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Smooth Muscle Myosin Heavy Chain Isoform Distribution in the Swine Stomach

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To evaluate the distribution of smooth muscle myosin heavy chain isoforms (SMB, with head insert), we examined frozen sections from the various regions of swine stomachs using isoform-specific antibodies. We previously reported variable SMB myosin heavy chain (MHC) expression in stomach cells that correlates with unloaded shortening velocities. This is consistent with the generalization of tonic fundic muscle having low expression and phasic antral muscle having high expression of the SMB MHC isoform. Using immunohistochemistry (IHC), we show a progression of the

SMB MHC from very low immunoreactivity in the fundus to very intense immunoreactivity in the antrum. In the body, the average level of SMB MHC immunoreactivity lies between that of the antrum and fundus. Intercellular heterogeneity was observed in all stomach regions to a similar extent. However, the intercellular range in SMB MHC immunoreactivity decreases from fundus to antrum. All stomach regions show isolated pockets or clusters of cells with similar SMB MHC immunoreactivity. There is a non-uniform intracellular immunoreactivity in SMB MHC, with many cells showing greater-intensity staining of SMB MHC in their cell peripheries. This information may prove useful in helping to elucidate possible unique physiological roles of SMB MHC.

Myosin, a hexamer composed of two myosin heavy chains (MHCs) and two sets of myosin light chains (MLCs), has several isoforms resulting from differences in each subunit. Alternate splicing of a single MHC gene results in four different MHC isoforms. Alternate splicing of the 3' end of the MHC gene codes for SM1 and SM2, which differ by 43 amino acids including a 34-amino-acid size difference in the COOH terminal of the myosin molecule (Babij and Periasamy 1989; Nagai et al. 1989). Alternate splicing of the 5' end of the gene results in SMA and SMB MHCs, which differ by the presence or absence of a 7-amino-acid insert at the 25–50-kD junction of the myosin molecule (Hamada et al. 1990; Kelley et al. 1993; White et al. 1993). Alternate splicing of a different gene results in LC17a or LC17b, which have the same molecular weight but differ at four of the last nine amino acid positions at the COOH terminus (Nabeshima et al. 1987; Lenz et al. 1989; Lash et al. 1990). These essential MLC isoforms are characterized by their isoelectric points; LC17a is the more acidic of the two species (Hasegawa and Morita 1992; see Eddinger et al. 2000 for nomenclature). Lastly, two different genes code for non-muscle (NM) and smooth muscle (SM) LC20, which are identical in size but differ at 11 residues (Taubman et al. 1987; Kumar et al. 1989; Gaylinn et al. 1989). The more basic of these two isoforms is characterized as the SM isoform, with the more acidic being the NM variety (Bárány and Bárány 1996).

Isoform content may be particularly important to smooth muscle physiology because isoform composition is the principle determinant of contractile characteristics in other contractile cells (Wagner 1981). Because a major difference between SM cells is contraction type, tonic

and phasic SMs are traditionally used as experimental models due to their different rates of contraction and their ability to maintain force. Tonic muscles develop sustained tone slowly when potassium-depolarized in vitro, whereas phasic muscles develop tone rapidly (Somlyo and Somlyo 1968). SMs from tissues such as the aorta and other large vessels and sphincters are characterized as tonic muscle, where slow, sustained contraction is needed for optimal organ function. Conversely, gizzard, intestine, and esophageal body (when it is smooth muscle) are primarily phasic muscle, where frequent, comparatively rapid contraction may be required for proper organ function. Special attention has recently focused on the 25–50-kD junction domain of myosin S1 head, the region of the MHC ATPase site. This is where the seven amino-acid insert resides and appears to be correlated with differences between tonic and phasic contraction (Hamada et al. 1990; Kelley et al. 1993; Rovner et al. 1997; Eddinger and Meer 2001).

The stomach is traditionally divided into three regions: the fundus, which comes directly off the esophagus; the body, which lies between the fundus and antrum; and the antrum, which encompasses the distal third of the stomach and feeds into the duodenum (Figure 1A). Histologically, all three stomach regions are composed of two separate discernible muscle layers, the muscularis mucosa and the more massive muscularis externa (Figure 1C) (Bockus et al. 1974; Jacobson et al. 1974; Schultz et al. 1989). Although there is no reported contractile difference between muscle layers, stomach motility changes as one moves along the greater curvature of the stomach (Jacobson et al. 1974; Schultz et al. 1989). In general, tonic contractions are localized in the fundus of the stomach, which regulates the reservoir functions of the stomach and adjusts the overall size of the stomach as it pushes food to the antrum (Schultz et al. 1989). Phasic contraction is localized to the antrum, where stomach contents are mixed and ground before being passed to the duodenum (Figure 1A) (Schultz et al. 1989). The body, which is the region between fundus and antrum, is traditionally considered a tonic muscle. However, it has been found that this region is a transition between antral and fundic extremes (Jacobson et al. 1974; Johnson et al. 1987; Schultz et al. 1989).

