked by c-KIT [114]. Unfortunately, we were unable to verify c-KIT staining in these cells due to the lack of cross-reactivity with the c-KIT antibodies tested. These data point to the need for more detailed analysis of gene expression to determine if they produce functionally relevant mitogens and morphogens similar to Paneth or Paneth-like cells in the mouse small intestine and colon.

Besides enabling detailed mechanistic studies in vitro, long-term crypt culture from the pig small intestine represents a significant advancement toward developing tissue-engineering strategies and stem cell-based therapies. Organoid units from primary small intestine have been placed on biodegradable scaffold tubes and then implanted into the omentum of an autologous host [25]. The engineered tissue demonstrated morphological characteristics of small bowel 7-weeks post implantation. While these studies used biopsied samples derived from resected tissue, it is likely that a therapeutic mass of tissue would be required to be clinically relevant. The culture method developed in this study will enable a
small number of crypts, perhaps from a biopsy, to be expanded *ex vivo* to increase the mass of epithelium required to test therapeutic strategies for tissue replacement. Detection of successful and functional tissue replacement is fundamental to monitoring outcomes of these new approaches to treat disease and injury to the intestinal epithelium. This new long-term crypt culture model also represents a significant advancement toward development of pharmaceutical screening modalities, stem cell therapy models, and tissue replacement strategies that can all be tested in a translationally relevant context. The comprehensive set of reagents identified, developed and validated will serve as a foundation for using the pig as a translational model to study stem cell-driven regeneration of the intestinal epithelium.

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**Growth Factors and Signaling Molecules**
Figure 1. **Markers to identify cells of epithelial origin.** Identification of intestinal cells of epithelial origin within the porcine small intestine and colon are shown. Immunostaining for EpCAM, a pan-epithelial transmembrane protein, demonstrated expression in the basolateral membrane of all cells in both the small intestine and colon arranged along the luminal monolayer of the epithelial mucosa. Immunostaining for Villin, a protein associated with the microvillar actin filaments, showed a gradient of expression localized to the apical border of small intestinal and colonic epithelial cells with increasing intensity in cells located closer to the lumen. All specific markers (red). Nuclei (blue). Scale bar 200µm, inset scale bar 50 µm.
Figure 2. Markers to assess proliferation and apoptosis. Identification of proliferative cells in different stages of the cell cycle and those undergoing apoptosis in porcine small intestine and colon are shown. All proliferative markers localized to the nuclei of positive epithelial cells. Immunostaining for PCNA, a general marker for cellular proliferation, demonstrated the greatest number of positive cells compared to the other markers of proliferation. Immunostaining for MCM2, a marker of cells at the G1 stage of the cell cycle, was localized to a subpopulation of cells within the crypt base. Immunostaining for BrdU, a marker of cells within the S stage of the cell cycle, was also localized to a subpopulation of cells within the crypt base. Immunostaining for pH3, a marker for cells between the G2 – M stage of the cell cycle was similarly localized but to fewer cells. Immunostaining for cleaved caspase 3, an indicator of apoptosis, marked a few expressing cells near the villus tip within small intestine and the luminal surface of colon. All specific markers (red). Nuclei (blue) Scale bar 200µm, inset scale bar 50 µm.
Figure 3. Markers to identify stem, progenitor, and transit amplifying cells.

Identification of stem, progenitor, and transit amplifying cells in porcine small intestine and colon are shown. Immunostaining for SOX9, a member of the SRY-family of transcription factors that is primarily expressed in CBC cells and transit-amplifying progenitor cells, is localized to the nuclei of all cells within the crypt base of both the small intestine and colon. Immunostaining for SOX9 demonstrates colocalization with the proliferative cells (PCNA+) at the crypt base. Immunostaining for HOPX, an atypical homeodomain containing protein, demonstrated marking of cells consistent in location and numbers with a ‘reserve’ IESC population. All specific markers (red or green). Nuclei (blue). Scale bar 200 µm, inset scale bar 50 µm.
Figure 4. Markers to identify the absorptive cell lineage. Identification of absorptive enterocytes in porcine small intestine and colon are shown. Immunostaining for enterocytes demonstrates sucrase isomaltase, a digestive enzyme, marking the apical brush-border of cells in the small intestine. Carbonic anhydrase, a digestive enzyme, positively identified absorptive cells in the colon with staining localized to the apical brush-border of these cells. All specific markers (red). Nuclei (blue). Scale bar 200µm, inset scale bar 50 µm.
Figure 5. Markers to identify the secretory cell lineage. Identification of secretory cells in porcine small intestine and colon are shown. Immunostaining for enteroendocrine cells, the hormone secreting cells important to gut homeostasis, using antibodies against CgA, SST, GLP-1 and GAST, demonstrated staining within the cytoplasm of positive cells of the small intestine. A similar pattern of staining was identified in the colon for CgA, SST and GLP-1. All specific markers (red). Nuclei (blue). Scale bar 200µm, inset scale bar 50 µm.
Figure 6. Markers to identify goblet cells. Identification of goblet cells in porcine small intestine and colon are shown. Goblet cells, the mucus producing cells, were identified by immunostaining for MUC2, a component of mucin, and UEA-1, a lectin that specifically binds to alpha-linked fructose receptors. The mucinous secretions of multiple cells in both the small intestine and colon were positively identified. All specific markers (red). Nuclei (blue). Scale bar 200µm, inset scale bar 50 µm.
**Figure 7.** Transmission electron microscopic epithelial characterization. The ultrastructural appearance and characterization of porcine small intestine and colonic epithelial cells are shown. All cell types were morphologically distinguishable. Crypt base columnar stem cells, goblet cells, enteroendocrine cells, and absorptive enterocytes are all marked with asterisks. ‘L’ indicates lumen. Small Intestinal images, scale bar 2 µm. Colon images, scale bar 5 µm.
Figure 8. *In vitro culture of porcine crypts.* The *in vitro* isolation, growth and maintenance of enteroids derived from porcine intestinal crypts are shown. On the day of collection (day 0) crypts maintain their morphologic appearance. As the enteroids develop they become enterospheres (day 2) and progressively enlarge and form complex structures with a pseudolumen and crypt-like structures (days 4, 8, 14, 21). Scale Bar 100 µm.
**Figure 9.** Markers to identify cell lineages within *in vitro* cultures. The identification of specific cell lineages within *in vitro* cultures of porcine crypts is shown. The existence of stem/progenitor and differentiated lineages were confirmed in enteroids utilizing the established genetic biomarkers for cell lineage identification: anti-SOX9 (stem/progenitor), anti-PCNA (proliferation), anti-CgA (enteroendocrine), anti-MUC2 (goblet) and anti-sucrase isomaltase (absorptive enterocyte) antibodies. All specific markers (red). Nuclei, blue. Scale Bar 50 µm, inset scale bar 10 µm.
REFERENCES


10.3390/ijms12031727.


100. Sangiorgi E, Capecchi MR. (2008) Bmi1 is expressed in vivo in intestinal stem cells. Nat Genet 40: 915-920. 10.1038/ng.165; 10.1038/ng.165.


CHAPTER V

CHARACTERIZATION OF DISCRETE EQUINE INTESTINAL EPITHELIAL CELL LINEAGES: A PLATFORM FOR FUTURE REGENERATIVE STUDIES
Abstract

Objective

The objective of this study was to characterize the normal equine small and large intestinal epithelium with an emphasis on the stem cell niche constituents.

Tissue Samples

Mucosal biopsies from the small and large intestine were obtained from 12 healthy horses of various age, breed, and sex euthanized for reasons unrelated to gastrointestinal disease or systemic disease.

Procedures

Intestinal biopsies were sharply dissected immediately following euthanasia. Specimens were prepared for immunohistochemical, immunofluorescence and transmission electron microscopic imaging in order to detect and characterize each epithelial cell type. Antibodies against protein biomarkers of cellular identification were selected based on known expression in other mammalian species.

Results

All intestinal epithelial cell types were positively identified with immunostaining and were further morphologically characterized using transmission electron microscopy. Some differences in biomarker expression and antibody cross reactivity were identified in equine tissue as compared to other species. However, each known type of epithelial cell was identified in equine tissue.
Conclusion and Clinical Relevance

Methodology presented in the present studies will enhance the detection of stem and progenitor cells as well as post mitotic cell lineages in biopsied equine intestinal tissues, which may have relevance to mucosal regenerative potential and survival in horses with colic.

