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Characterization of Bves expression during mouse development using newly generated immunoreagents

Travis K. Smith and **David M. Bader**

The Stahlman Cardiovascular Research Laboratories, Program for Developmental Biology, and Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee 37232-6300, USA

Abstract

Bves (blood vessel/epicardial substance) is a transmembrane protein postulated to play a role in cell-cell interaction/adhesion. It was independently isolated by two groups as a gene product highly enriched in the developing heart. Disagreement exists about its expression during development. Most notably, the expression of Bves in non-muscle cells of developing epithelia is disputed. Determining the expression profile of Bves is a critical initial step preceding the characterization of protein function in development and in the adult. We have generated new monoclonal antibodies against mouse Bves and used these immunoreagents to elucidate Bves expression in development. As expected, we detect Bves in myocytes of the developing heart throughout development. In addition, skeletal and smooth muscle cells including those of the coronary system express Bves. Finally, specific, but not all, epithelial derivatives of the three germ layers are stained positively with these monoclonal antibodies. Protein expression in cultured epithelial and muscle cell lines corroborate our in vivo findings. Taken together, these results demonstrate the expression of Bves in a wide range of epithelial and muscle cells during mouse embryogenesis and indicate a broad function for this protein in development, and show that these newly generated reagents will be invaluable in further investigation of Bves.

Keywords

Bves; muscle; epithelia; embryogenesis

INTRODUCTION

Bves was isolated independently by two laboratories (Reese et al., 1999; Andree et al., 2000) using subtractive hybridization screens for heart enriched gene products. Additional members of the gene family (Popdc2 and Popdc3) were also isolated (Andree et al., 2000). Sequence analysis of Bves revealed no conserved or predicted functional motifs and no homology to any previously identified protein. Three hydrophobic regions near the N-terminus were identified that have since been determined to be functional in anchoring the protein to the plasma membrane (Wada et al., 2001; Knight et al., 2003). Orthologous genes

Telephone: 615-936-1976, Fax: 615-936-3527, Email of corresponding author: david.bader@vanderbilt.edu.

have been identified in numerous invertebrates and vertebrates (Reese and Bader, 1999; Reese et al., 1999; Andree et al., 2000; Hitz et al., 2002; Ripley et al., 2006).

Controversy about the expression pattern of the Bves protein persists in the literature. Detection of Bves message through *in situ* hybridization, Northern blotting (Andree et al., 2000), or *lacZ* knock-in (Andree et al., 2002a) do not agree with detection of the Bves protein using multiple anti-Bves immunological reagents (Reese et al., 1999; DiAngelo et al., 2001; Wada et al., 2001; Osler and Bader, 2004; Ripley et al., 2004; Vasavada et al., 2004). While *in situ* hybridization and *lacZ* knock-in analyses have been interpreted as indicating that Bves is expressed preferentially in cardiac and skeletal muscle, analyses of protein expression indicate that Bves is expressed in many epithelial cell types as well. The first polyclonal antibody generated by our laboratory, D033, revealed expression in the proepicardium, migrating epicardium, epicardial-derived mesenchyme and smooth muscle cells of the cardiac arteries of the developing chicken heart (Reese et al., 1999). A second polyclonal antibody, B846, also revealed Bves expression in cardiac muscle and all epicardial/epicardially derived tissues listed above (Wada et al., 2001), as well as expression in various epithelial cell lines (Wada et al., 2001), epithelia of all three germ layers during early chick development, epidermis, gut endoderm (Osler and Bader, 2004), and epithelia of the lens, retina, and cornea (Ripley et al., 2004). A subsequent antibody against the *X. laevis* ortholog of Bves was developed, and has revealed highly similar expression in the frog (Ripley et al., 2006). A monoclonal antibody generated against the chicken Bves protein (DiAngelo et al., 2001) also demonstrated that Bves is expressed in skeletal muscle, cardiac muscle, brain, and epicardium (Vasavada et al., 2004). The monoclonal antibody generated by Duncan and colleagues clearly reacts with the chicken Bves protein in cardiac myocytes and transiently in the epicardium, but has not been reported to react with chicken Bves protein in other epithelial cell types (DiAngelo et al., 2001; Vasavada et al., 2004).

Here, we describe the generation of multiple new α- mouse Bves monoclonal antibodies that display reactivity with cardiac muscle, skeletal muscle, and epithelial cell types throughout embryonic development, as well as cultured epithelial and muscle cell lines. We also thoroughly examine the developmental expression profile of the mouse Bves protein using these and other previously generated α-Bves reagents. Thus, we provide a comprehensive description of Bves expression at the protein level in the mouse, which is lacking in the literature at this time. Our data clearly demonstrate that the Bves protein is present in developing muscle and epithelial cell types derived from all three germ layers. These studies are essential for a meaningful understanding of Bves function and to determine the role of Bves in mouse embryogenesis.