Eddinger and Meer (2001) reported that a regional distribution of SMB MHC expression in cells from the stomach correlates with their mechanical diversity. Tonic SM cells in the fundus contain on average less than 40% SMB MHC (range 12–100%), whereas phasic, antral SMs contain an average of 94% SMB MHC (range 80–100%). In addition, the unloaded shortening velocity of these cells correlates with the percent SMB MHC expressed (Eddinger and Meer 2001). Population means revealed that the average shortening velocity of fundic cells was one third that of antral cells and cell shortening velocities correlate directly to the percent SMB MHC in individual cells (Eddinger and Meer 2001). These findings are similar to data published at the tissue level in hypertrophied rat bladder (Sjuve et al. 1996; DiSanto et al. 1997) and at the protein level by Kelley et al. (1993) and Rovner et al. (1997).

This study was designed to define the histological distribution of SMB MHCs in the stomach regions previously examined by our lab. By examining single cells, it was technically impossible to determine a tissue's histological distribution of SMB MHC. Results from this study confirm the trend in SMB MHC immunoreactivity from low to high as one traverses along the greater curvature of the stomach from fundus to antrum. In addition, we report intercellular and intracellular heterogeneity in SMB MHC immunoreactivity. Cells with high SMB MHC immunoreactivity are equally likely to be next to a cell of similar SMB MHC immunoreactivity, or not, throughout all regions examined. Finally, we report intracellular heterogeneity as a tendency for SMB MHCs to be localized to the perimeter of a cell and otherwise randomly distributed within the cell.

Materials and Methods

Tissues were isolated from pigs killed in a local slaughterhouse. The stomachs were removed, cleaned of blood, adipose, and loose connective tissue, and dissected into fundus (region 2), body (regions 4–5), and antrum (region 8) (Figure 1A). These portions were frozen in liquid nitrogen-cooled 2-methylbutane, and stored at -20C until serially sectioned (approximately 4 µm).

The polyclonal SMB MHC antibody (gift of Dr. A.S. Rovner; University of Vermont) was generated in rabbit against the following peptide: glutamine-glycine-proline-serine-phenylalanine-alanine-tyrosine-glycine-glutamic acid-leucine-glutamic acid-cysteine. The working dilution for the SMB MHC antibody was 1:500 in 0.1% BSA. The specificity of this antibody has been characterized extensively (Szymanski et al. 1998; White et al. 1998). In addition, we have tested this antibody on ELISA and shown that the antibody has a 10–100-fold higher affinity for SMB over SMA (data not shown).

Sections from all tissue regions were labeled with the SMB MHC antibody following the methods of Harlow and Lane (1988). In brief, sections were fixed in 2% paraformaldehyde and permeabilized in 0.5% Triton X-100 for 10 min each. The preparation was blocked in 5 mg/ml BSA for 1 hr, after which the SMB MHC antibody was applied for 2 hr. Sections were washed and labeled using a Texas red-conjugated goat anti-rabbit secondary antibody (1:1000 in 0.1% BSA) for 1 hr (Jackson ImmunoResearch Laboratories; West Grove, PA). Cell nuclei were fluorescently labeled using DAPI (1:500 in 0.1% BSA) for 15 min.

PBS–Tween [(in g/liter) NaCl 8.0, KH_2PO_4 0.2, Na_2HPO_4 1.15, KCl 0.2; 0.1% Tween-20, pH 7.4] was used to dilute all solutions and to rinse the tissues between all steps except after BSA blocking, where no rinsing was performed. After the staining procedure, sections were mounted in media containing several flakes of *p*-phenylenediamine (anti-fading compound), 75% glycerol [(in mM) KCl 75.0, Trisbase 10.0, EGTA 2.0, MgCl_2 1.0, pH 8.5], covered with a cover-glass, and sealed with clear nail polish before being stored at 4C until being examined. Negative controls were done for each experiment by replacing primary antibodies with PBS–Tween or by using pre-immune serum (Figures 1B and 2).

