The Developmental Expression of Surfactant Protein-A (SP-A) in the Murine Intestinal Tract

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Abstract

Surfactant protein-A (SP-A) plays a critical role in the innate immune system and has well characterized effects in the lung where it attenuates inflammatory responses and controls invasion of bacteria. Extra-pulmonary sources of SP-A have also been indentified: SP-A mRNA and protein have been detected in the adult gastrointestinal (GI) tract, while significant levels of SP-A protein have been detected in amniotic fluid. To date it is not clear if newborn intestinal exposure to SP-A comes from ingested amniotic fluid or from production in the newborn intestinal tract. RNA *in situ* hybridization with a digoxigenin-labeled anti-sense SP-A probe was used to detect cells with the SP-A mRNA message in the intestinal tract of post-natal day (PND) 3 and PND 6 mice. Immunohistochemistry in PND 7 mice was used to determine intestinal SP-A protein expression. We report positive staining for SP-A protein in cells of the lamina propria of the intestinal tract, although results were mixed as some SP-A null intestinal tissue also stained positively. RNA *in situ* hybridization with a digoxigenin-labeled anti-sense SP-A probe hybridized to SP-A mRNA in the lamina propria and muscularis externa of the intestinal tract, the same cells that stained positively for SP-A protein. These results indicate that SP-A is likely produced in the intestinal tract in newborn mice.

Abbreviations

SP-A: Surfactant protein-A

GI: Gastrointestinal

PND: Post-natal day

LPS: Lipopolysaccharide

TLR: Toll-like receptor

RT-PCR: Reverse-transcriptase polymerase chain reaction

qPCR: quantitative polymerase chain reaction

NEC: Necrotizing enterocolitis

Ct: Cycle threshold

mRNA: messenger RNA

Introduction

Immune surveillance and protection of mucosal surfaces are essential for proper functioning of biochemical processes and cellular metabolism. A large portion of this homeostatic control is done by the immune system's array of cytokines and macrophages that respond to foreign particles inside the body. In the respiratory system, the removal of foreign particles is essential in maintaining proper exchange of gases and in keeping the work of respiration at a minimum.

Surfactant is essential in maintaining lung homeostasis and is a mixture of phospholipids and proteins that are secreted by type II alveolar cells in the lungs. Surfactant and its associated proteins reduce the surface tension at the alveolar surface, increasing lung compliance and reducing the work required to inspire (Widmaier, 452). Surfactant proteins can be broken down into two classes: hydrophobic and hydrophilic. Surfactant protein B and C comprise the hydrophobic class and aid phospholipids in spreading out on the alveolar surface to reduce surface tension. Surfactant proteins A and D comprise the hydrophilic class and have an additional immune function. These two surfactant proteins have a calcium dependent carbohydrate binding site that allows molecules to bind and facilitate a biochemical process important in the maintenance of a stable lung environment (Bourbon, 78-96).

Surfactant protein A is the most abundant of the surfactant proteins (Wright, 1997). In humans SP-A is encoded by two genes, SP-A1 and SP-A2, which share 97% of their nucleotide sequence (Floros, 2009); however, SP-A is encoded by only one gene in the mouse (Moore, 1992). SP-A has a molecular weight of 28-36 kDa depending on its various glycosylation patterns (Sueishi, 1981) and is part of the C-type collectin family having an N-terminal collagen-like region, an intermediary domain for interaction with phospholipids, and a C-

terminal lectin type domain for carbohydrate binding (Day, 1994). *In vivo* SP-A acts as an octadecamer and aggregates as six trimeric subunits (King, 1989) where interactions with either its globular heads or collagenous tails define its physiological function.

Surfactant protein A has been established as an essential component in the innate host defense system. Work done by Gardai et al. has shown that surfactant proteins A and D are able to cause either an increase or decrease in the inflammatory pathway response based on the binding pattern of the stimulus. Surfactant protein globular heads bind SIRPα and reduce the production of inflammatory cytokines through down-regulation of the NF-κB pathway; conversely, their collagenous tails can bind to CD91/calreticulin and stimulate an inflammatory response. When environmental stimuli such as rough lipopolysaccharide (LPS), cellular debris, or fungal elements interact with the globular heads of SP-A, SP-A's collagenous tails aggregate and bind to CD91/calreticulin, signaling an increase in cytokine production through the NF-κB pathway (Gardai, 2003).

Also important in modulating the immune response of SP-A is its interaction with toll-like receptors (TLRs) 2 and 4. Chronic exposure to endotoxins has shown increased gene expression of SP-A and increased inflammation (George, 2003). In addition, zymosan, a component of yeast cell walls, has been shown to directly bind to TLR-2 and stimulate an immune response. SP-A has been shown to regulate the binding of TLR-2 and zymosan, causing a down-regulation in the response of the immune pathway NF-κB (Sato, 2003). Similar responses have been observed with the stimuli peptidoglycan, a component of gram-positive bacteria cell walls (Iwaki, 2002) and with LPS (Yamada, 2006). LPS has been shown in the absence of SP-A to increase binding to Notch 1 of TLRs, increasing proteins and cytokines in the NF-κB pathway. This increase in cytokines leads to an increased inflammatory immune response

(Monslave, 2009). These studies all indicate the importance of SP-A in the regulation of the immune response to environmental stimuli.

The majority of literature regarding SP-A has focused on its function in the respiratory tract as demonstrated by the information presented above; however, extra-pulmonary sources of SP-A have also been identified. Paananen et al. identified SP-A in the pig Eustachian tube using Northern hybridization and RT-PCR, while Kobayashi et al. identified SP-A expression in the human Eustachian tube (Paananen, 1999; Kobayashi, 1992). Furthermore, MacNeill et al. (MacNeill, 2004) identified SP-A in the epithelium of the vagina through immunohistochemistry and Northern blot techniques.

Importantly, Rubio et al. demonstrated via indirect immunofluorescence, Western blotting, and cloning and sequencing that SP-A is expressed in the small intestine and colon of adult rats (Rubio, 1995). Elkiam et al. confirmed the presence of SP-A in the adult human intestinal tract by using Western blotting to locate SP-A in surfactant-like particles (Elkiam, 1997). Recently, using immunohistochemistry Luo has shown that SP-A is overexpressed in epithelial cells of the intestine and surface of villi in adult patients who suffer from Crohn's disease and ulcerative colitis (Luo, 2008). This finding complements typical findings in the respiratory tract of overexpression of SP-A induced by lung injury (Sugahara, 1996).