MATERIALS AND METHODS

Generation of α-Bves monoclonal antibodies

Antibodies were generated against the peptide DPTLNDKKVKKLEPQMS (amino acids 266–283 of mouse Bves) in collaboration with QEDBioscience (San Diego, CA) using standard methodology (Bader et al., 1982). Antibodies were initally screened using ELISA against the original peptide. Reactive clones were selected from this screen and were then subjected to screening using secondary immunofluoresence against COS-7 cells transfected

with Bves expression constructs. Reactive clones were further characterized using standard immunoblotting procedures against GST-fused Bves, Popdc2, and Popdc3. Once isolated, hybridomas were cultured and also injected into the peritoneal cavity of mice to generate ascites fluid. Five independent clones were used to generate ascites, and all five of these hybridoma lines will be deposited in the Developmental Studies Hybridoma Bank.

Antibodies

Primary antibodies against E-cadherin (Chemicon), ZO-1 (Zymed), sarcomeric myosin (MF20, DSHB), c-myc (Sigma), cytokeratin (Sigma), and GST (Amersham) were applied according to manufacturer's specifications. Alexa-488 and Alexa-568 conjugated secondary antibodies (Molecular Probes) were used at 1:4,000 dilutions for indirect immunofluoresence, and alkaline phosphatase conjugated secondary antibodies (Sigma) were dilluted 1:10,000 for immunoblotting. DAPI (4', 6-diamidine-2phenylidole-dihydrochloride; Roche) was used to visualize nuclei per manufacturer's specifications. When direct labeling of antibodies was necessary, Zenon Alexa Fluor labeling kit (Molecular Probes) was employed to label primary anitbodies according to manufacturer's specifications. The polyclonal antibody B846 has been previously described (Wada et al., 2001; Osler and Bader, 2004). Newly generated α-Bves monoclonal antibody ascites fluids were used at a 1:2,000 dilution for immunohistochemistry with both tissue sections and cultured cells. Samples were incubated in primary antibodies at 4°C overnight in a humidified chamber.

Western Blotting analysis

Hearts were excised from adult (~8 weeks) ICR mice (Jackson Labs), and dissected into small pieces. Tissue was then homogenized using extraction buffer (1× PBS, 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS) containing mammalian protease inhibitors (Roche Complete). Samples were then centrifuged at 21,000 × g for 30 minutes at 4°C. Supernatant was collected, and protein concentration assayed using Bradford assay (Biorad). 40 ug protein was diluted in SDS-PAGE sample buffer, and electrophoresed on a 10% SDS-PAGE gel at 125 V. Protein was then transferred to Immobilon-P membrane (Millipore), and then blocked in blocking solution (10% nonfat dry milk (Carnation), 100mM Tris Cl pH 7.5, 150 mM NaCl, .25% TritonX-100 (Sigma)) overnight at 4°C. Primary antibody (SB1, anti-Bves monoclonal Ab) was applied to blot at 1:2000 dilution (~1 ug/mL) for one hour at room temperature. Blot was washed 3 times with 1× TBST (100mM Tris Cl pH 7.5, 150 mM NaCl, .25% TritonX-100 (Sigma)), and then incubated with alkaline phosphatase conjugated secondary antibodies (Sigma) at manufacturer's recommended concentrations in blocking buffer for one hour at room temperature. Blot was washed 3 times with 1× TBST, and binding of secondary antibodies visualized using NBT/BCIP (Roche) according to manufacturer's specifications.

Tissue/cell preparation

Tissues were harvested, washed in cold PBS and then incubated in 20% sucrose in PBS overnight at 4°C. Tissues were then processed for frozen sectioning and immunohistochemical staining of using standard methodology (Bader et al., 1982; Wada et

al., 2003). Cultured cells were transfected with expression constructs using Fugene (Roche) transfection reagent according to manufacturer's instructions.

Immunofluoresence methods for α-Bves monoclonal antibodies

As this is the first communication employing these immunochemical reagents, a protocol generated for their use is provided. Briefly, tissue sections or cultured cells were fixed for 10 minutes in cold 70% methanol, washed three times with PBS, and permeabilized with 0.25% Triton X-100 in PBS for 10 minutes. Sections/cells were then washed with PBS three times, and non-specific binding was blocked by incubation with 2% bovine serum albumin in PBS for one hour at room temperature. Ascites fluids were applied at 1:2,000 dilutions for 1–16 hours at room temperature. No variation in background or staining intensity was observed over that period. Alexa-conjugated secondary antibodies (Molecular Probes) were added for one hour at room temperature according to manufacturer's specifications. Subsequent washing was standard.