During each experiment, sections from all three stomach regions were stained at the same time and under the same conditions. Similarly, to preserve the integrity of the experiment, strict standards were employed for all tissue visualization using an Olympus IX70 microscope with fluorescent capabilities. Micrograph exposure times were held constant between images in a set of slides to standardize

fluorescent intensities between micrographs. Neutral density filters were used as needed to limit brightness during DAPI visualization. By strictly adhering to these criteria, the only significant variable affecting micrograph light intensity was the actual labeling of SMB MHCs. Cell images were recorded using a Princeton Instruments(Princeton, NJ) digital camera linked to a PC loaded with IP Lab v 3.0 for Windows (Scanalytics; Fairfax,VA).

Results

Negative controls did not produce artifacts that could be mistaken for the varying trends in SMBMHC distribution; only slight background staining was observed in some stomach preparations (Figures 1B and 2). Negative controls using no primary antibody (Figures 1B and 2A) or pre-immune serum from the rabbit that produced the antibody (Figure 2B) showed little to no immunoreactivity compared to the fluorescent signal observed using the SMBMHC serum (Figure 2C). Some immunoreactivity was observable in the stomach mucosa: However, this staining was of a much smaller magnitude than in muscle cells in the muscularis mucosa and externa (data not shown). It is currently unclear if this is nonspecific binding, SMCs from the capillary and lymphatic systems, or if other cell types present can express SMB MHC.

Major differences in SMBMHC immunoreactivity are observable between the stomach regions. Although no absolute quantitation was attempted, stomach antrum showed greater reactivity of SMBMHC than stomach fundus or body (Figure 3, antrum vs body and fundus). SMBMHC immunoreactivity in the body appeared to range between fundic and antral extremes (Figure 3, body).

An intercellular mosaic pattern of SMBMHC immunoreactivity was observed (Figure 4A1 and 4A2). No significant homogeneity was observable even between adjacent smooth muscle cells, and cells showing high levels of SMB MHC immunoreactivity were equally likely to neighbor a cell of similar immunoreactivity, or not(Figures 3F–3L). This range in heterogeneity is most clearly observable in transverse sections of smooth muscle, where the limits of each cell are most discernible, and brightly illuminated cells appear randomly distributed

throughout the tissue (Figures 3H–3L and 4A1) but is also observable in longitudinal sections (Figures 4A2 and 4B). This range in heterogeneity, although present in all regions of the stomach, stands out most dramatically in the body and fundus. The range in heterogeneity of SMB MHC in the antrum appeared to be narrower because all cells in this region show relatively high levels of SMB MHC immunoreactivity. Although the distribution of SMB MHC-dense cells appeared to be random, small pockets of cells with similarly high or low levels of SMB MHC immunoreactivity were observed.

Intracellular heterogeneity, similar to intercellular heterogeneity, is present in cells from all tissue regions. A mosaic or “checkerboard” immunoreactivity of SMB MHC is discernible in transverse and longitudinal tissue sections. Although most intracellular heterogeneity appears to be randomly distributed, as reported for intercellular distributions, a tendency for high SMB MHC immunoreactivity to be localized in the periphery of muscle cells was frequently observed (Figure 4C).

Discussion

In this study we focused on the SMB MHC isoform to extend our previous studies on its distribution in the stomach. SMB MHC has been identified as a myosin isoform with unique contractile properties. At the protein level, SMB MHC is necessary and sufficient for myosin to propel actin two to three times more rapidly in in vitro motility assays and to cause a twofold increase in ATP hydrolysis over the SMA MHC isoforms (Kelley et al. 1993; Rovner et al. 1997). Shortening velocities in single cells were also found to correlate to SMB MHC composition (Eddinger and Meer 2001). Likewise, in tissue from the hypertrophied rat bladder, SMB MHC composition is decreased by 25% and results in a 50% reduction in mean shortening velocity and rate of force development (Sjuve et al. 1996; Gomes et al. 2000). In addition, increasing SMC MHC expression as one traverses along the arterial tree from the aorta to the saphenous vein correlates with an increase in myosin ATPase and unloaded shortening velocity (DiSanto et al. 1997; and our unpublished observations).

Although these correlations have been made at the protein, cell, and tissue levels, the magnitude of changes due to SMB MHC is not consistent among studies. Eddinger and Meer (1996, 1997, 2001), Bárány and Bárány (1996), and Rovner et al. (1997) suggest that the three-dimensional organization of the contractile proteins in cells may be critical in determining the functional significance of protein isoforms. Differences observed in contraction velocities for SMB vs SMA MHC between in vitro motility assays, and single-cell and tissue preparations may result from the differences in the three-dimensional organization of the proteins in these preparations. Additional differences between tissue and single-cell results could be due to differences in the morphological distribution of SMB MHCs. Because there are distinctive contractile properties of myosin and cells containing SMB MHCs, the organization of these cells and proteins is extremely important in understanding the coordinated contraction in cells and tissues as a whole.