Introduction

Studies have shown that intestinal ischemic injury that denudes greater than 50% of the glandular epithelium, such as occurs with large colon volvulus, is associated with a poor prognosis for survival (23). Such severe mucosal injury likely compromises the proliferative cell population that resides within the lower 50% of the glandular crypts. This population of cells is responsible for creating new epithelium every 3-5 days. However, research to explore this proliferative compartment of the intestinal mucosa in further detail has been lacking because, until recently, the technology to distinctly identify unique cell types did not exist. Protein biomarkers for intestinal epithelial stem cells have only been identified and described in rodents since 2007,(1) and in pigs in one recent study (12). The glandular epithelium is arranged in structures called crypts of Lieberkühn. At the base of the crypts are stem cells flanked by Paneth cells. Immediately adjacent to these cells are progenitor cells, and collectively this region of the crypts is termed the stem cell niche (4). To the author’s knowledge, no study has fully characterized the equine intestinal epithelium based on protein biomarker expression and ultrastructural cellular appearance.
Recent advances in the field of intestinal stem cell biology have enabled detailed study of the stem cell niche as the potential source of novel therapeutic targets to enhance intestinal mucosal regeneration (14, 15). The objective of this study was to characterize the equine normal intestinal epithelium with an emphasis on the stem cell niche constituents. Histopathology, immunofluorescence and electron microscopy were used to distinguish stem, partially differentiated and post-mitotic/fully differentiated epithelial cells.

**Materials and Methods**

*Ethics Statement*

All animal studies were approved by the Institutional Animal Care and use Committee at North Carolina State University.

*Animals and Sample Collection*

Tissues were obtained from healthy horses of various age, breed, and sex euthanized for reasons unrelated to this project. Sections from the gastrointestinal tract including the duodenum, jejunum, ileum, proximal (ascending) and distal (descending) colon were sharply dissected.

*Histological and Immunofluorescence Analyses*

Tissues were rinsed with a 1x phosphate-buffered saline (PBS). For immunohistochemical analysis, tissue was fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned (~5-8 µm thickness). Slides were stained with hematoxylin and eosin or toluidine blue to visualize crypt and villus morphology. For immunofluorescence, rinsed tissue was fixed in 4% paraformaldehyde (PFA) solution for 14-18 hours at 4°C. The tissue was
transferred to 30% sucrose for at least 24 hours at 4°C, embedded in optimal cutting
temperature (OCT) media, frozen and sectioned at 5-8µm thickness using a cryotome and
mounted on positively charged glass slides. Sections were washed three times with PBS to
remove OCT. When necessary, heat induced epitope retrieval (HIER) was performed by
placing the slides into reveal decloaker solution (Biocare Medical, Concord, CA) for 30
seconds at 120°C and then 90°C for 10 seconds, in a pressure cooker. The slides were
allowed to cool at room temperature for 20 minutes prior to continuing. Tissue
permeabilization was performed on all slides with PBS-0.3% Triton X-100 for 20 minutes,
washed twice with PBS and incubated in blocking medium (Dako, Carpinteria, CA). Primary
antibodies were applied to the tissue section in an antibody diluent (Dako) and incubated
overnight at 4°C. Dilutions for functional antibodies were as follows: αSOX9 (rabbit, 1:1000,
Millipore, Temecula, CA), αMucin2 (rabbit, 1:1000, Santa Cruz Biotechnology, Santa Cruz,
CA), αLectin from Ulex europaeus-Atto 488 conjugate (1:500, Sigma-Aldrich, St. Louis,
MO), αsucrose isomaltase (goat, 1:500, Santa Cruz Biotechnology), αCD44 (rat, 1:400,
Biolegend, San Diego, CA), αVillin (goat, 1:500, Santa Cruz Biotechnology), αCleaved
Caspase 3 (rabbit, 1:400, Cell Signaling Technology, Inc., Danvers, MA), αGlucagon (rabbit,
1:250, Santa Cruz Biotechnology), αCarbonic Anhydrase (goat, 1:250, Santa Cruz
Biotechnology), αProliferating cell nuclear antigen (mouse, 1:100, Millipore), αProliferating
cell nuclear antigen (mouse, 1:500, Abcam, Cambridge, MA), αSP-1 Chromogranin A
(rabbit, 1:1000, Immunostar, Hudson, WI), αBeta-catenin (mouse, 1:200, Cell Signaling
Technology, Inc.), αMinichromosome Maintenance Complex 2 (goat, 1:200, Santa Cruz
Biotechnology), and αPhospho-histone H3 (rabbit, 1:500, Millipore). All secondary antibodies (Invitrogen AlexaFluor 555 or Cy3) were diluted to 1:500 and counter stained with bisBenzimide H 33258 nuclear stain (1:1000, Sigma). Images were captured on an inverted fluorescence microscope (Olympus IX81, Tokyo, Japan) fitted with a digital camera (ORCA-flash 4.0 or -03G, Hamamatsu, Japan). The objective lenses used were X10, X20, and X40 with numerical apertures of 0.3, 0.45, and 0.6, respectively (LUC Plan FLN, Olympus, Tokyo, Japan). Immunohistochemically stained slides were imaged with an Olympus Bx45 microscope fitted with an Olympus DP72 camera. The objective lenses used were X10, X20, X40 and X60 oil with numerical apertures of 0.3, 0.7, 0.6 and 1.35 respectively (UPlanFLN, Olympus).

Appropriate antibody specificity to cellular biomarker was supported by known localization from published immunofluorescence imaging of porcine and mouse studies, as well as consistent repeatability of staining and appropriate localization along the crypt villus axis.

*Transmission Electron Microscopy (TEM)*

TEM imaging was performed by the laboratory for advanced electron and light optic methods at North Carolina State University. The tissue fixation, preparation and image acquisition were performed as previously published (4, 5, 8).
Results

Identification of Cells of Epithelial Origin

Overall background staining was deemed to be negligible, as determined by nonspecific IgG staining. Initially, it was important to distinguish cells of epithelial origin from those of mesenchymal and hematopoietic origin in the small intestine and colon of normal equine tissue. This was achieved using three different antibodies: Villin, CD44 and β-catenin. The antibody raised against human Villin localized to the apical brush border of equine intestinal epithelial cells as would be expected due to its known association with microvillar actin filaments (24). A gradient of expression was noted from the crypt base toward the lumen as has been described in other normal mammalian intestine (Fig 1) (24). Epithelial cell identification was also achieved in both the small and large intestine using an antibody raised against CD44, a receptor for hyaluronic acid that mediates cell-cell and cell-matrix interactions. Positive expression was identified within the cell membrane, as expected. However, additional antibody binding was noted with intense staining throughout the lamina propria. Finally, the basolateral border of all epithelial cells along the entire crypt villous axis was detected with an antibody raised again human β-catenin (Fig 1).

Detection of Proliferating and Apoptotic Cells

Following the general identification of cells of epithelial origin, we aimed to more specifically detect cells within discrete stages of the cell cycle. Immunostaining for protein biomarkers of cellular proliferation positively identified cells within the crypt, and their nuclear localization was confirmed my co-immunostaining with bizbenzamide. Proliferating
cell nuclear antigen (PCNA) was the most widely distributed within the crypt as compared to other markers of cellular proliferation (Fig 2). Minichromosome Maintenance Complex 2 (MCM2) is a marker of cells at the G1 stage of cellular proliferation and positively identified cells within the crypt base (Fig 2). Phosphohistone H3 (pH3) is expressed by cells between the G2 – M stage of the cell cycle and localized to the fewest number of cells in equine mucosa (Fig 2). Since the balance between the addition and loss of cells is critical to normal epithelial homeostasis and to studies that aim to evaluate changes within damaged and regenerating tissue, a marker for cellular apoptosis was also confirmed. An antibody against cleaved caspase 3 (Casp3) positively identified cells in both the small and large intestine. As is expected with normal tissue, few positive cells were identified and they were located near the luminal surface (Fig 2).