Generation of mammalian and bacterial expression constructs

Mammalian and bacterial expression constructs were generated using PCR amplification followed by cloning into pCI-neo (Promega) and pGex 5x-3 (Amersham). All expression constructs express the portion of the respective protein 3' to the hydrophobic transmembrane domains of Bves. A c-myc epitope tag was also added to the carboxyl terminus of all mammalian expression constructs to aid in monoclonal antibody characterization.

Culture of neonatal mouse myocytes

Hearts of N2 mice were harvested, mechanically dissociated, and placed in cold PBS. Hearts were placed in 5 mL 0.25% Trypsin-EDTA (Cellgro) for two minutes with rapid stirring using mechanical stir bar. 3.5 mL of Trypsin-EDTA was removed after two minutes, and replaced with 3.5 mL fresh Trypsin-EDTA. The first two fractions of Trypsin-EDTA were discarded, with fractions 3–8 retained and placed into a 10× volume of myocyte medium (DMEM: 4.5 g/L glucose, 0.0025M thymidine, 10 U/mL penicillin, 10 mg/mL streptomycin, 100 μg/mL gentamycin, 15% NuSerum (Collaborative Biomedical Products)). Myocytes were gently pelleted at 650 × G for seven minutes, then resuspended in growth media, counted using a hemacytometer, and plated on 100 mm plastic dishes at 3×10^6 cells per dish.

RESULTS

α;-Bves monoclonal antibodies recognize Bves specifically

Immunochemical reagents against mouse and chicken Bves have previously been generated and characterized (Reese et al., 1999; DiAngelo et al., 2001; Wada et al., 2001; Osler and Bader, 2004; Ripley et al., 2004; Vasavada et al., 2004). Recently, DiAngelo et al. (2001) have generated a monoclonal antibody against the intracellular C-terminus of chicken Bves. To this point, no monoclonal reagents against murine Bves exist and the characterization of protein expression in any organism is incomplete.

Specificity of α-Bves reactivity was initially established using transfection of COS-7 cells with a c-myc-tagged Bves expression construct followed by co-immunofluoresence using α-c-myc and putative Bves monoclonal antibody supernatants. All ten of the antibody-secreting clones that passed the initial screening process were reactive with transfected Bves (SB1 is given as an example in Figure 1A, i–iii). Anti-Bves antibodies were then tested for cross reactivity with other members of the gene family (Figure 1A, iv–ix) and a non-related protein, LEK1 (Figure 1A, x–xii) by transfection of COS-7 fibroblasts with c-myc tagged Bves, Popdc2, and Popdc3. All antibodies generated were found to react in a similar manner: specifically with Bves and not with other Popdc family members or the unrelated LEK1 protein.

α-Bves monoclonal antibodies were then tested for specificity in immunoblotting assays. GST-fusion expression constructs of each Bves family member were generated, and purified protein was subjected to standard immunoblotting procedures. SB1 is used here as an example. Again, α-Bves monoclonal antibodies were found to react specifically with Bves, and were unreactive with Popdc2 (Figure 1B) and Popdc3 (data not shown).

α-Bves monoclonal antibodies were also tested for ability to bind Bves in affinity chromatography assays. Sepharose bound antibodies were reacted with purified GST-mBves119–369. SB1 and SB2 were found to bind GST-mBves119–369, while IgG did not (Figure 1C, 1D). These data demonstrate the reactivity and specificity of these reagents for Bves amongst Popdc family members.

α-Bves monoclonal antibodies detect Bves in epithelial cell lines

Analyses of Bves expression in the chicken utilizing *in situ* hybridization techniques differ from results obatined using immunochemical methods (Reese et al., 1999; Andree et al., 2000; Wada et al., 2001; Osler and Bader, 2004; Vasavada et al., 2004). While both methods provide evidence for expression in muscle cell types, *in situ* hybridization assays and immunochemical assays do not agree concerning expression in epithelial cell types. Utilizing cell lines allows the examination of Bves expression in a clonal cell population consisting of a single cell type. Having verified specificity of antibodies SB1–SB5, we then tested the newly generated antibodies on a variety of cell lines (Figure 2). α-Bves monoclonal antibodies revealed expression in cell lines derived from the rat epithelial epicardium (line EMC, Figure 2A), differentiated mouse skeletal myoblasts (line C2C12, Figure 2B), human corneal epithelium (line HCE, Figure 2C), and canine kidney epithelial cells (line MDCK, Figure 2D). Intense staining in the lateral compartment of the cell membrane is observed in all epithelial cell lines tested thus far. Punctate intracellular staining is also observed in these cell lines (see arrowheads, Figure 3C) and in the C2C12 muscle cell line (Figure 2D). These patterns are in general agreement with the subcellular distribution detected with polyclonal reagents (Reese and Bader, 1999; Wada et al., 2001; Osler and Bader, 2004). Therefore the SB monoclonal series recognizes Bves in a variety of cell types across a spectrum of mammalian species and cell lines, in agreement with previously generated reagents.