It has been suggested that differences in contractile force and shortening velocity may result, at least in part, from variants in isoforms from other parts of the myosin molecule, such as LC17a/b or SM1/2. Correlations between LC17a and an increased rate of contraction have been reported in tissue (Helper et al. 1988; Malmqvist and Arner 1991; Hasegawa and Morita 1992; Szymanski et al 1998). Experiments designed to specifically control for LC17 isoforms at the protein level by exchanging pure LC17b onto expressed heavy meromyosin containing the seven-amino-acid insert, and altering endogenous ratios of LC17a/b, did not result in any change in myosin in vitro motility (Kelley et al. 1993, Rovner et al. 1997), nor were they confirmed in a single-cell study by Eddinger et al. (2000). These latter results bring into question any causal relationship for the MLC17a/b isoforms with shortening velocity.

Hewett et al. (1993) and Cai et al. (1995) reported correlations between SM1/2 and maximal shortening velocity in tissue preparations. Other attempts to correlate SM1/2 MHC isoforms with actin motility and ATP hydrolysis, however, failed to establish such a clear relationship (Packer et al. 1991; Kelley et al. 1993; Meer and Eddinger 1997). Tissue such as the chicken gizzard, with relatively high ATPase activity, exhibit a preponderance of SMB MHC without

increased expression of SM1 or SM2 (Kelley et al. 1993). Therefore, it appears that SMB MHC is of uncontroversial importance among other studied myosin isoforms in regards to rate of ATP hydrolysis and contraction velocity.

Although this study is the first to show the histological distribution of SMB MHC in the various regions of the stomach, heterogeneity has been previously suggested by other studies. Eddinger and Meer (2001) reported SMB MHC heterogeneity in single cells from the stomach using RT-PCR. Likewise, tissue heterogeneity has been identified in tissues such as chicken gizzard, mature bovine vascular SM, opossum lower esophageal sphincter, and opossum esophageal body (Frid et al. 1994, 1997; Eddinger and Meer 1996; Szymanski et al 1998).

This study shows that SMB MHC immunoreactivity increases as one moves along the greater curvature of the stomach from fundus to antrum, consistent with Eddinger and Meer (2001). This trend is most apparent in the muscularis mucosa (Figures 3A–3C), where expressed levels of SMB are less than that observed in the muscularis externa (Figures 3D–3L). In addition to these regional differences in SMB MHC immunoreactivity, heterogeneity was observed between smooth muscle cells in all stomach regions. Appearing randomly dispersed, cells with high levels of SMB MHC immunoreactivity were found near cells with great or little to no discernible SMB MHC reactivity (Figures 3G–3L, 4A1, and 4A2). Intracellular heterogeneity is also observable in SM cells from all stomach regions (Figures 3G–3L). Although this variability can take on a mosaic pattern (Figure 3J), many cells showed high SMB MHC immunoreactivity at their peripheries (Figure 4C). This isoform arrangement could aid in maintaining coordinated contraction of an entire cell.

Small pockets or clusters of cells with high levels of SMB immunoreactivity were observed in longitudinal sections (Figure 4B) and in transverse sections (Figures 3G–3L) in all three regions of the stomach. A possible physiological role for this heterogeneity might be to increase the rate of SM cell activation. SM cells expressing greater amounts of the SMB MHC isoform can shorten more rapidly than cells with low levels of this isoform (Eddinger and Meer 2001). Preferential innervation of these cells would result in relatively rapid shortening of these cells and stretching of neighboring cells. These neighboring cells could then be activated by any of a number of proposed mechanotransduction mechanisms, including mechanosensitive ion channels, membrane-bound enzymes, or modulation of their cytoskeleton (Hill et al. 2001). Evidence for all these mechanisms working in vascular and enteric SM exists (Brookes et al. 1999; Farrugia et al. 1999; Phillips and Powley 2000). Therefore, selective innervation of SMB MHC-rich cells that shorten quickly could, via stretch-activated mechanotransduction, activate neighboring cells, resulting in more rapid tissue activation. This mechanism could be in parallel to electrical and chemical diffusion for inter-cellular coupling and activation.