While a small number of studies have demonstrated SP-A protein in the intestines, others have failed to demonstrate intestinal protein expression (Korfhagen, 1992; Floros, 1986, Madsen, 2003). Additionally, others have only examined adult animals without examining newborns. SP-A has been shown to be developmentally regulated in the lung with SP-A transcripts being detected at gestational day 15 in the mouse and decreasing by post-natal day 5 (Korfhagen, 1992; Wong, 1996). In humans, SP-A is not expressed until the 33rd week of gestation in type II

alveolar cells; however at as early as 13 weeks of gestation, SP-A was able to be traced in undifferentiated tracheal epithelium (Khoor, 1993). SP-A expression has been shown to peak just before birth, with a decrease of SP-A expression in the first few months of life (Stray-Pederson, 2008). This down-regulation of SP-A after birth is important in understanding its differential effects in newborns, and may contribute to different phenotypes in newborns, especially in the gastrointestinal tract.

Previous work in the George laboratory has shown that null SP-A newborn mice reared in a bacterial-laden corn dust environment experience intestinal inflammation, peritonitis, and death within the first two weeks of life in the absence of respiratory inflammation. Heterozygote breeding strategies produced pups (SP-A +/-) that were able to generate SP-A while the mother (SP-A -/-) was not. The ability of the heterozygous pup to produce SP-A provided itself with protection in the corn dust environment (George, 2008). This indicates an importance for endogenous production of SP-A.

Recent work by George et al. has shown that SP-A null mice exhibit abnormal patterns of bacterial colonization. Specifically, SP-A null mice are colonized by more aerobic gram-positive bacteria compared to matched wild-type pups. qPCR demonstrated that SP-A null mice are colonized with more total bacteria at 14 days of life. qPCR also showed a trend for null mice to be colonized with less potential commensal bacteria (*Lactobacillus/Enterococcus*) by 48 hours of life, although not significant. Additionally, null pups have more non-*E. coli* gram-negative bacteria (George, 2010).

Certain polymorphisms of the SP-A genes are associated with respiratory distress syndrome in neonates (Haataja, 2000). Additionally, many pre-mature infants born before the age of surfactant synthesis are predisposed to respiratory distress syndrome (Hallman, 1988;),

while SP-A null mice have been shown to be susceptible to bacterial invasion in the lung (Levine, 1997). Likely, similar effects may be noticed in areas such as the gastrointestinal tract. One of the most prevalent diseases among pre-mature infants is that of necrotizing enterocolitis (NEC), at an incidence of 2-5% (Wiedmeier, 2008). NEC is multi-factoral disease characterized by ischemic necrosis. Contributing factors include a less-developed immune system, a more permeable epithelial barrier due to incorrect tight junction function, and disordered peristalsis (Mayer, 2003; Salzman, 1998; Muresan, 2000; Berseth, 1989). With these factors affecting the pre-mature infant, abnormal bacterial colonization occurs (Israel, 1994).These patterns of abnormal bacterial colonization observed in NEC are consistent with patterns of abnormal colonization observed in our SP-A null newborn mice (Claud, 2001).

Collectively, these results suggest that SP-A does play an important role in the gastrointestinal tract, especially in newborn mice. For this reason it is important to understand the physiological role of SP-A in the developing gastrointestinal tract and the source of newborn gastrointestinal SP-A. SP-A protein has been found in the amniotic fluid, the presumed source to be the fetal lung, where concentrations increase throughout pregnancy (Miyamura, 1994). Additionally, SP-A gene expression has been found in the newborn gastrointestinal tract (George, 2008).

This thesis examines the expression of SP-A protein in the gastrointestinal tract via immunohistochemistry, and the specific location of SP-A gene expression via RNA *in situ* hybridization, with the hypothesis that SP-A gene and protein expression occur in the newborn murine gastrointestinal tract and have physiological significance. The presence of SP-A in the developing intestinal tract likely serves a similar innate immune function that mirrors its effect in

the lung: SP-A can act as an opsonin, coating bacteria as a signal for phagocytosis thereby regulating microflora colonization and preventing colonization by pathogenic strains.

Methods

Animals Mice of the C3HeB/FeJ background served as the wild-type control animals. Mice with an SP-A knock-out mutation on a C3H/Hen background were originally obtained from Dr. Frank McCormack, University of Cincinnati. The SP-A knock-out mutation was backcrossed 10 generations from mice of the C3H/Hen background onto the C3HeB/FeJ background to obtain SP-A null mice on the same background as the wild-type controls. Mice were housed in plastic cages with micro-isolate lids and maintained by the animal care staff at the University of Minnesota. Mice had access to food and water *ad libitum*.

SP-A genotyping Genotyping of breeders and offspring ensured correct maintenance of the strains. DNA was obtained from ear punches of each mouse and lysed in Direct PCR for Tails lysis buffer (Viagen) with proteinase K. Primers were designed to amplify a region of the SP-A gene that spanned exons 3 and 4 and intron 3 that corresponded to the region of the neomycin insert in the knock-out mice (Forward: GCA GAG ATG GGA GAG ATG GTA TCA A; reverse: ATG GAC CTC CAT TAG CAT GTG GGA). Primers for the SP-A null mice corresponded to the neomycin insert in the SP-A gene. (Forward: TGA ATG AAC TGC AGG ACG AG; reverse: ATA CTT TCT CGG CAG GAG CA).

Tissue Collection SP-A wild-type and SP-A null mice at post-natal days (PND) 3 – 7 were sacrificed for tissue collection by an overdose of pentobarbital (FatalPlus). Adult wild-type and SP-A null tissue was also collected to serve as controls. Tissue was embedded in aluminum foil molds with O.C.T. compound (TissueTek) and flash frozen in liquid nitrogen or a dry ice/acetone mixture and stored at -80 °C until use. Lung tissue for immunohistochemistry was

inflated with an O.C.T./PBS (70/30) mixture before being flash-frozen. Tissue collected specifically for RNA *in situ* hybridization was flash-frozen within 5 minutes of euthanasia of the animal to ensure preservation of RNA within the cells. All materials used in tissue collection for RNA *in situ* hybridization were pre-treated with RNAse Away (Molecular Bio Products) or RNAse Zap (Ambion) and rinsed with DEPC-H_2O to eliminate RNAse contamination.

Immunohistochemistry Tissue was sectioned at 5 μm and thaw-mounted onto FisherBrand SuperFrost Plus glass slides. Slides were air-dried at room temperature for 20 minutes before being stored in sealed slide boxes at -80 °C until use. Slides were fixed in cold acetone for 2 minutes before being quenched in 0.3% hydrogen peroxide in methanol for 30 minutes. A Rabbit VectaStain ABC Elite Rabbit IgG kit was used for blocking and detection. The antibody used was a Santa Cruz Biotechnology rabbit polyclonal IgG anti-SP-A antibody (H-148) used at a dilution of 1:100 for intestine and 1:250 for lung sections. Staining was detected with diaminobenzidine (DAB) and cells were counter-stained with 40% hemotoxylin before being dehydrated and coverslipped.