Bves is expressed in mouse epidermis throughout development

While epithelial and muscle cell lines clearly express Bves, these cell lines may not reflect embryonic expression of the protein. To test whether Bves protein is present in developing epithelia derived from ectoderm, frozen sections of mouse embryos at various stages of development were examined for Bves expression using the SB1 antibody. Expression of Bves protein was observed in multiple epithelial tissues. Using epithelial markers cytokeratin and ZO1 to verify staining patterns, we determined that Bves is expressed in the epidermis of the mouse throughout development (Figure 3). At E12.5, Bves is expressed in the apical portion in the epidermal layer. At low power magnification in figure 3, the general colocalization of SB1 and cytokeratin is observed (Figure 3, A–C). At E12.5, Bves is also detected in the apical regions of the developing epidermis. Interestingly, while both SB1 and the polyclonal B846 both recognize Bves protein in the epidermis (Figure 3, D–F), the distribution pattern of SB1 labeling is somewhat broader in the epidermis than that of the polyclonal antiserum. As development proceeds, a high degree of colocalization of Bves and ZO1 is observed in the epidermal layer in the lateral membrane (Figure 3, G–I), while no Bves expression is observed in the endothelium of the subdermal blood vessels (see ZO1 positive cells indicated by arrowheads Figure 3, G and I). Near the end of gestation, an increase in intensity of staining is observed, and the pattern of Bves distribution appears to be wider than at earlier points of development. The monoclonal antibody SB1 appears to be reactive with Bves protein in very apical portions of epidermal cells. This is exemplified by comparison of the epidermally-expressed E-cadherin and Bves (Figure 3, J–L). While both are clearly expressed in this epithelium and exhibit domains of overlap, the major deposition of Bves is apical to E-cadherin. Taken together, the data demonstrate Bves expression in ectodermally derived epidermis of the mouse.

Bves is expressed in developing cardiac and skeletal muscle

The first generation polyclonal antibody D033 (Reese et al., 1999) did not react with cardiac muscle. However Andree et al (2000) clearly demonstrated that Bves mRNA was expressed at high levels in cardiac myocytes using *in situ* hybridization. In addition, DiAngelo et al (2002) used an α-Bves monoclonal antibody to show protein expression in avian cardiac myocytes. Subsequent polyclonal antisera from our laboratory also demonstrated Bves expression in the heart (Osler and Bader, 2004). To determine the expression pattern and subcellular localization of the Bves protein in the mouse heart, an analysis at various embryonic stages using SB1 was undertaken. At E12.5, Bves staining using the SB1 antibody is observed in a uniform subcellular pattern in cardiac myocytes (Figure 4A). At later stages and in the adult, staining is most intense at the myocyte periphery (Figure 4B). Polyclonal B846, which has previously been used to study Bves expression in epithelial cells (Osler and Bader, 2004), also reacts with cardiac myocytes. As previously seen in epidermis (Figure 3), the pattern of localization revealed by B846 in myocytes varies from that of SB1. Consistent with these results, SB1 recognizes a broader distribution of Bves in the myocardium, labeling the entire periphery of the cell (lateral and longitudinal surfaces). A similar pattern is seen in the chicken during embryonic stages using the polyclonal antisera B846. Bves is seen to localize around the entirety of the myocyte (data not shown). However, B846 only recognizes Bves at the intercalated disc, and not on lateral surfaces

(see Supplementary Data). These results are consistent with observations in epithelia, where B846 appears only to recognize Bves protein at points of cell-cell contact, as the intercalated disc is the only point of cell-cell contact for the cardiac myocyte. Additionally, Bves localizes to nascent points of cell-cell contact in reaggregating myocytes (see Supplementary Data). This result in highly consistent with observations regarding Bves localization during nascent contact formation in cultured epithelia (Osler et al., 2005).

It is of interest to note that SB1–5 also detect Bves expression in not only in cardiac myocytes, but also in smoooth muscle cells of the coronary system (as seen by colocalization with α-smooth muscle actin, Figure 4B) in agreement with our previous studies (Reese et al., 1999; Wada et al., 2001). Andree et al (2000) report Bves mRNA expression in somites and delveoping skeletal muscle. In agreement with those studies, SB1–5 also detects Bves protein in developing skeletal muscle (Figure 5). In skeletal myocytes, as confirmed by colocalization with the α-MHC monoclonal antibody MF20, SB1 reveals strong Bves expression. The polyclonal α-Bves reagent B846 also recognizes this Bves expression in skeletal myocytes. Note that the epithelial epidermis (arrowheads, Figure 5) is positive for SB1 while MF20 labeling is negative; however both antibodies are positive for adjacent skeletal muscle. Interestingly, Bves expression is also seem in the epithelial somite early during musculogenesis (data not shown).