It is possible that this electromechanical model could present a way for a contractile stimulus to spread more rapidly than by relying on chemical diffusion or enteric input alone. With this in mind, a limited number of cells expressing high levels of SMB MHC could recruit many other cells with which they associate. A greater demand for such stimulation could be thought to exist in thicker, more muscular tissues, such as the antrum, in which diffusion would be a less desirable way to transmit contractile stimulus. Further research in this area is needed to test this hypothesis.

The observation of heterogeneous distribution of SMB MHCs in the stomach may require reinterpretation of previous data reporting the ratio of SMA/SMB MHCs in the stomach as one value rather than a series of values from tonic and phasic regions of this organ. Traditionally, the stomach has been considered a homogeneous tissue, and this oversimplified assumption has been shown not to be the case. Future research can take SMB MHC heterogeneity in the stomach into

account by recognizing the organ's full isoform complexity.

Heterogeneity in protein isoform expression is known to differ in certain diseased states, such as arteriosclerosis or hypertension in vascular SM (Frid et al. 1997; Shanahan and Weissberg 1998). Variable distribution of SM isoforms may help to govern patterns of abnormal cell proliferation (Frid et al. 1997). Were it known that a given SM isoform was linked to a greater incidence of cell migration or abnormal cell proliferation, the distribution, concentration, and location of this isoform would be diagnostically and clinically important. The stomach is an organ containing regions of SM tissue with variable SMB MHC content. In this respect, the stomach may be an excellent model in which to predict and study the effects of differential isoform content.

This study has mapped out the histological distribution of SMB MHCs in three regions of the swine stomach. SMB MHC shows the least immunoreactivity in tonic fundic cells and increases as one moves along the greater curvature of the stomach to the body and antrum. Random intercellular heterogeneity with areas of cells expressing similar amounts of SMB MHCs was observed in all three stomach regions. A greater range in heterogeneity was observed in the fundus, while a smaller range in cells expressing high levels of SMB MHC was observed in the antrum. Intracellular heterogeneity was also observed, with a tendency for SMB MHC to be located around the periphery of a cell. This study is important in providing basic information regarding the distribution of the SMB MHC isoform, and allows hypotheses to be made and tested regarding the physiological significance of heterogeneous expression of SMB MHC.