Detection of Specific Epithelial Cell Types

Stem and progenitor cells are a specific subclass of proliferating cells. These were clearly identified deep within the crypt base with a marker against Sex Determining Regions Y-box 9 (SOX9), a member of the SRY-family of transcription factors. This protein is also expressed within the nucleus and immunostaining colocalized with bisbenzamide (Fig 3). Unfortunately, other antibodies against biomarkers of stem and progenitor cells, including antibodies against LGR5, OLFM4, CD24 and HOPX were not found to cross react with equine tissue (Table 2). However, antibodies raised against protein biomarkers to distinguish mature, fully differentiated cells did cross react. These cells are broadly classified as either absorptive or secretory but can then be further subdivided.
Small and large intestinal absorptive enterocytes normally express digestive enzymes within the brush border that can be used as protein biomarkers of cellular identification. An antibody raised against human sucrase isomaltase (SIM) in the small intestine and carbonic anhydrase II (CAII) in the large intestine localized to the apical border of these cells (Fig 4). The secretory cell lineage is subdivided into enteroendocrine, goblet and Paneth cells. Enteroendocrine cells make up a minority of cells along the entire length of the small and large intestine and secrete hormones packaged within small cytoplasmic granules located near the basement membrane. Chromogranin A (CgA) is commonly used as a general marker of all subtypes of enteroendocrine cells because of its expression within the membrane of these granules (9). Positive identification of these cells was successfully achieved with fluorescence labelling within the cytoplasm of cells distributed throughout the length of the intestinal tract (Fig 5) (2). Further positive confirmation of this cell type within equine intestinal epithelium was achieved using TEM images by the appearance of cells with small, cytoplasmic, electron dense granules located basolaterally (Fig 6). A subtype of enteroendocrine cells, the L-cell, which secretes glucagon-like peptide (GLP), was also identified using an antibody against glucagon (Fig 5). Another important intestinal secretory cell type is the mucus producing goblet cell. Mucus secretions serve an important function in mucosal barrier protection and aid in nutrient absorption. Additionally, it provides a target within the cytoplasm of these cells for positive identification. An antibody raised against MUC2 marked goblet cells in both the small and large intestine (Fig 5). Interestingly, positive immunostaining for MUC2 was localized to cells within the crypt base, a location
where goblet cells are uncommonly found. However, in some species Paneth cells have been described to contain clear mucinous appearing vacuoles (3, 20). Paneth cells are secretory cells only found in the small intestine that have recently gained attention due to their proposed role in intestinal stem cell maintenance especially during times of injury (6, 19). This cell type is generally described as located deep within the crypt base, adjacent to stem cells and identified histologically by eosinophilic stained cytoplasmic granules (7). In the horse, we found that the degree of eosinophilic staining by H&E varied by individual however faint eosinophilic granular material could be identified (Fig 7E). Paneth cell identification was further supported using toluidine blue with large granules within the Paneth cells staining dark blue (Fig 7F). Identification of these cells was ultimately confirmed with TEM imaging where cells containing electron dense granules were visualized adjacent to thin crypt based stem cells (Fig 6). However, in order to help more clearly distinguish Goblet and Paneth cells we tested an antibody against *Ulex europaeus* agglutinin-1 (UEA-1) a lectin that binds to alpha-linked fructose receptors located on the cell surface glycoproteins and glycolipids. In the mouse this antibody is used to detect both goblet and Paneth cells (25). In the pig, this antibody appears to bind Paneth-like cells in the small intestine and goblet cells throughout the small and large intestine (12). However, in the horse, only goblet cells in the small intestine appeared to bind this antibody (Fig 7B). Additionally, no positive staining was appreciated within the crypt base, where Paneth cells reside although cells along the rest of the crypt villous axis were positively identified (Fig 7B). In order to confirm the cellular identity as either goblet or Paneth cell, a colocalization
experiment with MUC2 and UEA-1 was performed (Fig 7D). UEA-1 co-localized with MUC2 in most of the cells along the crypt villous axis except for an obvious lack of co-localization within the cells closest to the base of the crypt (Fig 7D). Additionally, no positive UEA-1 staining was appreciated within the large intestine (image not shown). More clear identification of Paneth cells was attempted using antibodies against lysozyme and cKIT (17). However, neither antibody cross reacted with equine intestinal tissue despite multiple attempts. Therefore, although Paneth cells can be clearly identified in equine tissue, their protein biomarkers are different from other species.

Discussion

Multiple approaches to positively identify equine epithelial cell types were pursued in this study because of the innate advantages and disadvantages to each technique. Establishing what is normal using each modality is important in order to provide the platform for future studies. For example, studies are ongoing that use these tools to characterize the changes in the intestinal epithelium following ischemic injury (11, 21). These studies aim to improve prognostic capabilities and identify possible novel therapeutic targets for equine cases of colic in the future.

Colic is a major cause of morbidity and mortality in the horse. The USDA's National Animal Health Monitoring System reported colic second only to old age as the leading cause of death in horses (22). Mortality in these cases is associated with a breakdown of the mucosal barrier of which the intestinal epithelial cells are an important component. These cells create a single layer which simultaneously forms a barrier, transports nutrients, and
undergoes self-renewal initiated within the stem cell compartment of the crypts. The undifferentiated stem cells that are located at the base of the crypts, and progenitor cells adjacent to the stem cells, make up the stem/progenitor cell population. The remaining epithelium is then made up of mature, post-mitotic cell types that include absorptive enterocytes, goblet cells and Paneth cells. We initially aimed to generally identify cells of epithelial origin. However, an antibody against the protein biomarker commonly used, EpCAM, did not cross react with equine tissue. Therefore, antibodies raised against other biomarkers were tested. As described previously, CD44 is a cell surface receptor that mediates cell-cell and cell-matrix interactions. Contrary to what is described in other species where CD44 expression is restricted to the stem cell zone (13), CD44 expression in horse small and large intestine appeared evenly distributed along the crypt villous axis. However, the basolateral localization in these positive cells was consistent with what is described in other species (13). The broad expression within the nonepithelial cells in the lamina propria and submucosa is also consistent with what is described in other species (13). It is well known that species variations exist. However, it is interesting that this difference in the horse exists since the increased level of CD44 expression in the crypt base in other species is attributed to its proposed role in stem cell maintenance. β-catenin also plays an important role in stem cell niche homeostasis through its interaction with the Wnt pathway. This pathway interaction is thought to be critical to the maintenance of stem cells in an undifferentiated state (16). Upon Wnt pathway activation, β-catenin translocates from the cell membrane to the nucleus. Despite this, only basolateral membrane localization was
appreciated in our immunofluorescence images within the small and large intestine even in crypt base cells (Fig 1). Both antibodies against CD44 and β-catenin may prove invaluable in future studies to monitor stem cell regulatory pathways.

The potential to identify cells undergoing apoptosis serves as a useful tool in studies that examine cellular injury. The antibody against CASP3 was therefore tested and positively identified a few expressing cells at the villus tip within the small intestine and luminal surface in the colon (Fig 2) (10). Our study confirms the utility of the CASP3 antibody in both equine small and large intestinal mucosa however it should be noted that this protein biomarker has been previously used in equine studies (18).

The predominant subtype of epithelial cells is enterocytes. These cells have the important function of absorbing nutrients, electrolytes and water. We were able to successfully identify these cells in both the small and large intestine. Additionally, cells of secretory cell lineage were also clearly identified. Enteroendocrine cell hormone secretion serves a multitude of physiologically necessary functions despite that fact this population represents the fewest number of cells. Goblet cells, on the other hand, are broadly distributed with the greatest cell number within the colon. The mucins that these cells produce are integral to normal intestinal function by providing protection of the epithelial surface as well as aiding in absorption. MUC2 marked an abundant numbers of mucous producing goblet cells along the entire intestinal length as well as within cells extending to within the crypt base (Fig 5 and 7). The identity of these crypt based mucin producing cells within was pursued using an antibody against UEA-1. In murine and human tissue, UEA-1 is used to
identify both goblet and Paneth cells (25). Interestingly, in the horse, UEA-1 antibody reactivity appeared restricted to goblet cells in the small intestine (Fig 7). This has not been described in any other species. We have therefore distinguished a clear difference between the expression of this epitope in equine cells compared to other species. Unfortunately, we were not able to identify an antibody for solely Paneth cell identification since neither antibodies against lysozyme or cKIT cross-reacted with equine tissue. However, the presence of Paneth cells can be visualized in multiple ways including co-localization studies with UEA-1 and MUC2 as well as with H&E and TEM imaging.

Ultimately, we believe that the knowledge gained from this study is critical to the future of equine medicine in the treatment of colic as the ability to monitor the preservation or loss of stem and progenitor cells as well as measure changes in stem cell regulatory pathways and post mitotic cell lineages will likely influence our appraisal of mucosal regenerative potential and survival in horses with colic.
Table 1. Functional Antibodies in Normal Equine Intestine

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<td>Cleaved Caspase 3</td>
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Table 2. Non-functional Antibodies in Normal Equine Intestine

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<td>Epithelial</td>
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Figure 1. Biomarkers to detect epithelial cells. Intestinal epithelial cells are identified within the equine small and large intestine. Immunostaining for Villin, a protein associated with the microvillar actin filaments, is localized to the apical cellular border. Immunostaining for CD44 and β-catenin, both membrane bound proteins, are identified on the basolateral cellular border. All specific markers (red). Nuclei (blue). Scale bar 200 µm, inset scale bar 50 µm.
Figure 2. Biomarkers to detect proliferating and apoptotic cells. Proliferating cells in different states of the cell cycle and those undergoing apoptosis in equine small and large intestine are shown. Immunostaining for PCNA, a general marker of proliferations, identified the greatest number of cells along the crypt. MCM2, is a marker of cells in the G1 stage of the cell cycle, and immunostaining was restricted to the base of the crypt. pH3, a marker of cells between the G2- M stage of the cell cycle identified the fewest number of cells. All markers of proliferation co-localized with bizbenzamide, the nuclear stain. CASP3 indicates cells undergoing apoptosis and is usually restricted to cells near the luminal surface in healthy tissue. Few expressing cells are present in both the small and large intestinal tissue. All specific markers (red). Nuclei (blue). Scale bar 200 µm, inset scale bar 50 µm.
Figure 3. Biomarker to detect stem/progenitor cells in crypt base. Detection of stem/progenitor cells in equine small and large intestine are shown. Immunostaining for SOX9 is primarily expressing crypt based stem and early progenitor cells and is localized to the nucleus. All specific markers (red). Nuclei (blue). Scale bar 200 µm, inset scale bar 50 µm.
**Figure 4.** Biomarkers to detect absorptive enterocytes. Equine small and large intestinal absorptive enterocytes are identified. Immunostaining for brush border digestive enzymes, sucrase isomaltase (SIM) and carbonic anhydrase II (CAII) positively identify enterocytes of the small and large intestine, respectively. All specific markers (red). Nuclei (blue). Scale bar 200 µm, inset scale bar 50 µm.
Figure 5. Biomarkers to detect secretory cells. Identification of secretory cell in equine small and large intestine are shown. Mucus producing goblet cells are positively identified with an antibody against MUC2. Enteroendocrine (EE) cells are identified using antibodies against protein biomarker CgA and glucagon-like peptide (GLP). All specific markers (red). White arrows identify positive immunostained cells. Nuclei (blue). Scale bar 200 µm, inset scale bar 50 µm.
Figure 6. Ultrastructural appearance of equine intestinal epithelium. Transmission electron microscopic images of the crypt base in the small and large intestine. Morphologic characteristics of each cell type are clearly distinguishable. Crypt base columnar stem cells, paneth, cells, goblet cells, enteroendocrine cells, and absorptive enterocytes are all marked with asterisks. Note paneth cells are not found in the large intestine. ‘L’ indicates lumen. Scale bar 2 μm (top panels), scale bar 5 μm (bottom panels).
Figure 7. Positive identification of equine paneth cells. Identification of paneth cells using co-immunofluorescence (A-D), hematoxylin and eosin (H&E; E) and toluidine blue (F) staining is shown. Both goblet and paneth cells are MUC2 positive cell in equine small intestine. However, UEA-1 immunostaining is restricted to small intestinal goblet cells as demonstrated by colocalization of color (yellow) proximal to the crypt base. All specific markers (red or green). Nuclei (blue). Scale bar 200 µm, inset scale bar 50 µm.
REFERENCES