Expression of Bves in the epicardium has been debated and remains controversial at this time. While *in situ* hybridization studies did not yield significant signal in the epicardium (Andree et al., 2000), monoclonal (DiAngelo et al., 2001) and polyclonal antibodies (Reese et al., 1999; Wada et al., 2001; Osler and Bader, 2004) detect transient or sustained expression of Bves in the epicardium. Thus, it is important to note that our initial observations using the reagents and methods described here do not detect Bves protein in the definitive epicardium, even though these antibodies are highly reactive with the epicardially derived EMC cell line (Figure 2A).

Bves is expressed in epithelia of the lung and esophagus during development

We next tested SB1 antibody reactivity in endodermally derived epithelium using lung and gut epithelium as examples of this cell type. Bves is detected in the epithelial components of the digestive tract and lung during development. Particularly, strong expression in the esophagus and main bronchi is observed at E14.5 (Figure 6). As confirmed by co-expression of E-Cadherin, these cells are the epithelial linings of these passageways. However, the expression of Bves in the respiratory system appears to be restricted to the trachea and larger bronchi, while expression in smaller airways is not observed at high levels. Comparison of Bves and E-cadherin staining demonstrates that Bves protein distribution is not uniform in the epithelium of the respiratory system, while expression in the esophagus appears to be more evenly distributed. These data clearly demonstrate the expression of Bves in endodermally derived epithelium.

DISCUSSION

For a meaningful understanding of protein function during development or in the adult, it is essential to determine the domain of expression of the protein. The expression of Bves has

been a topic of debate since the initial experiments characterizing the gene. The expression of Bves has been previously examined using polyclonal antisera in the developing chick and frog (Reese et al., 1999; Wada et al., 2001; Osler and Bader, 2004; Ripley et al., 2004; Vasavada et al., 2004; Ripley et al., 2006), *in situ* hybridization in the develoing chick (Andree et al., 2000) and frog (Hitz et al., 2002; Ripley et al., 2006), *lacZ* knock-in (Andree et al., 2002a; Andree et al., 2002b), RT-PCR (Wada et al., 2003; Osler and Bader, 2004), and Northern blotting (Reese et al., 1999; Andree et al., 2000; Andree et al., 2002a). However, despite the utilization of these many methods in several model systems, disagreement in the literature still persists regarding the expression pattern of this protein. While some reports indicate that the expression of the protein was either restricted to or highly enriched in cardiac, skeletal, and smooth muscle (Andree et al., 2000; Andree et al., 2002a; Andree et al., 2002b; Hitz et al., 2002); other reports have strongly supported a broader expression pattern that extends to many epithelial cell types (Reese et al., 1999; Wada et al., 2001; Wada et al., 2003; Osler and Bader, 2004; Ripley et al., 2004; Vasavada et al., 2004). Here, using newly generated monoclonal reagents, we show that Bves is expressed in tissues derived from all three germ layers, various epithelia and in epithelial cell lines, and in smooth and striated muscle.

Our goal was to develop reagents to more precisely determine the expression pattern of the Bves protein, and to examine the expression of Bves during mouse development. As expected, expression of Bves was observed in skeletal and cardiac muscle (Figure 5 and Figure 4, respectively). This is in agreement with previous analyses of Bves mRNA expression and with antibody studies from the Duncan and Bader laboratories (Reese et al., 1999; Andree et al., 2000; DiAngelo et al., 2001; Wada et al., 2001; Osler and Bader, 2004). In contrast, we have reported that our original D033 polyclonal serum, while clearly reacting with the protein (Reese et al., 1999; Osler and Bader, 2004), does not recognize Bves in heart muscle. In addition to striated muscle staining, the SB antibody series recognizes Bves expression in some but not all smooth muscle populations. Clearly, coronary smooth muscle is intensely stained by SB antibodies (Figure 4) in agreement with our previous polyclonal data.

The major point of disagreement at this time concerning the expression of Bves is whether it is present in developing and adult epithelia. Using newly generated monoclonal antibodies, we were able to clearly demonstrate protein expression in many endodermally- and ectodermally-derived epithelia. Still, not all epithelia in the developing or adult organism exhibit antibody reactivity. This may be due to variation in isoform production and/or the simple lack of expression. The localization of protein revealed by the SB1 antibody is highly similar to that observed using previously characterized mono- and polyclonal antibodies with the notable exception of epicardial staining. Additionally, our present studies definitively detect the protein in numerous cell lines of epithelial origin (Figure 2). The subcellular distribution of Bves protein revealed by the SB series generally but not completely follows the pattern of staining observed with polyclonal B846 (Wada et al., 2001; Osler and Bader, 2004). This staining is abundant at points of cell-cell contact, consistent with the hypothesis that Bves plays a role in cell-cell adhesion/interaction (Wada

et al., 2001; Osler and Bader, 2004). We have also observed Bves at points of myocyte-myocyte contact in reaggregation assays, which further supports this hypothesis.