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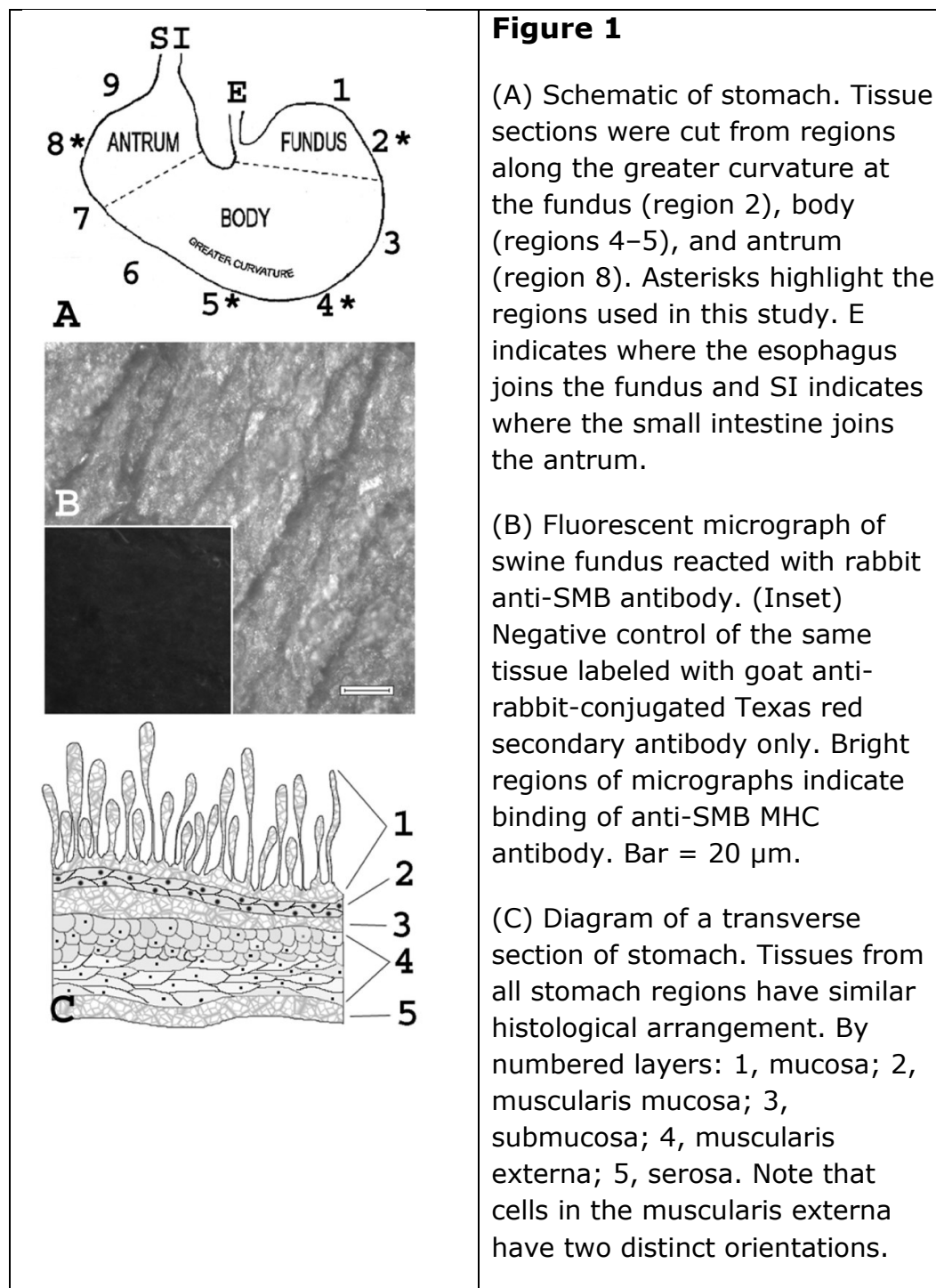
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