Probe Synthesis for RNA in situ hybridization: Total RNA was isolated from adult wild-type lung with a Qiagen RNeasy Micro Isolation kit. RNA was reverse transcribed to cDNA with a Bio-Rad iScript cDNA synthesis kit and amplified via PCR. Specific SP-A primers spanning exon 5 and 6 of the SP-A gene were designed (sense: 5'-GCT TAC CTG GAT GAG GAG-3', corresponding to bases 1862 - 1879 in exon 5 (Korfhagen, 1992); antisense: 5'-ACA AAT GGC CAG CCG GTA CTG CA-3' (Wong, 1996), corresponding to bases 2629-2651 in the sixth

exon) to produce a 438 bp segment of DNA. The amplified DNA was transformed into competent TOP10F' *E. coli* using a PCRII-TOPO vector (Invitrogen) with the Rapid One Shot chemical transformation protocol. The transformed vector was isolated with a Promega Wizard Plus SVMiniprep DNA purification system and purified with a ChromaSpin STE-100 spin column (Clonetech). Sequencing confirmed the correct orientation of the insert. The vector was linearized with XbaI to make the antisense probe or with HindIII to make the sense probe. Linearized templates were incubated with the appropriate RNA polymerase (Sp6 for antisense or T7 for the sense probe) and an rNTP mix with digoxigenin-labeled UTP (Roche) at 37°C for 2 hours. Additional polymerase was added 1 hour into the incubation time, and the reaction was stopped with 0.5M EDTA. Transcribed probes were purified with a glycogen carrier on a ChromaSpin STE-100 spin column (Clonetech) and tested for digoxigenin incorporation with an anti-digoxigenin antibody (Roche) on a nitrocellulose blot. Probes were stored at -80°C in RNasin and 100mM DTT. A cμ probe for detection of B cells was graciously given by Angela Panoskaltsis-Mortari, PhD, to serve as a control probe for the RNA *in situ* hybridization procedure.

RNA in situ hybridization The *in situ* hybridization protocol was adapted from a previously published protocol developed by the Panoskaltsis-Mortari laboratory (Panoskaltsis-Mortari, 1995)**.** Briefly, tissue was sectioned with a cryostat at 4 μm, thaw-mounted on FisherBrand SuperFrost Plus glass slides and air-dried for 30 seconds before being fixed in 3% paraformaldehyde in DEPC-treated PBS. Slides containing intestinal tissue were washed with 0.2 M HCl for 8 minutes to inhibit endogenous alkaline activity. Tissue was hybridized with heat-denatured (80°C) antisense SP-A or sense SP-A (lung and intestinal tissue) or a cμ probe

(spleen), sealed with a coverslip and incubated at 50 °C overnight in a humid chamber. The following day excess probe was degraded with 40ug/mL RNase A before immunological detection. Tissue was blocked with 2% Normal Horse Serum and incubated with an anti-digoxigenin antibody (1:500 dilution) at room temperature for 2 hours. The antibody was detected with an NBT/BCIP solution with 2mM levamisole overnight at 4°C. Slides were mounted in aqueous mounting medium (AquaMount).

Results

Immunohistochemical staining for SP-A in newborn tissues.

Intestinal tissue from wild-type post-natal day 7 mice probed with a polyclonal anti-SP-A antibody stained positively in the lamina propria of intestinal villi in all 5 tissue samples. No staining was seen in the epithelial cells of the villus. Staining occurred in regions of the small intestine with little to no background staining (Figure 1). Adult wild-type lung tissue served as a positive control and stained positively in type II alveolar cells. No staining occurred in negative-control wild-type lung (Figure 1) when primary antibody was not added. However, 2 of 5 PND 7 SP-A null intestinal tissue samples showed positively staining cells in the lamina propria when probed with a polyclonal anti-SP-A antibody. Positively stained cells occurred in the lamina propria of the intestinal villus with no staining in the epithelial cells of the villus (Figure 2). Cells stained with similar intensity and frequency in the two SP-A null samples as stained in wild-type tissue.

An SP-A anti-sense probe was successfully designed

Isolating total RNA from adult-wild type lung and amplifying the subsequent cDNA produced a probe of 438 bp. After transforming the probe into competent *E. coli*, extracting the vector, and linearizing the vector, the Sp6 promoter produced a digoxigenin-labeled anti-sense SP-A probe that stained positively when probed with an anti-digoxigenin antibody and detected with an NBT/BCIP solution on a nitrocellulose blot. Adult wild-type lung tissue stained positively for hybridization of the anti-sense SP-A probe to messenger RNA with staining in type II alveolar cells (Figure 3). Positive staining also occurred with the anti-sense SP-A probe in post-natal day 6 lung tissue (Figure 4). Adult wild-type spleen served as a control for the RNA *in situ*

hybridization procedure, and positive staining occurred when probed with a cμ probe (Figure 3). The cμ probe stained for B cells.

SP-A mRNA was detected in cells of the newborn intestine

Intestinal tissue of wild-type mice post-natal days 3 and 6 stained positively for SP-A mRNA when probed with a digoxigenin-labeled anti-sense SP-A probe. Positively stained cells were found in the lamina propria of the intestine (Figure 4) and in the muscularis externa (Figure 5). Not all cells in the lamina propria stained, nor did all sections of lamina propria in various villi. No staining was observed in the epithelial cells of the small intestine. When intestinal tissue was probed with a digoxigenin-labeled SP-A sense probe (negative control), no staining occurred (Figure 6).

Discussion

In the lung SP-A protein is critical for normal immune function of attenuating inflammatory responses from bacteria and other pathogens. SP-A recognizes and binds to bacteria through its carbohydrate recognition domain. This domain recognizes bacterial proteins such as LPS and peptidoglycan and can stimulate immune cells through interactions with TLRs (Yamada, 2006; Iwaki, 2003). These interactions are important in maintaining a stable lung environment void of pathogens.

Additionally, SP-A is expressed in extra-pulmonary mucosal sites such as the vagina (MacNeill, 2004), Eustachian tube (Paananen, 1999; Kobayashi, 1992), and intestinal tract (Rubio, 1995; Elkiam, 1997) where it likely serves a similar function of preventing abnormal bacterial colonization and invasion of pathogens. Importantly, Rubio et al. have demonstrated the evolutionary development of SP-A. An SP-A like molecule reactive with an antibody raised against rat pulmonary SP-A was found in teleost fish swim bladders and intestines; however, no such molecule was found in fish gills. This finding signals an evolutionary development whereby present-day pulmonary surfactant likely evolved from this phylogenetically older form absent from pulmonary structures (Rubio, 1996).