The expression pattern revealed by investigation of Bves mRNA expression and *lacZ* knock-in is often different from the pattern revealed through examination of the protein expression. Both *in situ* analyses (Andree et al., 2002a) and *lacZ* knock-in assays (Andree et al., 2002b) reveal very little epithelial expression. However, three independently generated polyclonal antisera (against chick Bves, mouse Bves, and frog Bves) from our laboratory reveal Bves expression in a variety of epithelial tissues, along with striated and smooth muscle types. In addition, the Duncan laboratory has generated a monoclonal antibody against chick Bves that also recognizes expression of Bves in the epicardium of the chicken at E6. Examination of Bves expression in other epithelia using this antibody has not yet been published. Still, the present data clearly detect Bves in a variety of epithelial cell types. The discrepancy between mRNA expression analysis and analyses using immunoreagents may be due to a low level of Bves message in non-muscle cell types that makes mRNA detection difficult. Accordingly, when using either immunochemical or riboprobe assays to determine expression of a protein, negative results should be interpreted cautiously as many factors can affect the results of these assays independent of message/protein presence.

These antibodies clearly demonstrate that Bves is expressed in a variety of epithelial and muscular cell types, and that Bves protein expression extends to derivatives of all primordial germ layers. Understanding of the expression pattern of the protein is necessary for developing and understanding the function of the protein. Such broad expression within the organism and in species ranging from invertebrate to human indicates that the function of the Bves protein will likely be more general in nature, including muscle and non-muscle cell types.

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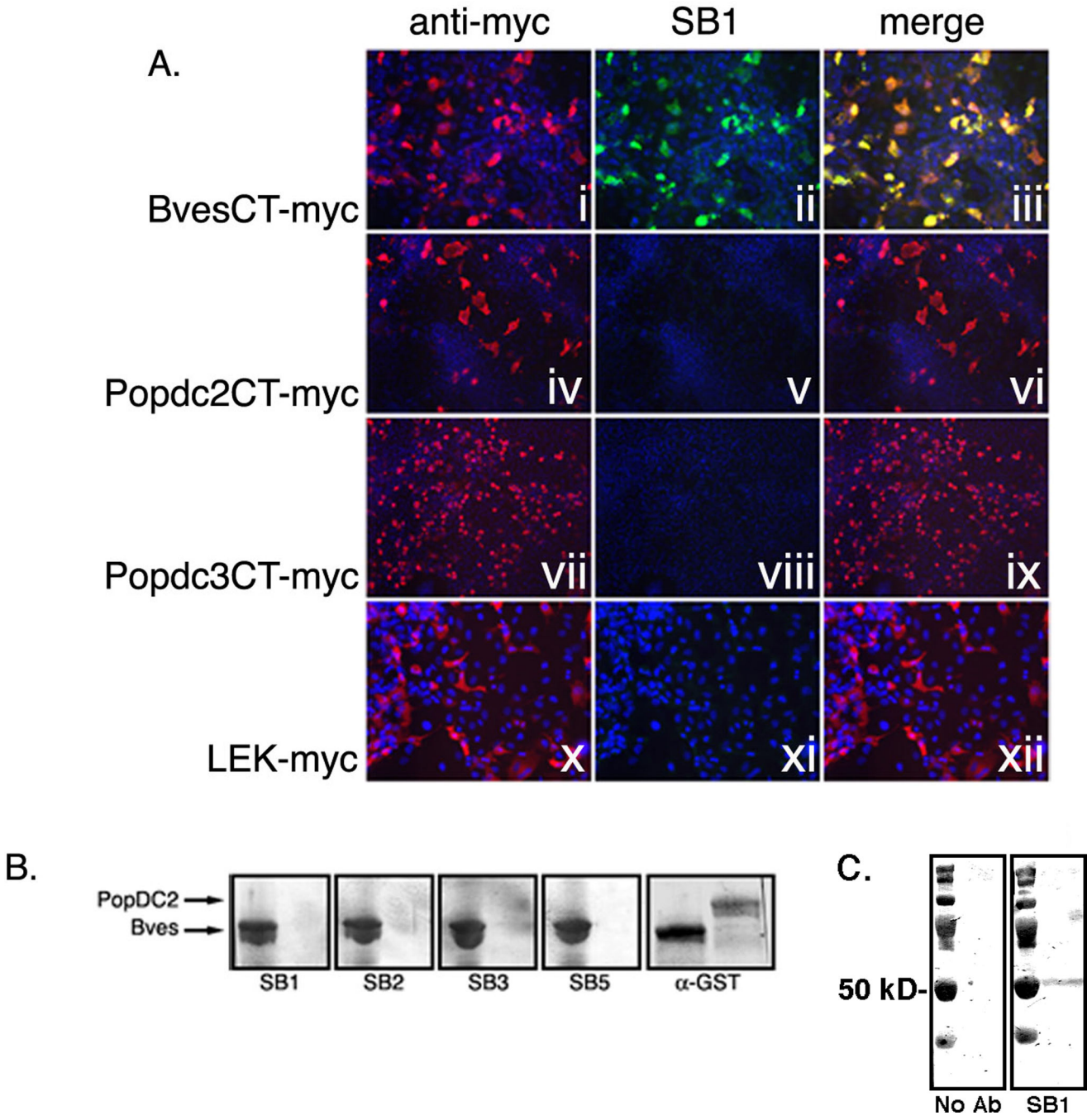

Figure 1.