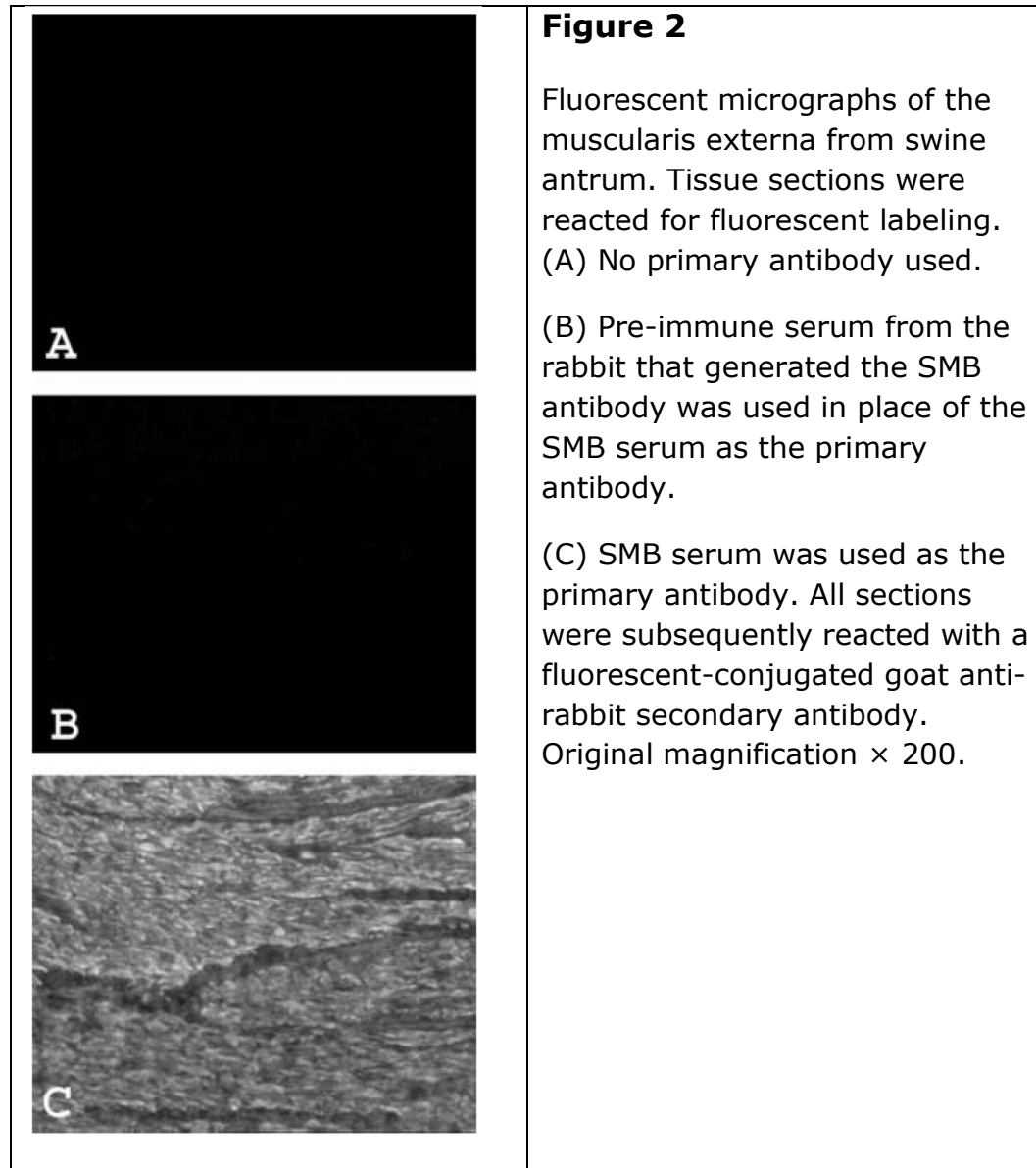
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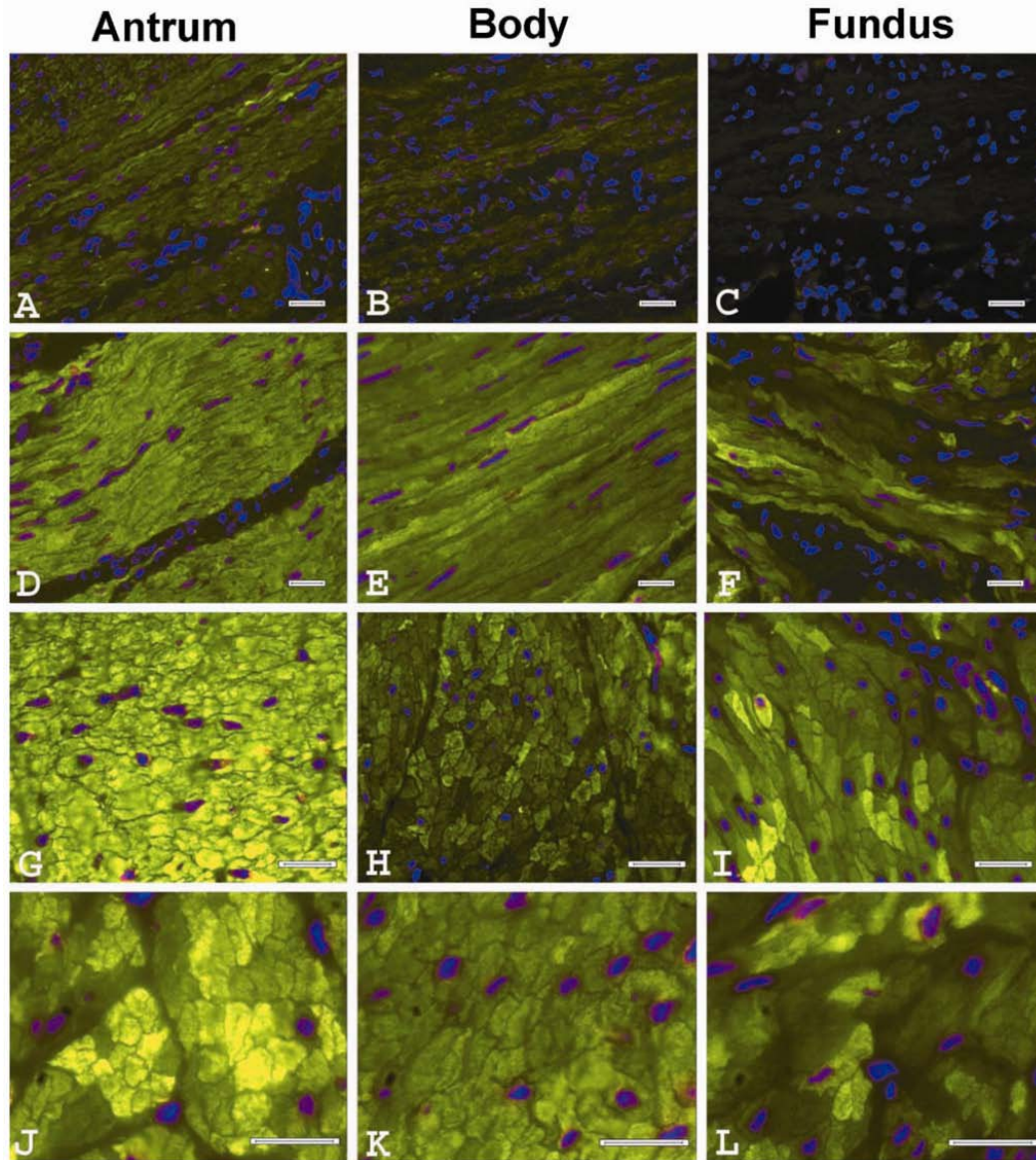
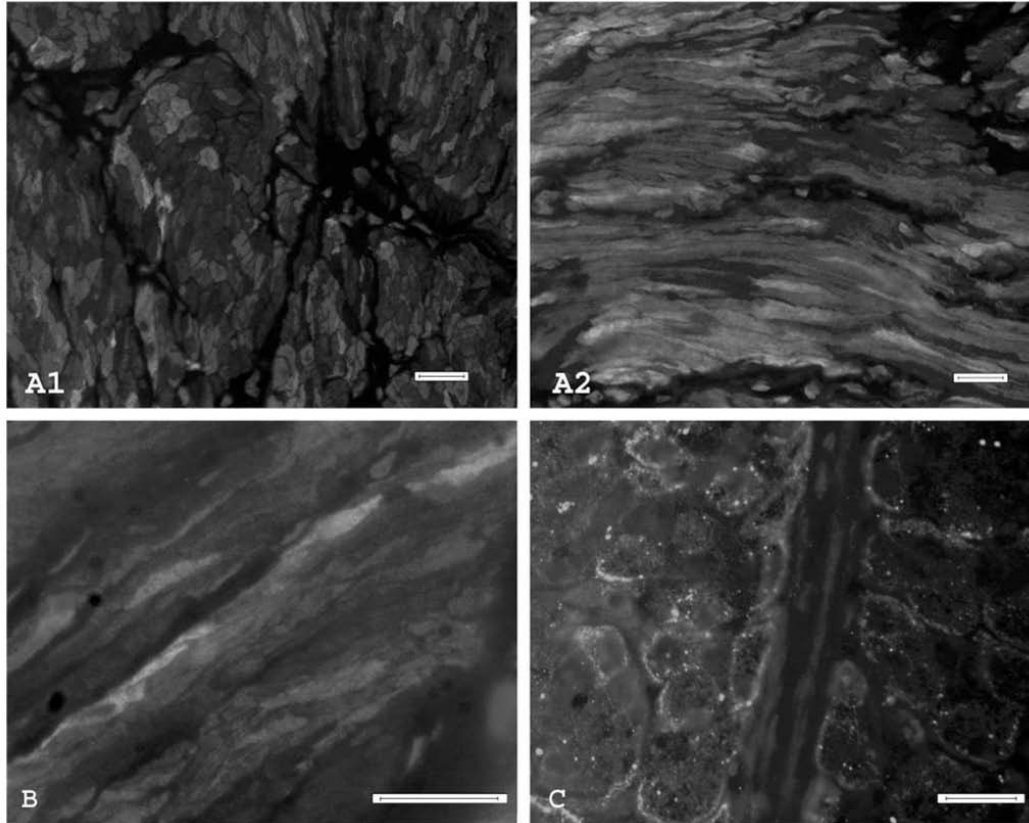