This thesis is the first time that the expression of SP-A in the newborn murine intestine has been examined. Previous literature has focused on expression in the adult murine intestine with little finding of the SP-A protein in adult intestine; however, SP-A may be developmentally expressed here. In the lung, SP-A expression peaks shortly after birth before decreasing to steady-state levels present in the adult. In murine lung, SP-A transcripts can be detected at gestational day 15 with a peak at post-natal day 5 (Korfhagen, 1992; Wong, 1996). It is possible that SP-A expression in the intestine follows a similar pattern.

Immunohistochemistry in post-natal day 7 wild-type mice showed positively stained cells for the SP-A protein in the lamina propria of the intestine. The location of the positively stained cells was unexpected as Rubio had shown positive staining in adult intestinal epithelial cells (Rubio, 1995). Additionally in the vagina, MacNeill has shown that squamous epithelial cells stain positively for SP-A protein (MacNeill, 2004).

The location of SP-A protein that we found in cells other than epithelial cells begs the question of how SP-A protein can exert its effect on the colonization of bacteria if expression is not in surface cells where direct contact can be made. A possible explanation involves the strength of immune response exhibited by layers of the intestinal tract. The thin mucosal layer of epithelial cells is rather weak in exhibiting an immune response; therefore, the lamina propria that lies directly beneath the epithelial layer of cells serves as a second line of defense for the multiple pathogens the intestinal tract encounters. Many cells found in the lamina propria are specific to the immune system—T cells, B cells, dendritic cells, and macrophages. Additionally, myofibroblasts and fibroblasts in the lamina propria can function as part of the innate immune system by expressing TLRs (Otte, 2003). These cells can make up 30% of the mononuclear cell population of the lamina propria and function as antigen presenting cells (Saada, 2006). Intestinal stromal cells have also been shown to express TLRs, indicating action by the innate immune system in the lamina propria (Walton, 2009). SP-A has been shown to interact with TLRs in the lung (Sato, 2003; Iwaki, 2002; Yamada, 2006), and it may serve a similar function in the intestine by being present in the lamina propria.

Other research has shown that intestinal stromal cells may be important in stimulating T regulatory cells to suppress the inflammatory response. Powell suggests that intestinal stromal cells may be important in both stimulating and suppressing an immune response depending on

other immune factors present (Powell, 2011). This hypothesized dual role of intestinal stromal cells mirrors the dual role SP-A plays in the lung—it can stimulate or suppress immune function based on the way its globular head or tails bind to other components (Gardai, 2003). SP-A presence in this region likely further enhances the innate immune function of the intestinal tract; however, the exact type of cells that stained positive for SP-A have yet to be determined.

Additionally, some tissue sections (2 of 5) in SP-A null PND 7 mice also stained positively for SP-A protein expression via immunohistochemistry. All mice genotyped indentified with correct expression of either the neomycin insert or wild-type SP-A gene, so loss of the neomycin insert could be ruled out as a possibility for this phenomenon; however, the anti-SP-A antibody used was a polyclonal antibody. Likely, one of the other clones in the antibody not entirely specific for SP-A was the cause of this unexpected staining. Furthermore, Stahlman et al. have shown that human SP-A contains a major blood group epitope. Polyclonal and monoclonal anti-SP-A antibodies developed from surfactant isolated from a blood type A individual were able to aggregate erthyrocytes of blood type A. In their study, positive immunohistochemistry staining for SP-A occurred in tissues where blood group antigens are distributed such as Brunner's glands and duodenal epithelium, Lieberkuhn's glands in the small intestine, acini and ducts of the pancreas, and uterine and cervical tissue (Stahlman, 1992).

Since the staining in the PND7 null SP-A intestinal sections and wild-type tissue sections occurred in the same area, it is likely that the reactivity seen may mirror the findings of Stahlman. Many species such as the mouse, sheep, goat and rabbit used to generate antibodies may have A antigen which exhibits similar homology to the human blood A antigen (Franks, 1969). This could produce non-specific staining for SP-A, as possibly seen in my results.

Consequently, this further complicates the elucidation of SP-A protein expression in the intestine.

SP-A gene expression does exist within the intestinal tract of newborn mice, although at significantly lower levels than in the lung. RT-PCR determined SP-A intestinal gene expression in newborn mice to be at a Ct of 30.22, where the upper limit of detection was 40 cycles. Newborn lung showed much higher expression of SP-A at a Ct of 20.23 (George, 2008). These results indicate that SP-A is expressed in the newborn intestine, albeit at a level approximately 5000 times less than in the lung.

With the non-definite protein expression results obtained, the exact cells that contain the SP-A mRNA message were determined. RNA *in situ* hybridization provided precise staining of cells that contain the message, and the invention of non-radiolabled probes made the procedure less hazardous. A 438 bp anti-sense digoxigenin-labeled SP-A probe was designed that corresponded to the carbohydrate recognition domain of the SP-A mRNA message, spanning exons 5 and 6.

Practice trials of the RNA *in situ* hybridization showed the digoxigenin-labeled anti-sense SP-A probe bound cells in adult wild-type lung tissue and PND 6 wild-type lung tissue, likely corresponding to type II alveolar cells. The exact nature of the positively stained cells could not be determined by morphology as the lung tissue was not inflated. For preservation of the mRNA message, we elected to flash-freeze the tissue immediately instead of inflating the lung. Practice trials with a positive control (cμ probe staining for B cells in adult wild-type spleen) for the *in situ* procedure also confirmed the workings of the *in situ* protocol.

RNA *in situ* hybridization with an anti-sense SP-A probe in intestinal tissue showed cells that stained positively. Cells that stained positively were found in the lamina propria of the

intestinal villi—the same location that SP-A protein was detected via immunohistochemistry. Additionally, positive staining cells for the SP-A mRNA message occurred in what is likely the muscularis externa.

Mikkelsen et al. have shown that the muscularis externa in its subserosal layer contains macrophage like cells (Mikkelsen, 1985). Additionally, Poindexter et al. have shown that human defensins are normally present in the muscularis externa and are upregulated in the muscularis externa after a burn injury (Poindexter, 2009). These findings indicate a role for the muscularis externa in immune protection. Furthermore, in the lung Kresch et al. have shown that SP-A is important in the release of neutrophil chemotactic factor (Kresch, 2010), and Jouet et al. have shown that in canine ileitis, polymorphonuclear lymphocytes increased in both the lamina propria and the muscularis externa (Jouet, 1995). This finding that SP-A is important in recruitment of neutrophils and that neutrophils are present in the lamina propria and muscular externa during inflammation further augments the role of SP-A in gastrointestinal immune function.