11. Gonzalez LM, Stranahan LW and Blikslager AT. The proliferative pool of stem cells are decreased by large colon volvulus in horses. 128, 2014.


CHAPTER VI

MUCOSAL STEM CELLS ARE DECREASED IN HORSES WITH LARGE COLON VOLVULUS
Abstract

Background
Mucosal regeneration following large colon volvulus (LCV) likely depends on stem cells that reside in the crypts. However, the impact of ischemic injury on crypt stem cells has not been studied.

Objectives
To determine (1) the impact of ischemic injury from LCV on mucosal stem cells and (2) if an association exists between stem/progenitor cell number and survival.

Methods
Pelvic flexure biopsies from horses with LCV and horses with non-strangulating colonic lesions (controls) were collected. Antibodies against biomarkers specific to stem/progenitor cells including: Sex Determining region Y-box 9 (SOX9), Proliferating Cell Nuclear Antigen (PCNA), and Phosphohistone 3 (PH3), were used for immunohistochemical analyses. Statistical comparisons were performed using one way ANOVA. A receiver operator curve was created to determine a cutoff value for stem cell number associated with survival.

Results
There was a significant reduction in the number of stem/progenitor cells (SOX9+, PCNA+, PH3+) in the crypts of horses with LCV (P<0.05). Among LCV cases, there were significantly fewer stem/progenitor cells in horses undergoing resection (P<0.001). Based on the receiver operator curve, a cutoff value of >36 SOX9+ crypt cells predicted survival with a
sensitivity and specificity of 63% (95% CI; 38%-84%) and 62% (95% CI; 24%-91%), respectively.

Conclusions

LCV significantly reduces stem/progenitor cell numbers in the crypt. Interestingly, the number of stem cells was particularly low in horses undergoing resection, suggesting clinicians are resecting more severe cases. Collection of additional cases may lead to identification of a critical number of stem cells associated with survival in LCV.

Introduction

Colic is the single-most fatal disease process in the horse. Large colon volvulus (LCV) represents one of the most severe diagnoses with a reported mortality rate ranging from 35-83% [1-9]. The intestinal tract is lined by a single layer of columnar epithelium, which simultaneously forms a barrier, transports nutrients, and undergoes self-renewal every 3 to 5 days. This regenerative process depends on stem and proliferative cells that reside in the crypts of Lieberkuhn. All of these energy intensive functions make the intestinal epithelium particularly sensitive to ischemia. Research studies have been performed to investigate the severity of injury following strangulation for varying periods of time in order to understand the poor survival rate [10, 11]. These findings included ultrastructural evidence of progressive cell death, and loss of epithelium from the basement membrane as a result of ischemia and subsequent reperfusion. Of particular relevance was the finding that horses with LCV and loss of greater than 50% of the glandular epithelium had a poor prognosis for survival [12, 13]. Since intestinal stem cells are contained within the lower portion of the
glandular crypts, this histologic study implies that horses with a full complement of these regenerative cells can survive, but this has not be specifically addressed. This is because the methodology to label intestinal stem cells in equine tissue has only recently been developed [14] (Figure 1). The two main objectives of this study were to determine: (1) the impact of ischemic injury associated with LCV (≥360°) on the epithelial stem and proliferative pool of cells and (2) if there is an association between a critical number of stem and proliferative cells remaining following injury and patient survival. We hypothesize that the regenerative potential and viability of strangulated intestinal mucosa is dependent on a critical number of stem and proliferative cells within the colonic mucosa.

**Materials and Methods**

*Study population and inclusion criteria*

Adult horses admitted to the Equine and Farm Animal Veterinary Center at North Carolina State University, as well as Peterson & Smith and Chino Valley Equine Hospitals between February 2006 and March 2013 undergoing exploratory celiotomy. Pelvic flexure biopsy samples from all cases with pelvic flexure enterotomy were banked for future studies. Survival data was obtained for all horses included in the study, including survival from surgery and until discharge (short-term survival). However, statistical analysis to identify the critical number of stem/progenitor cells required for survival was only performed on pelvic flexure biopsies from 33 clinical cases of LCV of ≥ 360° that recovered from surgery and cases in which an enterotomy was performed for non-strangulating cases of colic (controls; n=15).
Specimen preparation

Pelvic flexure mucosal biopsies were collected at the time of enterotomy and placed in 10% neutral buffered formalin solution. Tissues were paraffin-embedded, sectioned at 5-8 µm thickness and mounted on positively charged glass slides.

Immunofluorescence

Sectioned were routinely deparaffinized sequentially washing in xylene, 100% alcohol, 95% alcohol, 80% alcohol, 70% alcohol and finally in PBS. When necessary, heat induced epitope retrieval (HIER) was performed by placing the slides into reveal decloaker solution (Biocare Medical, Concord, CA) for 30 seconds at 120°C and then 90°C for 10 seconds, in a pressure cooker. The slides were allowed to cool at room temperature for 20 minutes prior to continuing. Tissue permeabilization was performed on all slides with PBS-0.3% Triton X-100 for 20 minutes, washed twice with PBS and incubated in blocking medium (Dako, Carpinteria, CA). Antibodies against biomarkers specific to stem cells localized to the crypt base, sex determining region Y- box 9 (SOX9) and all proliferating cells, Proliferative Cell Nuclear Antigen (PCNA), and Phosphohistone 3 (PH3), were used to quantify differences in cell populations between groups. All secondary antibodies (Invitrogen AlexaFluor 555 or Cy3) were diluted to 1:500 and counter stained with bisBenzimide H 33258 nuclear stain (1:1000, Sigma). Slides were imaged with an Olympus IX73 microscope fitted with an Olympus DP80 camera. The objective lenses used were X10, X20, X40 and X60 oil with numerical apertures of 0.3, 0.7, 0.6 and 1.35 respectively (UPlanFLN, Olympus).
**Statistical Analysis**

One way ANOVA with a Tukey-Kramer pair-wise comparison was used, and statistical significance was set at P<0.05. A receiver operator curve was created to determine a cut-off value of cell number that would predict survival with maximum sensitivity and specificity.

**Results**

There was a significant reduction in the number of stem (SOX9+) and proliferating (PCNA+, PH3+) cells in the crypts of horses with LCV (P<0.05) (Figure 1B, 1C, 1D). Among LCV cases, there were significantly fewer stem/progenitor cells in horses undergoing resection (P<0.001) (Figure 2). Based on the receiver operator curve, counting SOX9+ and PCNA+ cells is highly sensitive but not specific for predicting death in cases of LCV. A measure of <14 SOX9+ cells was 84% sensitive (95% CI; 60.42-96.62) and 38% specific (95% CI; 8.52 - 75.51) for predicting death in cases of LCV (Figure 3A). A measure of <69 PCNA+ cells within a biopsy specimen was 90% sensitive (95% CI; 55.50 - 99.75) and 26% specific (95% CI; 11.11 - 46.28) for predicting death in cases of LCV (Figure 3B).