Figure 3

Fluorescent micrographs of tissue regions arranged by column (from left to right: antrum, body, and fundus). All sections are from swine stomach and labeled with a rabbit anti-SMB IgG coupled with a fluorescent-conjugated goat anti-rabbit secondary antibody. Green indicates binding of anti-SMB MHC antibody; blue indicates binding of DAPI (nuclear stain). Micrographs in A–F are longitudinal sections; G–L are transverse sections. Micrographs A–C are of the muscularis mucosa. Micrographs D–L are of the muscularis externa. Note dramatic

decrease in expression of SMB as one progresses from the antrum to the fundus (A–C). Heterogeneity is especially pronounced in micrographs D–L of longitudinal and transverse sections of the muscularis externa. Bars = 20 μ m.

Figure 4



Fluorescent micrographs of swine stomach tissue reacted with anti-SMB MHC antibody. Bright regions indicate immunoreactivity. (A) Fundus: heterogeneity is clearly observable in both transverse (A1) and longitudinal (A2) sections of all stomach regions. Heterogeneous immunoreactivity is observed, with some clustering of cells with similar levels of SMB MHC expression. (B) Fundus: example of heterogeneity in a longitudinal section at higher magnification. (C) Antrum: cells in the muscularis externa. This transverse section illustrates intracellular heterogeneity with increased immunoreactivity of the SMB antibody around the periphery of many cells. Bars = 20 μ m.