Fewer cells stained positively for the SP-A mRNA message than stained positively for SP-A protein expression. Likely, sporadic staining present in the *in situ* procedure was a result of RNAse contamination at some point in the procedure or degradation of RNA from time of removal of the intestine to time of flash-freezing. It is also likely that the observed increase of cells positive for SP-A protein was the result of the polyclonal anti-SP-A antibody used. The polyclonal antibody is able to bind multiple B cell epitopes of an antigen, not all necessarily specific for SP-A. Nonetheless, positive staining for both SP-A mRNA and SP-A protein corroborate initial experiments in the George laboratory that showed SP-A gene expression in newborn intestine via RT-PCR.

Conclusions and Future Directions

The finding that similar cells stained positively for both mRNA and protein SP-A expression is good evidence that SP-A is expressed in the newborn murine intestinal tract. Here SP-A most likely exerts a similar immune function as it does in the lung. SP-A functions as an opsonin, binding to bacteria through its carbohydrate recognition domain to signal the pathogen for phagocytosis by other immune cells. Without proper SP-A expression in the intestine, bacterial clearance is likely to be delayed and diminished, accounting for the previous abnormal colonization patterns observed by the George lab (George, 2010).

These results, however, are not without inconsistencies. For this reason, the exact location of intestinal SP-A expression still remains elusive, and it is important to further investigate its location. The use of a monoclonal anti-SP-A antibody in immunohistochemistry may solve the inconsistencies of positive staining in SP-A null intestinal tissue. Additionally, creation of an antibody from surfactant protein-A void of blood group A antigens may inhibit cross-reactivity. Stahlman et al. demonstrated that when SP-A was treated with collagenase, their monoclonal anti-SP-A antibody that had shown cross-reactivity with blood-group antigens no longer reacted (Stahlman, 1992). This indicates the important of collagenase treatment of SP-A before creation of an anti-SP-A antibody to eliminate possible cross-reactivity.

An additional avenue to support intestinal SP-A protein expression involves stimulation of SP-A protein production *in vitro*. Intestinal sections at various post-natal days of life could be cultured in the presence of factors known to upregulate SP-A expression such as keratinocyte growth factor (Haddad, 2003) or lipopolysaccharide (Sugahara, 1996). Isolation of SP-A from the culture would provide supporting evidence of SP-A production in the gastrointestinal tract.

Furthermore, investigation into other sources of newborn intestinal SP-A exposure is necessary. These may include ingestion of amniotic fluid *in utero* or circulation of pulmonary surfactant. By determining the exact source of intestinal SP-A, its impact on gastrointestinal physiology and clinical significance can be more fully understood.

Figures

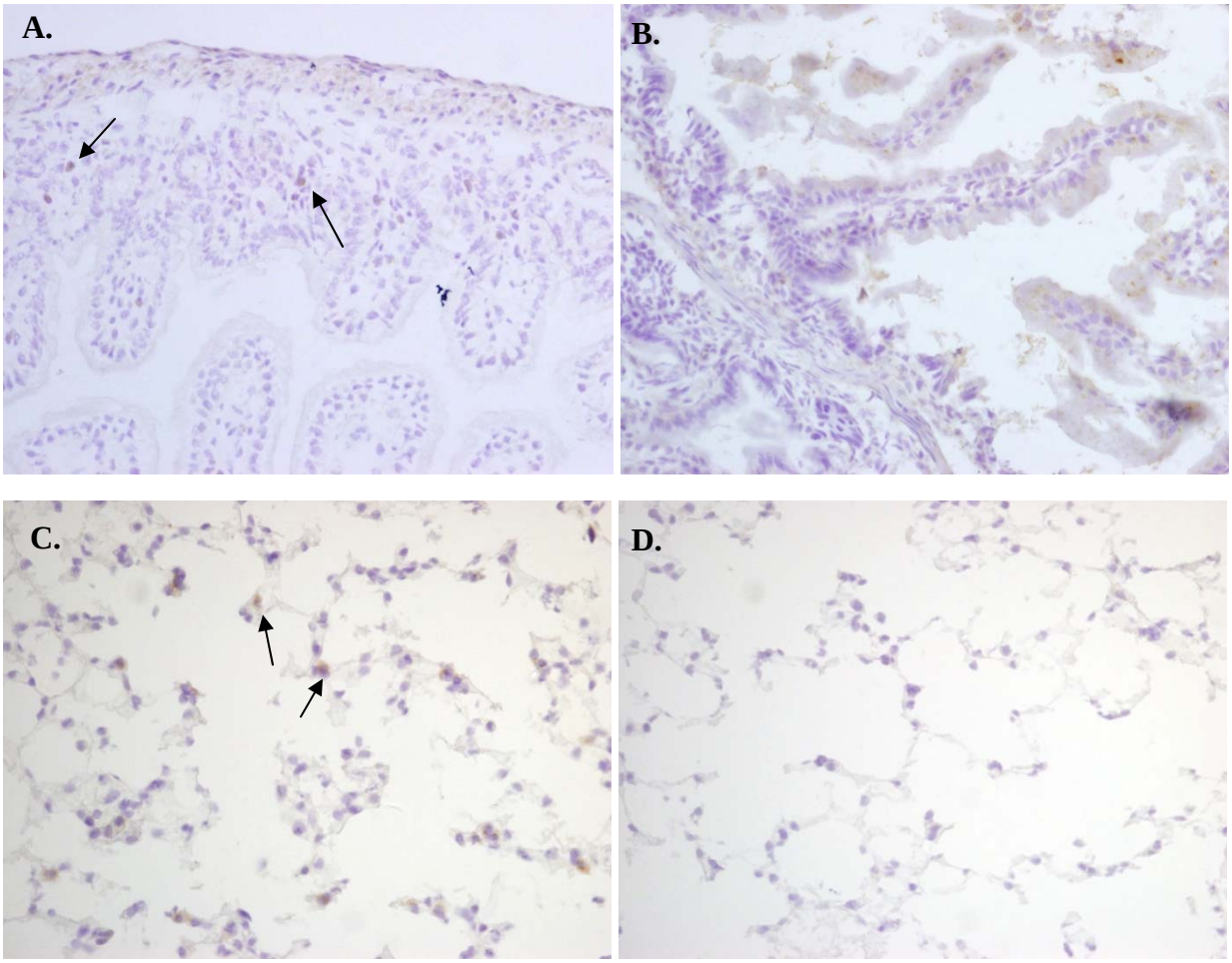

Figure 1. Immunohistochemistry for SP-A. **A.** SP-A staining in a PND 7 wild-type mouse intestinal tract. Stained cells were seen in the lamina propria. **B**. SP-A staining in a PND 7 SP-A null mouse intestinal tract. Only background staining in visible. **C**. SP-A staining in wild-type adult lung tissue (positive control). Alveolar cells (type II pneumocytes) stained positively. **D.** SP-A staining in an adult wild-type lung without addition of SP-A antibody (negative control). No staining occurred in the negative control.