Characterization of α-Bves monoclonal antibodies. SB1 seen to be reactive with COS-7 cells transfected with c-myc tagged Bves expression constructs, but no labeling of cells transfected with other members of the protein family (PopDC2 and PopDC3) is observed (Fig. 1A). SB family monoclonal reagents are also Bves specific in immunoblotting assays against GST-tagged fusion proteins. No reactivity with PopDC2 (Fig. 1B) or PopDC3 (data not shown) is observed. SB1 reacts with a single protein in immunoblotting assays of adult mouse heart (Fig. 1C). Reactive band is visualized at approximately 53 kD.

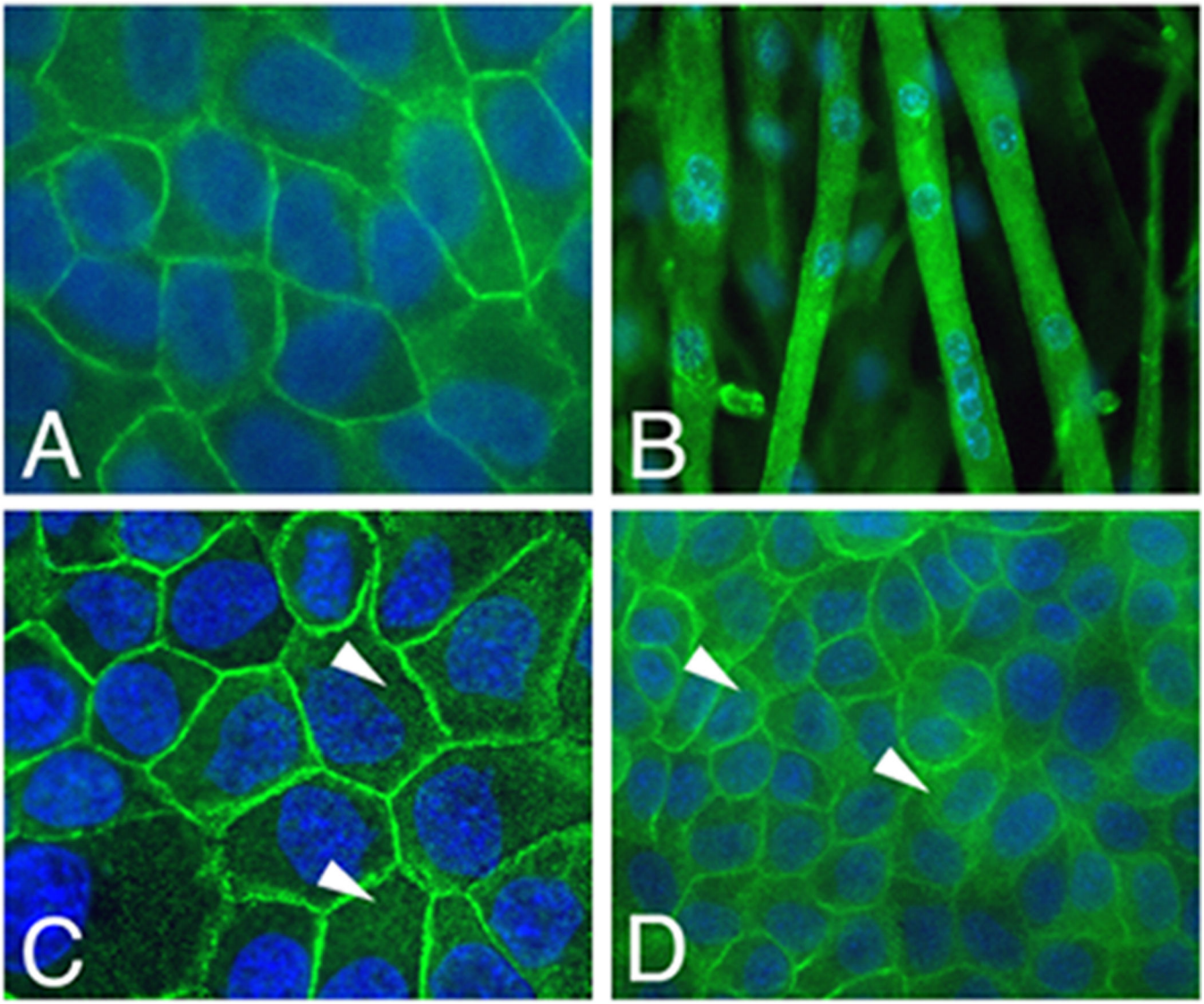

Figure 2.