**Discussion**

Colic continues to be an important area of research due to the impact on horse owners and the need to improve clinical outcome. Colic that results from ischemic injury, in particular, remains a significant source of interest because of the high mortality rate of cases as compared to other forms of colic. Recent advances in the field of intestinal stem cell biology have provided the impetus to explore this area for potential new therapeutic interventions to treat intestinal injury [15-17]. We believe that a more in depth assessment of the impact of
ischemic injury on equine intestinal mucosa is warranted, both for the clues it may provide to formulate an accurate prognosis, but also because it may reveal potential pathways to explore in order to direct future therapies. Thus far we can conclude that ischemic injury resulting from LCV significantly reduces the number of stem/progenitor cells in the crypt. Interestingly, the number of stem cells was lower in horses undergoing resection, suggesting clinicians are resecting more severe cases. No association with survival has yet been appreciated. However, the small sample size of LCV cases limits the statistical power of the study and evaluation of additional samples may lead to identification of a critical number of stem cells associated with survival in cases of LCV.
Figure 1. Immunofluorescent comparison of stem/progenitor cell populations between control and LCV cases. Immunofluorescence demonstrates positively marked stem (SOX9⁺) and proliferating cells (PCNA⁺, PH3⁺) in large colon crypts of control horses (left) and cases of LCV (right) (A). Number of positive stem and proliferating cells in control versus LCV cases (B, C, and D). Error bars indicate standard error. One-way ANOVA indicates significantly more cells in control cases vs. LCV cases (p < 0.05).
Figure 2. Immunofluorescent comparison between cases of LCV with and without surgical resection. Cells expressing the biomarker SOX9 are decreased in mucosal biopsies from LCV cases that underwent colonic resection (p<0.001).
Figure 3. Receiver Operator Curve prediction of death in cases of LCV. (A) A measure of <14 SOX9⁺ cells was 84% sensitive (95% CI; 60.42-96.62) and 38% specific (95% CI; 8.52 - 75.51) for predicting death in cases of LCV. (B) A measure of <69 PCNA⁺ cells within a biopsy specimen was 90% sensitive (95% CI; 55.50 - 99.75) and 26% specific (95% CI; 11.11 - 46.28) for predicting death in cases of LCV.
REFERENCES


CHAPTER VII

DISSERTATION SUMMARY AND FUTURE DIRECTIONS

Research from this dissertation provides the platform for future studies to use large animal models for the study of intestinal stem cell biology. Additionally, the direct impact of ischemia-reperfusion injury on the stem cell zone was evaluated. However, studies are currently underway to elucidate the mechanism of resistance to injury and the response of IESCs during repair (Figure 1, Appendix 1). Additional work is utilizing tissue from veterinary clinical cases to determine the impact of naturally occurring ischemic injury on the IESCs populations (Appendix 2). This combined work aims to define mechanisms and mediators that improve IESC-mediated repair following ischemic injury. The long-term goal is to define new therapeutic targets to enhance translational approaches for clinical (veterinary and human) cases of intestinal I/R.
**Figure 1.** Proposed mechanism of IESC resistance to ischemic injury mediated by QSCs.

The intestinal epithelium functions under a condition of ‘physiologic hypoxia’ with the least amount of oxygen available to the epithelial cells closest to the lumen (A). IESC populations show differenting resistance to I/R injury and HIF pathway activation in IESCs mediates this resistance and/or regeneration of severely injured epithelium after I/R (B).
APPENDICES
Appendix A.

INTESTINAL STEM CELL RESPONSES TO ISCHEMIC INJURY IN A LARGE ANIMAL MODEL (K01 Application)

RESEARCH STRATEGY

(A) Significance:

Ischemic intestinal injury that results in short bowel syndrome (SBS) is the most common cause of intestinal failure in both children and adults (29). Total parenteral nutrition (TPN) is currently the treatment of choice but is fraught with complications including venous thrombosis, infection and organ failure (29). Intestinal transplantation is an alternative therapeutic intervention but has 40% mortality in allograft recipients within five years (1). Preventing SBS by minimizing the need for intestinal resection would reduce or negate the need for TPN or intestinal transplantation. Rodent models of intestinal ischemia show architectural preservation of some crypts, where the intestinal epithelial stem cells (IESCs) reside (25, 36, 56, 72). The ability of the IESC compartment to expand after intestinal resection, radiation injury, and doxorubicin treatment has been demonstrated (20, 21, 35, 76). Available but controversial evidence suggests the existence of two IESC populations: Lgr5-enriched crypt based columnar (CBC) (8) and quiescent or reserve population (QSC) enriched for Hopx, Bmi1, Lrig or markers of secretory progenitors (14, 49, 61, 75). Current evidence suggests that both IESC types contribute to regeneration after injury in a context dependent manner but relative roles are still debated. To our knowledge, no studies have specifically focused on the impact of ischemic insult on IESCs or evaluated the contribution of CBC versus QSC to the reparative response after ischemia. Murine models have
contributed much to our knowledge of IESC but non-rodent models are highly desirable for translational studies and development of new therapies (4, 57). Injury models that closely recapitulate human disease, such as porcine ischemia/ reperfusion (I/R) injury will facilitate IESC targeted therapies to improve treatment and outcome of this devastating disease (4, 12, 57). The proposed research is expected to advance our knowledge of IESC populations in a non-rodent, porcine model and identify the intracellular pathways critical to I/R, resistance, injury or repair. This contribution is significant because it will provide a new clinically relevant, non-rodent model to test new clinical therapies for I/R and potentially other intestinal diseases.

(B) Innovation:

The need for non-rodent animal models to improve translational research is well accepted. The limited availability of human derived tissues requires that information gained from animal models be extrapolated to humans. Despite major advances using murine models and increasingly accepted potential therapeutic benefits of stem cell or stem cell targeted therapies, much is still needed to advance these to clinical reality (50, 63). Key physiological differences between rodents and humans confound extrapolation of findings in rodents to humans (41, 57, 59, 82). Compared with rodents, the pig has an overall size and genomic homology more similar to humans. Pigs and humans share common mucosal barrier physiology, food intake (both omnivores) and enteric microbiota composition (41, 54, 57, 79). These similarities have made the pig a desirable translational model for the study of many intestinal diseases yet little emphasis to date has been placed on porcine IESCs (6, 10,
23, 59, 82). My recent publication, Gonzalez et. al., “Cell Lineage Identification and Stem Cell Culture in a Porcine Model for the Study of Intestinal Epithelial Regeneration” describes development and validation of tools for analysis of porcine IESCs (28). This study also describes successful long-term culture of porcine crypts into fully differentiated enteroids providing a new platform for mechanistic studies and future translational work including stem cell engraftment and transplantation. Such studies are technically challenging in rodents due to limited IESC availability and have inherent limitations in translatability to humans and large animals (5).

The proposed model of temporary vascular occlusion creates an environment of hypoxia, inflammation, cell infiltration and compromised intestinal barrier function, as occurs in clinical cases of I/R (11, 12). The length of porcine intestine is sufficient to permit vascular occlusion of differing durations to be applied to multiple segments within a single animal. Additionally, the large amount of available tissue allows molecular and translational studies of porcine IESC that are difficult in rodents due to the low yields of IESC that represent only a small fraction of total epithelial cells. Additionally, the in vitro IESC culture model allows the direct analysis and manipulation of IESC and their progeny under hypoxic conditions. The proposed research is innovative because it utilizes a large animal injury model that recapitulates the disease process noted in clinical cases of intestinal I/R. Furthermore, the highly controlled manner in which I/R or hypoxia can be induced facilitates studies of the mechanisms, mediators and ultimately therapies for I/R induced injury. This
The project represents a departure from the focus on mouse models in the field of IESC biology and is expected to contribute to the advancement of clinical therapy.

(C) Approach

This proposal will test the central hypothesis that distinct IESC populations show differing resistance to I/R injury and that HIF pathway activation of IESCs mediates regeneration of severely injured epithelium after I/R.

Aim 1. To test the hypothesis that IESC expressing biomarkers of CBCs or QSCs show differential resistance to I/R injury and different contributions to the subsequent repair and regenerative responses. Working Hypothesis: Cells expressing biomarkers of the QSC population will demonstrate an increased resistance to I/R injury and will preferentially contribute to provision of new stem and transit amplifying cells during repair and regeneration.

Introduction: CBC type IESCs were originally identified by Cheng and Leblond in 1974 based on their cellular morphology (15, 16). Lgr5 and subsequently Ascl2, Olfm4 and Sox9 were shown by lineage tracing or IESC culture to mark multipotent CBCs (7, 8, 14, 24, 35, 66, 73, 74, 76, 81, 84). A more quiescent or reserve population of IESCs (QSC) able to be activated upon injury or during regeneration has been reported to be marked by Hopx, Bmi1, mTert, Lrig1 and enteroendocrine markers or secretory progenitor biomarkers and also...
shown to be multipotent by lineage tracing or culture (8, 14, 24, 30, 51, 61, 71, 75, 76, 81, 84). The objective of the current aim is to define the fate of the CBC and QSC populations. Additionally, considering what is known regarding the importance of the hypoxia inducible factor (HIF) pathway in conferring resistance of cells to hypoxic injury, the time course of HIF pathway activation following I/R injury and during repair will be defined (17, 26, 42, 64).

The *rationale* for this aim is to define the mechanisms of epithelial injury, specifically in the IESC populations, during intestinal I/R in a controlled translationally relevant non-rodent model. The proposed model of temporary vascular occlusion re-creates the loss of intestinal blood perfusion, allows for reperfusion and permits recovery, all of which occur in clinical cases of ischemic disease (11, 12).