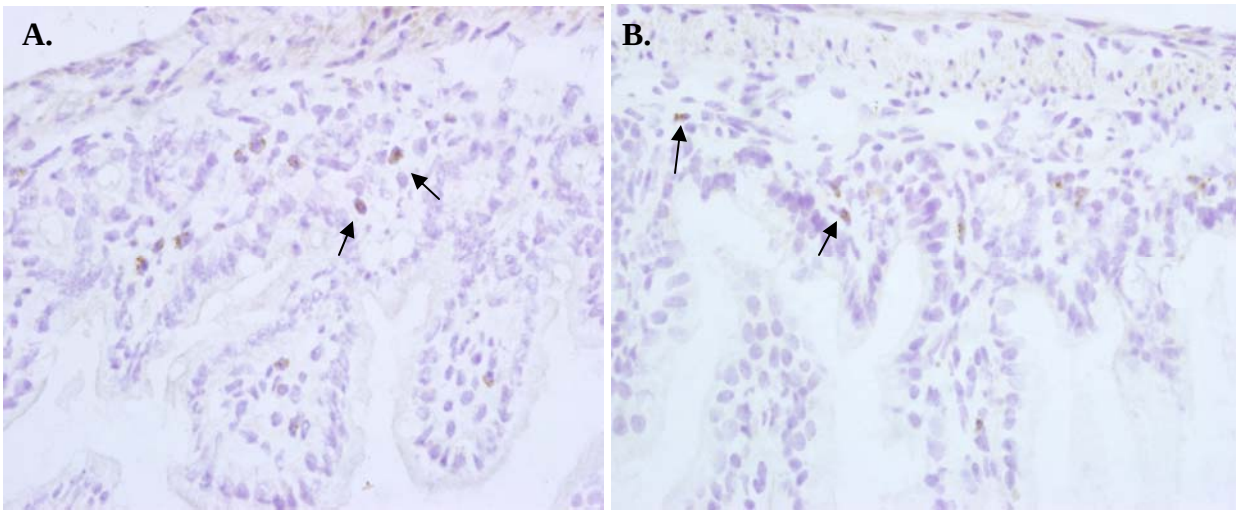

Figure 2. Immunohistochemistry comparing wild-type and SP-A null intestinal staining. **A.** SP-A staining in a PND 7 wild-type intestine. Cells of the lamina propria stained positively. All 5 samples showed positively staining cells in the lamina propria. **B.** SP-A staining in a PND 7 SP-A null intestine. Staining of cells in the lamina propria also occurred in 2 of the 5 SP-A null intestinal tissue samples. When staining occurred in SP-A null intestine, it occurred with the same intensity and frequency as in wild-type tissue.

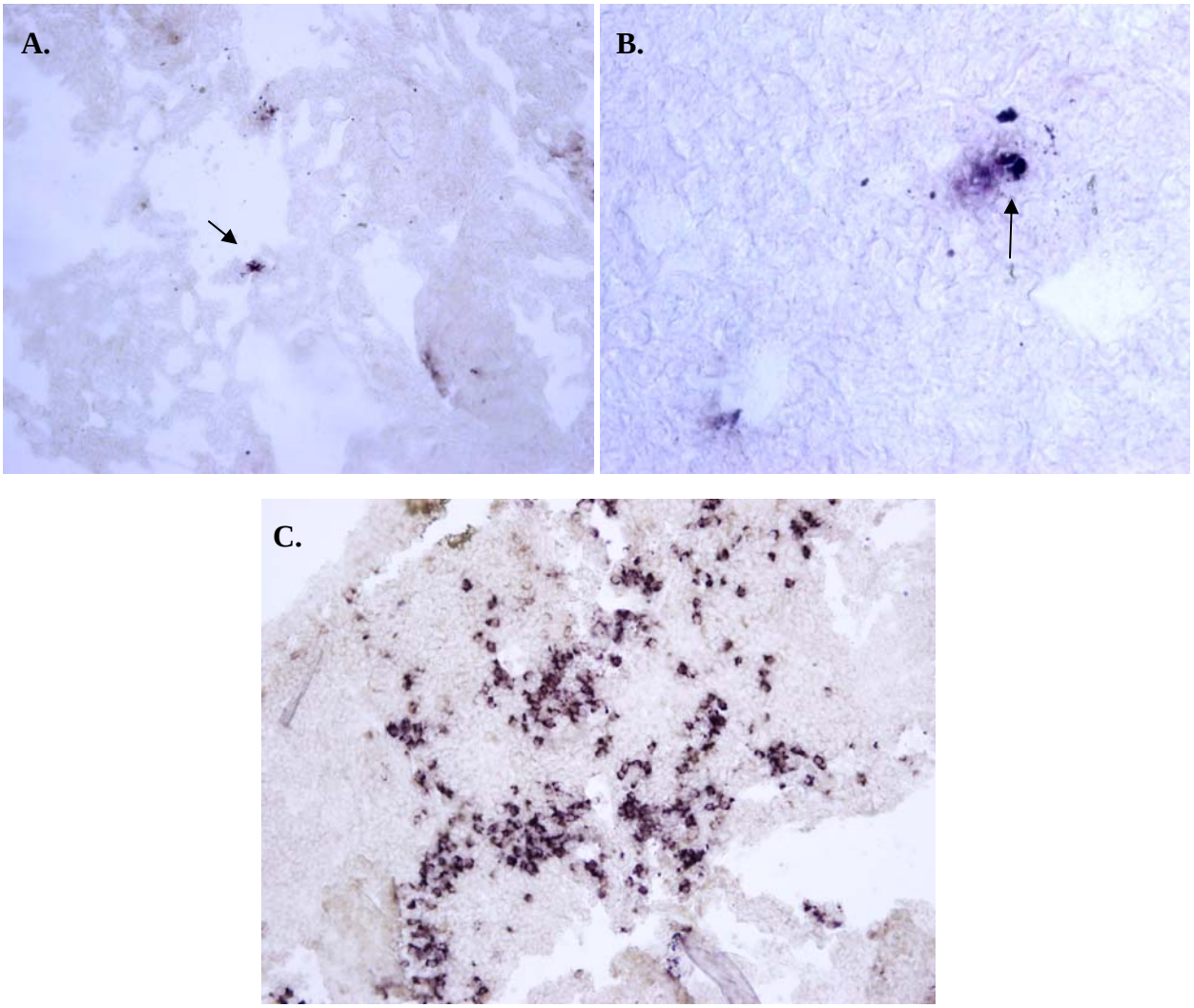

Figure 3. RNA *in situ* hybridization of adult lung and spleen. **A & B.** Adult wild-type lung tissue stained positively for messenger RNA when probed with an anti-sense digoxigenin-labeled SP-A RNA probe, detected with an anti-digoxigenin antibody, and stained with NBT/BCIP. Positive staining indicated successful design of the anti-sense SP-A probe. **C**. Adult wild-type spleenic tissue served as a positive control for the RNA *in situ* hybridization procedure. A cμ probe hybridized to B cells in the spleen.

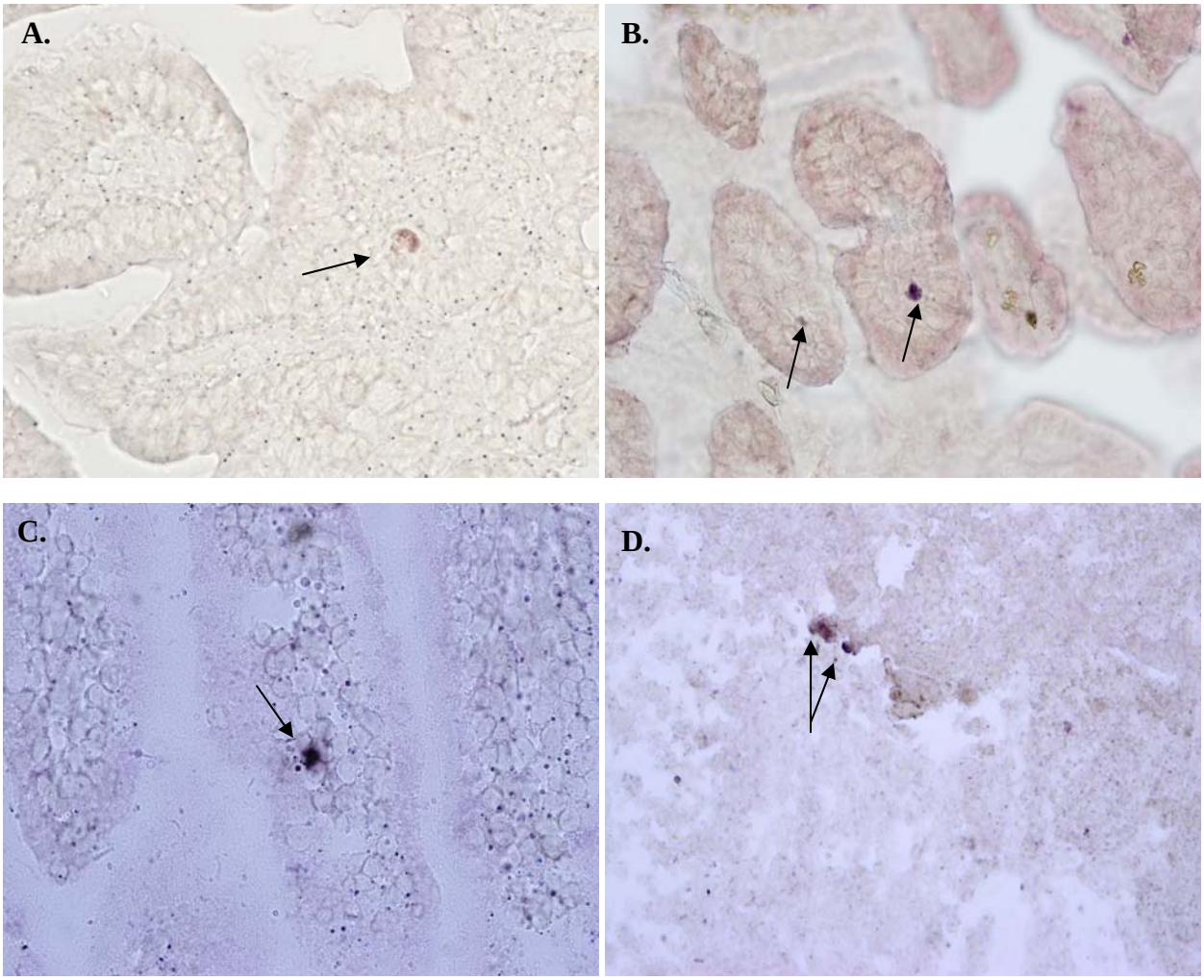

Figure 4. RNA *in situ* hybridization in a post-natal day 6 wild-type mouse. **A, B & C.** Intestinal tissue stained positively for SP-A messenger RNA when probed with an anti-sense digoxigenin-labeled SP-A RNA probe, detected with an anti-digoxigenin antibody, and stained with NBT/BCIP. Staining occurred in the lamina propria of intestinal villi. **D**. Lung tissue also stained positively for SP-A messenger RNA when probed with an anti-sense digoxigenin-labeled SP-A RNA probe.