**Training Goal:** Obtain new expertise in techniques to evaluate IESC, apoptosis, HIF pathway and proliferation in a clinically relevant porcine model of I/R.

**Preliminary Data:** In jejunal segments, mesenteric vessels were surgically clamped for 2 or 3hr and resected or were reperfused for 1hr then resected for analyses (n=3) (Figure 2A & B). To investigate reparative processes, jejunal segments in additional animals were subjected to 2 or 3 hours of ischemia followed by 18 hr of recovery (n=3) (Figure 2C). Grade of injury and changes in IESC populations were assessed histologically. Increased durations of vascular occlusion increased measured indices of tissue injury (% epithelial loss, injury grade) in a time-dependent manner (P<0.05) (2, 11, 12, 59). 1hr reperfusion increased indices of injury following 3hr of ischemia (P<0.05). Despite the severe loss of surface epithelium
and crypt damage following ischemic injury, by 18hr there was clear restitution of villus epithelium although villus length and crypt depth were still decreased (P<0.05) (Figure 2). Figure 3 demonstrates immunofluorescence for and co-staining of PCNA (proliferation marker), HOPX (QSC) and SOX9 (IESC/early progenitors). Note extensive colocalization of PCNA/SOX9 at the crypt base and many fewer HOPX⁺ cells of which approximately 20% are PCNA labeled. This validates SOX9 and HOPX as marking distinct populations. These cells were quantified in control jejunum, after 2 or 3hr of ischemia and after ischemia and 1hr reperfusion, and revealed decreases in SOX9⁺ cells at 2h (77±10%) and 3h (64±15%) (p<0.05) decline in PCNA cells after 3hr I/R (52±12%) and no change in HOPX⁺ cells at any time point (p>0.05). Following 18hr recovery, the PCNA⁺ cell number in tissue subjected to 3hr ischemia remained significantly decreased compared to control. However, the SOX9⁺ cell number had returned to that of control tissue. Interestingly, no significant change in the number of HOPX⁺ between injury groups as compared to control tissue was noted by the 18hr recovery point.

The QSC capacity to proliferate was confirmed in control tissue in a colocalization study that demonstrated few HOPX⁺ (QSC)/PCNA⁺ (proliferative) cells near the crypt base in uninjured intestine (Figure 3). No HOPX⁺/PCNA⁺ cells were identified in acutely injured tissue in any pig evaluated. However, proliferative QSC (HOPX⁺/PCNA⁺) were identified in 18hr recovered segments subjected to 1, 2, or 3 hr ischemia.
These preliminary data suggest that the SOX9+ cell population is able to recover to the level of the control tissue by 18hr of recovery despite the significant loss of these cells following acute injury. In contrast, HOPX+ IESC appear resistant to injury and activated following injury. Further work will test increased durations of ischemia, which may establish when the SOX9+ cells can no longer recover and if HOPX+ cells increase their proliferative rate. Additional recovery points will be evaluated to understand the contribution of each IESC population to complete mucosal regeneration, which has previously been shown to take 144hr (11).

**Research Design:** A porcine mesenteric vascular occlusion model that recapitulates clinically relevant I/R will be used to compare the susceptibility or resistance of the two IESC populations to injury and the time course of proliferation of each population during repair. **Approach:** In Aim 1, I will use biomarkers of QSC and CBC in quantitative histologic, protein and mRNA analyses to confirm preliminary data indicating loss of CBC and maintenance of QSC. Combined analyses of the two IESC populations with analyses of the apoptosis biomarker activated caspase 3 (CASP3), hypoxia biomarkers (HIF1α) and EdU will directly test if I/R differentially affects the two IESC populations and the time course of HIF pathway activation. Importantly, Dr. Lund’s research expertise includes well-established methods for analyzing apoptosis (62, 80). Additionally, Dr. Blikslager’s experience utilizing porcine I/R models and the facilities at NCSU provide the tools necessary for successful completion of this aim (3, 11, 12).
Methods: **Experiment SA1.a.** Use biomarkers of QSCs and CBCs in quantitative histologic, protein and mRNA analyses to confirm preliminary data for I/R induced loss of CBCs but preservation of QSCs. Yorkshire crossbred pigs will be used in terminal surgeries where ischemic injury will be induced by clamping jejunal mesenteric vessels. Within a single animal, two 10cm segments per time point will be isolated and clamped for 2, 3 or 4hr respectively (6 segments). Vessels will be un-clamped from one of the two segments per time point of ischemia and reperfused for 1hr. An additional segment will be identified at the start of surgery, as an internal normal control. Animals will be euthanized and tissue collected from individuals grouped by duration of ischemia with and without reperfusion. An advantage of the porcine model is availability of large amounts of tissue that readily permit multiple analyses in the same segment of intestine (jejunum). Hematoxylin and eosin stained sections will be scored using previously established parameters, including: % epithelial loss, injury grade and, crypt depth (2, 11, 12, 59). Immunohistochemistry will be used to quantify changes in number of specific cell types. For quantitative analysis, 12 crypts and villi per treatment, sectioned in the sagittal plane, will be selected for analysis (21, 59). Since antibodies specific for biomarkers for the porcine CBC cell population have not been identified, *in situ* hybridization will also be used. *In Situ* probes for Lgr5 and Olfn4 will be designed and labeled with digoxygenin and used to positively identify and quantify the CBC cells (8, 81). Sense strand probes will be generated and will serve as negative controls. Crypts or epithelial cells will be isolated by standard conditions or FACS (EPCAM⁺). Western blot analysis will quantify biomarkers of specific cells (SOX9, HOPX) or processes
(PCNA) between experimental groups. qRT-PCR will quantify gene expression for the following genes: QSC (Hopx, Bmi1, mTert), CBC (Lgr5, Olfm4, Ascl2, CD24, Sox9) and for normalization (Gapdh, 18S), all validated for porcine tissue in our previous study (28). mRNA levels of cell specific markers will be normalized to EPCAM (epithelial cell marker) to establish the relative expression of IESC markers in the total epithelial cell population. This accounts for the fact that immune infiltration could indirectly lead to altered levels of IESC markers evaluated by biochemical methods. The \( \Delta \Delta \text{Ct} \) method will be used to measure relative changes in gene expression. **Analysis:** Statistical differences between control and ischemic-injured tissue will be performed using a 2 way ANOVA, (effects of treatment or time, and interactions) with \( P<0.05 \) considered significant. Post hoc pairwise analyses between groups or times will be performed using Tukey’s test. We will test if there is a correlation between cell count and grade of injury using linear regression and Pearson correlation coefficient.

**Experiment SA1.b.** Directly examine whether ischemia selectively increases apoptosis or activates HIF pathway in CBCs versus QSCs using activated caspase 3 or HIF pathway biomarkers combined with specific biomarkers of the two IESCs during and following ischemia and reperfusion. Immunostaining of tissue obtained from SA1.a. will be used to co-localize markers of apoptosis (CASP3) and hypoxia–stabilized nuclear HIF1\( \alpha \) (anti-HIF1\( \alpha \); Abcam ab16066, confirmed reactivity to pig) with SOX9 or HOPX to distinguish the two IESC subtypes. **Analysis:** Statistical analyses as described for experiment SA1.a. will test for differences between the subtypes of IESCs and their expression of CASP3 or HIF1\( \alpha \). To
directly evaluate impact of ischemia on IESC function, crypts will be isolated from control jejunal segments and jejunal segments after 2 or 3 hr of ischemia. These will be cultured under conditions previously reported in the presence of Edu. Yield of enterospheres/enteroids over 0-12 days after plating will be assessed to test if ischemia affects the ‘stemness’ or enterosphere/enteroid forming ability of crypts, a surrogate measure of stemness. Enteroids will be harvested and stained for HOPX, SOX9, EdU or PCNA to assess if ischemia selectively affects the number or proliferation of particular IESC during enteroid growth. Depending on the outcome of the studies, enteroids will be harvested to examine IESC biomarkers at the level of RNA or biomarkers of HIF pathway. **Analysis:** Labs of my mentors (Lund, Magness, Henning) have expertise in quantitative analyses of enteroids. Drs. Lund and Magness have optimized high throughput fluidigm-based qRT-PCR to permit examination of multiple genes (48 genes x 48 samples) in small RNA yields from enteroids, and I will gain experience in this methodology.

**Experiment SA1.c.** Define the time course proliferation of QSCs versus CBCs during I/R and repair using co-localization with EdU, PCNA and phosphohistone 3B to mark different phases of cell cycle. Co-localize the ‘s’ phase marker EdU, the G2-M phase marker phosphohistone 3B (pH3B) and PCNA (general proliferation marker) with biomarkers of QSCs and CBCs. Tissue will be derived from Experiment SA1.a. Based on preliminary results, at least 3hr of vascular occlusion will be performed using the methods described in experiment SA1.a. Pigs will be euthanized with a lethal dose of pentobarbital (100mg/kg, IV) for tissue collection at times 18, 48, 72 and 144hr to study crypt and epithelial repair and
regeneration. These times are chosen based on unpublished observations that proliferation is maximal by 72-144hr. At each time point, tissue will be obtained from non-ischemic segments of jejunum within treated pigs. EdU (100mg/kg ip) will be administered 90min prior to euthanasia. Tissue will be collected for histologic, protein, flow cytometric and mRNA analyses. Immunostaining will be used to measure cell proliferation by detection of EdU and co-localization with markers of QSCs (HOPX\(^+\)) and CBC-type IESCs (SOX9). Western blot will quantitatively compare changes in expression of cellular biomarkers of CBC, QSC and proliferation. **Analysis:** Data analysis will be performed using two-way ANOVA with Tukey’s post-hoc tests as previously described.