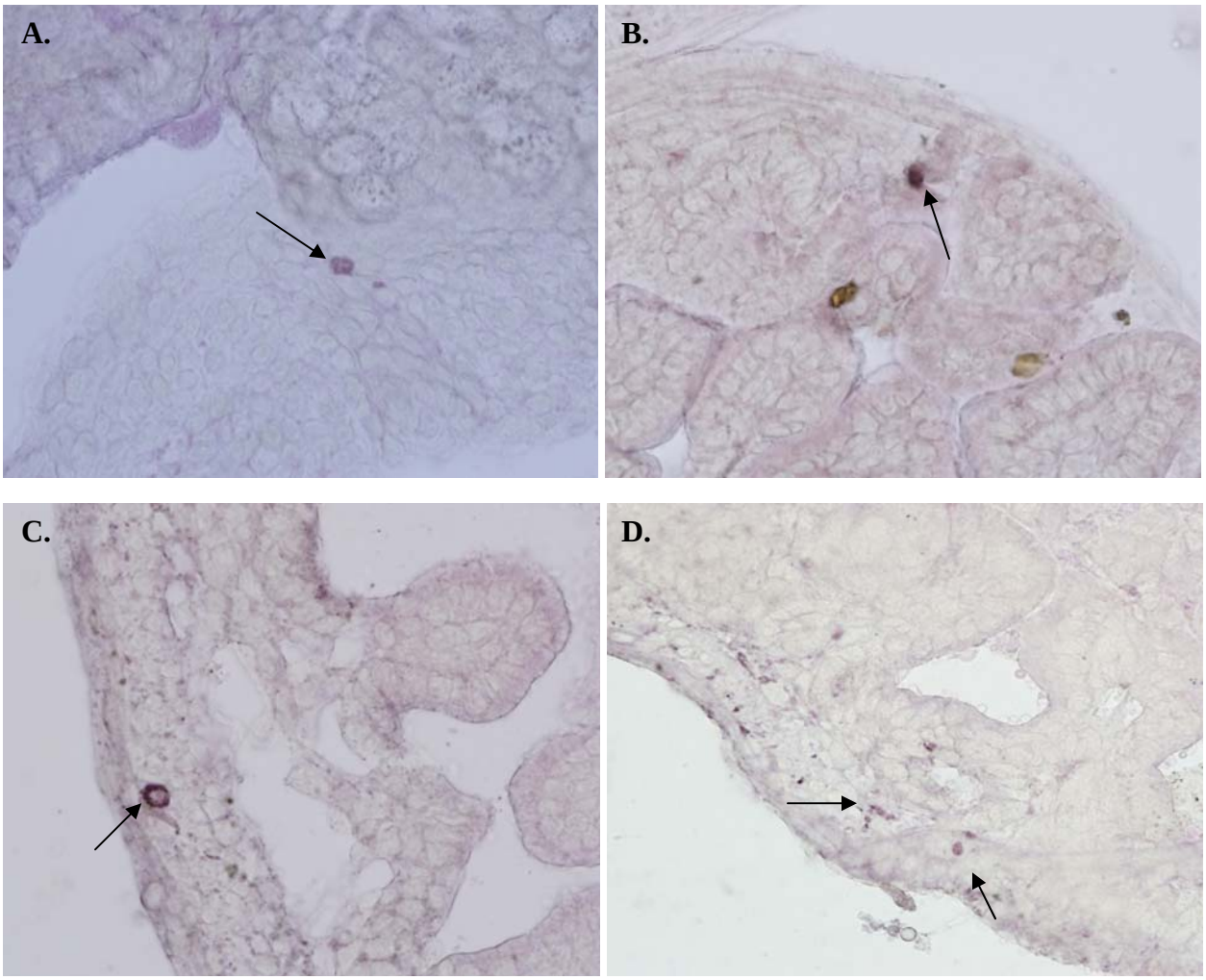

Figure 5. Additional RNA *in situ* hybridization staining. **A &B.** Cells in the muscularis externa also stained positively when probed with a digoxigenin-labeled SP-A antisense RNA probe in post-natal day 3 wild-type intestinal tissue. **C & D.** Positive staining in the muscularis externa also occurred in post-natal day 6 wild-type intestinal tissue when tissue was probed with a digoxigenin-labeled SP-A antisense RNA probe.

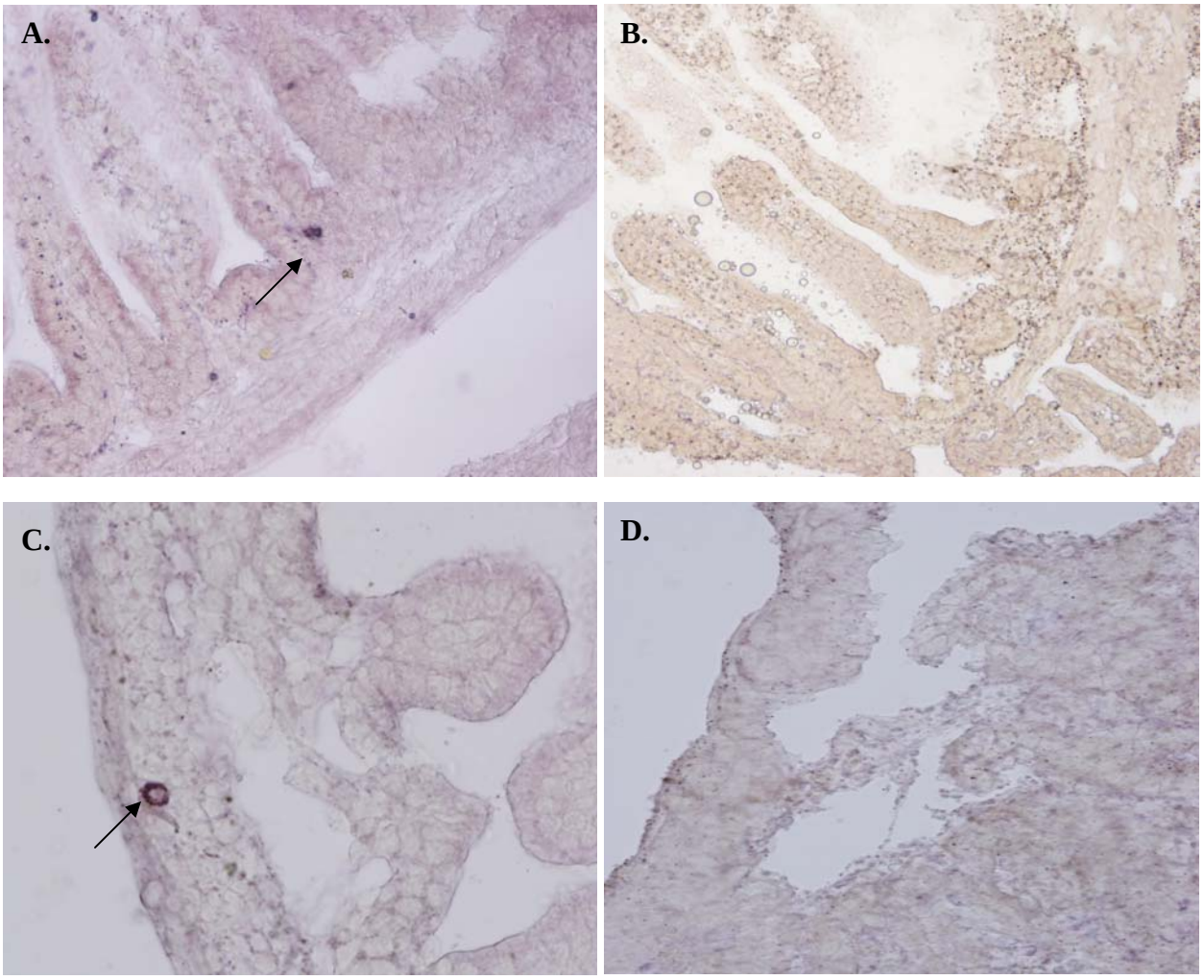

Figure 6. Comparison of RNA *in situ* hybridization antisense and sense probes. Both SP-A antisense and sense digoxigenin-labeled probes corresponded to the same region in the SP-A gene, exons 5 and 6. The sense probe matched the SP-A mRNA message (negative control) and was therefore unable to hybridize. **A.** Positive staining with the anti-sense SP-A probe in the lamina propria of a PND 6 wild-type mouse. **B.** No staining occurred in the lamina propria when a PND 6 wild-type mouse intestinal section was probed with the SP-A sense probe. **C.** Positive staining with the anti-sense SP-A probe in the muscularis externa of PND 6 wild-type mouse. **D**. No staining occurred in the muscularis externa when a PND 6 wild-type mouse intestinal section was probed with the SP-A sense probe.

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