**Experiment SA1.d.** Define the time course of HIF pathway activation following I/R and during repair. Crypts or epithelial cells will be isolated by standard conditions or FACS (EPCAM\(^+\)) from tissue derived from SA1.a. A custom porcine RT\(^2\) Profiler PCR array, available through Qiagen (Valencia, CA), will be used to evaluate the expression of at least 84 genes involved in hypoxia-related signaling. Genes directly involved in the response to hypoxia (Arnt2, Hif1\(\alpha\), Hif2\(\alpha\)), oxidative stress (Cat, Gpx1) as well as those involved in apoptosis (Bax, VegfA), signal transduction (Il1A, Igfbp1), cell growth (Igf1, Igf2, Il6) and metabolism (Mt3, Nos2) are in this array. The RT\(^2\) Profiler will be used as cell numbers are not limiting for the yield of total RNA. Across aims 1 and 2 I will gain expertise in both RT\(^2\) Profiler and fluidigm technologies to compare strengths and limitations of each. **Analysis:** qRT-PCR data will be analyzed as described in SA1.a. Key HIF pathway mediators altered in
I/R or repair will be examined for expression in SOX9+ or HOPX+ IESC/progenitors by
immunofluorescence, to assess if there are cell-type specific differences.

**Expected Outcomes Aim 1**: Based on preliminary data, I predict that HOPX+ cells will be
preserved after I/R and that at early times during recovery increases in proliferating HOPX+
cells will be observed. Similarly, I predict that a greater number of SOX9+ versus HOPX+
cells will be co-labeled with apoptosis markers after I/R. Proliferating SOX9+ cell numbers
may also increase during recovery since available evidence predicts that SOX9 marks both
CBC and QSC. However, a different time course of proliferation of SOX9+ versus HOPX+
cells will indicate differential contributions to early repair. Crypt culture/enteroid studies will
provide new information as to whether I/R or ischemia/recovery lead to altered intrinsic
ability of IESC to survive, grow and yield enteroids. Based on findings during regeneration
after radiation, I predict that crypts isolated during recovery will yield more or larger
enteroids (76). If HOPX+ cells predominate or are preferentially expanded, this would
provide evidence for key roles in survival and repair after I/R. Defining which HIF pathway
mediators are activated after ischemia versus those during repair will provide new insights in
a clinically relevant model and guide *in vitro* studies (*or in vivo* studies if time permits) to
manipulate specific mediators and test functional outcome. These findings will be significant
because they will indicate whether IESC and which IESC or a particular HIF pathway
mediator might be an optimal target for potential therapeutic applications.

**Potential Problems/Alternative Strategies and Future Directions – Aim 1**: The lack of an
IESC reporter pig precludes the use of many techniques, such as lineage tracing, currently a
gold standard for the study of stem cells in mouse models. Dr. Piedrahita (Research Advisory Committee member; Director of Center for Comparative Medicine and Translational Research) is already developing pig LGR5-reporter pigs which will become available over the course of the study to confirm key data. However, an alternative strategy available to define or confirm IESC response and contribution to repair and to assess particular mediators up-regulated in these cells during I/R is the use of Sox9-EGFP, Lgr5-EGFP or Lgr5-LacZ reporter mice or Bmi1-YFP reporter mice which are all in use in my mentors laboratories (24, 25, 31, 78, 81). Histologic, flow cytometric, gene microarray analysis and qRT-PCR as well as enteroid culture are ongoing within the Lund and Magness laboratories (25, 76, 78). Dr. Blikslager has developed a mouse I/R model, very similar to the porcine model, which could be used and undertaken as an additional training goal.

C.1. **Aim 2. To define the role of the HIF pathway within the IESC populations during ischemia, reperfusion, and subsequent regeneration.** Working Hypothesis: IESCs are activated to self-renew and differentiate during hypoxic injury through a mechanism dependent upon HIF pathway activation.

**Introduction:** The gastrointestinal epithelium is unique in that the heterogenous population of IESCs, progenitors and differentiated lineages must function normally in an environment with a steep oxygen gradient. The cells closest to the crypt base are perfused with more oxygenated blood while those closest to the lumen of the GI tract are in a hypoxic environment (Figure 1). The HIF pathway is a key regulator of cell function under conditions of both normal “physiologic hypoxia” and during disease (64).
Under normoxic conditions, HIF mediated gene transcription is inhibited. During hypoxia, HIF-1/2α are stabilized, translocate from the cytoplasm to the nucleus and induce transcription of target genes that protect against injury (40). Essential functions of genes transcribed following HIF pathway activation include those related to glucose metabolism, mitochondrial function, cell survival, apoptosis, and resistance to oxidative stress.

The objective of this aim is to utilize an *in vitro* culture platform to test if the HIF pathway is critical to resistance of IESCs to hypoxia associated injury. To achieve the objective outlined in Aim 2, I will test the working hypothesis that IESCs resist apoptosis or are activated to proliferate during hypoxic injury through a mechanism dependent upon HIF pathway activation. Use of *in vitro* culture systems permit functional tests of specific pathways in the survival, proliferation, expansion or differentiation of the IESCs (58, 65, 66, 77).

The **rationale** for this aim is to define the time course of HIF activation by hypoxia and recovery and establish optimal pharmacologic or genetic HIF pathway inhibitors/activators in a cell line. The impact of hypoxia and recovery will subsequently be tested in enteroids using the optimal times and most effective HIF pathway modulators established. Using this approach, potential HIF pathway targets that confer resistance and/or activation of IESCs will be identified. Successful completion of this aim in an *in vitro* environment will define and prioritize whether specific HIF pathway mediators may be valuable to promote protection or repair of ischemic injured IESC and intestinal epithelium *in vivo*.

**Training Goal:** To develop expertise in manipulating cell lines or IESC in culture to elucidate signaling pathways or intermediates that may be tested as future therapeutics in *in
vivo models. I will also be exposed to high throughput technologies. These include the use or exposure to of Fluidigm technology (Magness and Lund labs), microarray analysis (Lund lab) and RNAseq (available at UNC and NCSU and ongoing in the Lund and Magness labs). These cutting edge technologies permit experiments specifically tailored to porcine mRNAs and mediators and amenable to small or large quantities of RNA and even single cells (Magness lab).

**Preliminary Data:** The maintenance of epithelial barrier function depends on the tightly controlled balance between cellular proliferation, differentiation and apoptosis. The in vitro 3D culture of IESCs has only recently been possible (65, 66). In these cultures, IESC initially divide to form an enterosphere, enriched in stem and progenitor cells. These grow into complex enteroids that contain IESC, progenitors and all post-mitotic lineages found in the normal epithelial lining. Therefore, enteroid culture allows the unique opportunity to evaluate both stem cell enriched cultures (48hrs post-plating) as well as more complex enteroid structures containing IESC and mature cell types. I have already developed the method of porcine derived, long term enteroid culture and optimized reagents for evaluating gene and protein expression in enterospheres/enteroids (28). Additionally, I have designed and validated efficiencies (>92%) of qRT-PCR primers for key HIF pathway related genes including, Hif1α, Vegf and Itf and sequenced the products to ensure specificity. Gene expression within porcine enteroids was confirmed for all biomarker genes proposed for use within this grant including the biomarkers for IESC, progenitors or post-mitotic lineages such as goblet (MUC2) and enteroendocrine (Chromogranin A/B) cells.
Initial experiments will use the non-transformed cell line, IPEC-J2, derived from porcine jejunum. PCR has confirmed expression of markers of CBC and QSC in these cells. Importantly, similar to “physiologic hypoxia” found in vivo and in other cell lines (44), basal HIF-1α was detectable in IPEC-J2 cells cultured in normoxic conditions. Additionally, one of its downstream target genes, intestinal trefoil factor (Itf) was also expressed (26). As in the crypts, IPEC-J2 contain a small subpopulation of HOPX⁺ cells and greater SOX9⁺ and PCNA⁺ cell numbers. Ongoing studies are evaluating relative proportions of cells expressing HOPX, SOX9, PCNA or markers of differentiated lineages in subconfluent, exponentially growing, IPEC-J2 cells or confluent monolayers which will be important background information for study design.

**Research Design:** Utilize *in vitro* culture models subjected to hypoxia to model ischemic injury and directly test the survival or proliferative capacity of IESCs as well as assess the impact of HIF pathway modulation on IESC preservation and activation. **Approach:** In aim 2, I will use a commercially available hypoxia chamber (Modular Incubator Chamber; Billups Rothenberg, Inc.), available at UNC or in the Blikslager lab, to induce hypoxic injury (1% O₂) in cell culture and assess HIF pathway biomarkers within the IESC populations. Markers of cell identity, apoptosis and proliferation, will be assessed as described in Aim 1. Markers of HIF pathway activation related to glycolysis and increased mucosal barrier protection against ischemic injury including, inducible nitric oxide synthase (iNos), cyclooxygenase-2 (Cox-2)) (13, 32), vascular endothelial growth factor (Vegf) (39, 45, 48, 55, 69), Glut-1 and Glut-4(19), Mucin-3 (47), intestinal trefoil factor (Itf) (26), CD73 (70) and multidrug
resistance gene-1 (Mdr1) (18), will be assessed. HIF pathway stabilizers (43) and inhibitors (44) will be tested for their effectiveness in HIF pathway manipulation in initial experiments using IPEC-J2. The most effective and specific modulators of HIF pathway will then be used in IESC cultures. The custom porcine RT² Profiler PCR array designed from S.A.1d (Qiagen) or fluidigm platform will be used to evaluate the expression of IESC biomarkers or genes involved in hypoxia-related signaling. The Lund and Magness labs have demonstrated that Fluidigm is amenable to small yields of RNA from enteroids or single IESC. This permits analyses of up to 48 or 96 genes in 48-96 samples depending on the fluidigm chip used.

Methods: **Experiment SA2.a.** Use cell culture and a novel pig in vitro enteroid system exposed to hypoxia to model ischemia induced injury and recovery and directly test the proliferative capacity of the IESCs. Initial experiments will evaluate the time course of hypoxic injury (1% O₂; 0-8 hr) (44) and recovery (0-72 hr) on apoptosis (CASP3) or proliferation (EdU) of HOPX⁺ or SOX9⁺ cells in IPEC-J2 culture (available from the Blikslager lab). Histologic, protein, and gene expression assays at each time point post hypoxia will be used to define the acute response to varying durations of injury and recovery and the impact on cellular function. Selected time points will be used in subsequent experiments of hypoxia and recovery using porcine enterospheres at 48hr after initial crypt culture or fully mature cultured enteroids from longer-term culture (28, 83). **Analysis:** Data analysis will be performed using a one-way ANOVA with post-hoc Tukey’s test for pairwise comparisons of times or treatments.
**Experiment SA2.b.** Assess the impact of HIF pathway stabilization (via DMOG or PHD inhibitor AKB-4924 media supplementation or expression plasmids or lentivirus encoding stable HIF1/2α) and inhibition (via lentiviral-mediated HIF-1α, HIF-2α or HIF-β knockdown or media supplementation with ascorbate) on the preservation and activation of IESCs to self-renew and differentiate under hypoxic conditions *in vitro*. Initial experiments will use IPEC-J2 cell culture to establish optimal pharmacologic or genetic inhibitors and activators at the optimal times established in SA2.a. Cells collected at time 0 will be used to measure baseline levels of HIF pathway and IESC biomarkers. Stabilization of the HIF pathway will be achieved, pharmacologically or by transfection of a plasmid encoding stabilized HIF-1α and HIF-2α (containing a mutated oxygen-dependent degradation domain), as described (44). An HRE-containing luciferase reporter plasmid will be used as a control. The plasmids will be transfected into the IPEC-J2 or enteroid cultures prior to hypoxic injury. My mentors have achieved plasmid transfection into enteroids by electroporation and transfections should be feasible in the cell line. However, lentivirus will also be tested as a potentially more efficient system or one that may be necessary for enteroids. To define the impact of reduced HIF availability on IESC preservation and activation, baseline HIF activity will be reduced by supplementing the media with ascorbate (1mM; Sigma-Aldrich, St Louis, MO), a cofactor for an enzyme that initiates HIF1/2α degradation. Additionally, lentiviral particles encoding a panel of short hairpin RNAs directed against porcine HIF-β, HIF-1α or HIF-2α (MISSION TRC, Functional Genomics University of Colorado, CO)(44) will be used. Nontarget shRNA-encoding lentiviral particles will be used as controls. Successful stabilization and inhibition
of HIF activity will be confirmed with Western Blot and qRTPCR. Analysis: Data analysis will be performed using a one-way ANOVA with Tukey’s adjustment for multiple comparisons.

**Expected Outcomes Aim 2:** The Lund and Magness laboratories have extensive experience with cell culture models and pharmacologic or genetic pathway manipulation, which will ensure successful completion of this aim. We predict that HIF1α, HIF2α or both will affect IESC survival or proliferation and may preferentially affect HOPX+ IESC. Additionally, identification of downstream mediators will provide new insights into pathways involved in IESC preservation or regeneration, and therefore potential means to manipulate those pathways to hasten repair *in vivo* either in these studies if time permits or in the future.

Depending on the outcome of S.A.1D and initial aims with hypoxia challenge we may also test manipulation of specific downstream HIF pathway mediators in epithelial cells or crypts isolated from pigs after I/R to assess if they improve enterosphere/enteroid survival and growth. Such follow up experiments may be particularly important since protective (34, 60) and injurious (22, 38) roles of HIF pathway activation have been reported. This may reflect an *in vivo* role of HIF pathway in enhancing inflammatory responses to bacterial translocation in ischemic injury. The initial experiments in the cell line and enterosphere/enteroid cultures will assess HIF pathway roles in the absence of ongoing injury or bacterial translocation. Studies in crypts derived from pigs exposed to I/R will be useful since the associated injury will expose IESC to bacteria and inflammatory mediators. It will be mechanistically very interesting if HIF pathway modulation has different effects in IESC
from uninjured versus I/R injured intestine. Successful pharmacologic manipulation of specific mediators in porcine enteroids and demonstration of functional effects would be a major advance to facilitate mechanistic and drug discovery in intestine of a clinically relevant large animal model.

**Potential Problems/Alternative Strategies and Future Directions – Aim 2:** The HIF pathway is well defined and there are multiple sites that may be targeted to promote or inhibit downstream gene expression (9, 64, 68). We recognize that plasmid or lentivirus mediated manipulations may be challenging in enterospheres/enteroids but this is an area of rapid progress in the field (46, 67) and in the Magness and Lund labs and will add to my technical expertise. RNA-Seq technologies are being used in mouse injury and regeneration models in the Lund and Magness labs. If data suggest this is warranted, excellent facilities at UNC and NCSU would permit application of RNAseq to this pig model in the future. An important future goal for this aim is to translate the findings of the *in vitro* studies to the *in vivo* mesenteric vascular occlusion model. There are HIF pathway activators that have demonstrated protective effects in cases of renal ischemia-reperfusion injury (33, 37) and in Dr. Colgan’s models (43, 64). Additionally, with the projected availability of a LGR5-reporter pig key findings can be directly applied to the I/R model expressing an IESC reporter. Exposure to gene editing technology such as TALEN or CRISPR (27) to develop genetically modified pigs is an additional training goal and is available to me in the Piedrahita lab. Through the completion of this aim I will be well poised to develop
independent research directions to translate my findings and approaches to future in vivo therapeutic studies.


45. Kido M, Du L, Sullivan CC, Li X, Deutsch R, Jamieson SW and Thistlethwaite PA. Hypoxia-inducible factor 1-alpha reduces infarction and attenuates progression of cardiac


Appendix B

STEM CELLS ARE RETAINED IN REDUCED NUMBERS IN EQUINE STRANGULATED SMALL INTESTINE

Presented at Eleventh Equine Colic Research Symposium Dublin, Ireland 2014

Background

Small intestinal strangulating obstruction results in progressive destruction of mucosal architecture, progressing from the villi toward the base of the crypts where stem cells are thought to reside. Identification of stem cells following injury may provide clues to the regenerative potential of ischemic-injured tissues.

Objective

To characterize (1) epithelial cell types within normal equine mucosa and, (2) changes to the stem cell zone in ischemic-injured small intestine.

Methods

Mucosal biopsies were collected from healthy horses (n=10) and from resected small intestine from horses with strangulating obstruction (n=6). Histomorphometry and immunofluorescence using antibodies against select cellular markers were performed. Proliferating cells were labeled using an antibody against Proliferating Cell Nuclear Antigen (PCNA), and stem cells were labeled with an antibody against Sex Determining region Y-box 9 (SOX9). Statistical comparisons were performed using one way ANOVA (P<0.05 considered significant).
Results

All lineages of epithelial cells were identified in normal tissues, including stem cells and post-mitotic cell types. Evaluation of strangulated tissue revealed severe injury to the mucosa, although proliferating (PCNA+) and stem cells (SOX9+) were retained within the crypts. The margins of resected intestine appeared histologically normal, but there was a significant decrease in the number proliferating cells (PCNA+) and stem cells (SOX9+) (P<0.05) as compared to normal tissue.

Conclusions

The retention of proliferating and stem cells within strangulated tissue indicate marked resistance of these cells to ischemia. Nonetheless, the significant reduction in stem cells within the resection margins suggest injured bowel may benefit from therapies aimed at increasing activity of remaining stem cells following strangulation obstruction.