Bves is expressed in muscle and epithelial cell line. Bves expression (green) is observed using indirect immunofluoresence and monoclonal antibody SB1 (green) with DAPI (blue) staining for nuclei. Peripheral expression is observed in the EMC (epicardial mesotheial cells, Fig 2A) cell line, the HCE (human corneal epithelial, Fig. 2C) cell line, and the MDCK (Maldin Darby Canine Kidney, Fig. 2D) cell line. A broader staining pattern is seen in the differentiated mouse C2C12 mouse myoblast cell line (Fig. 2B).

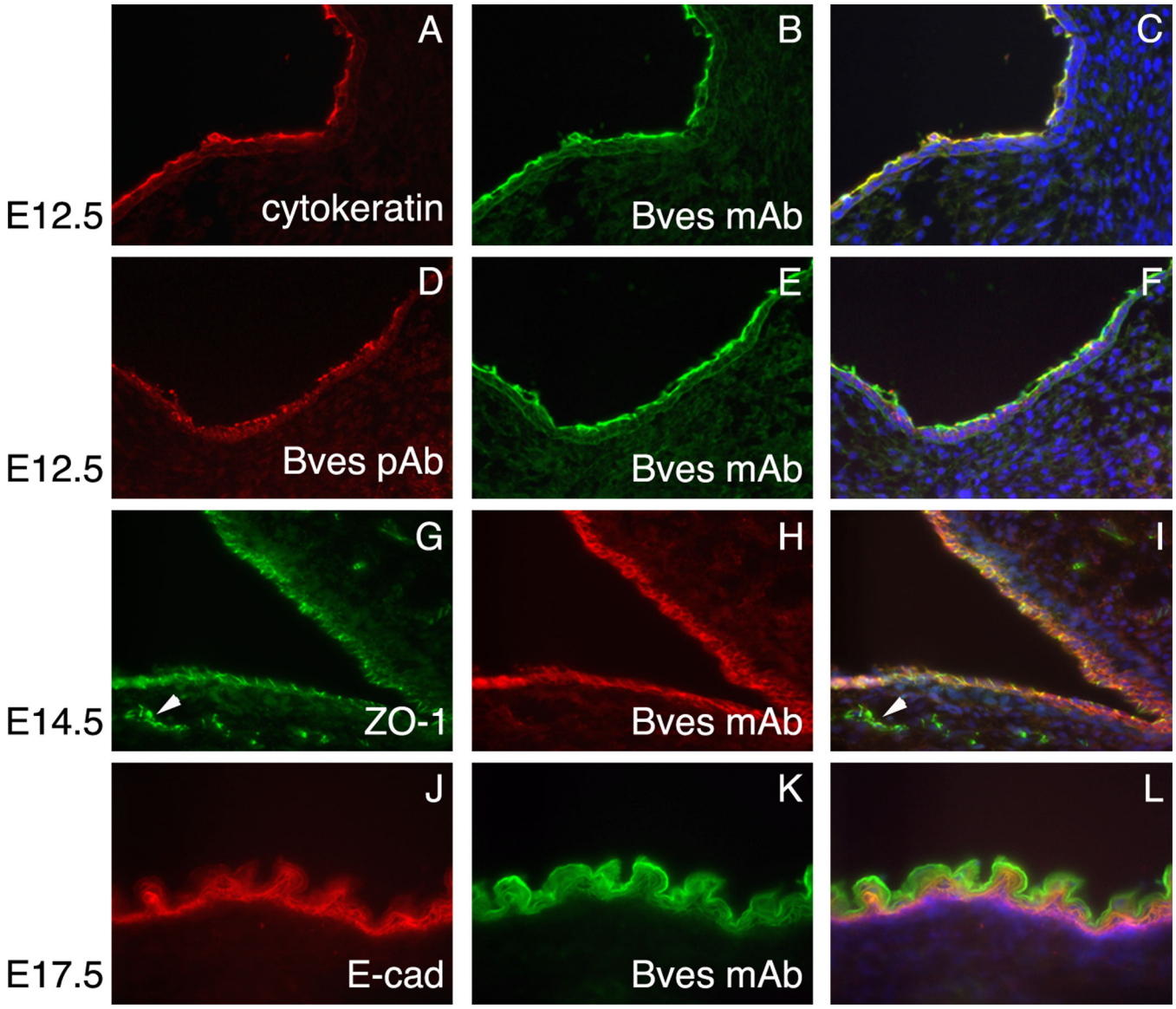

Figure 3.

Bves expression in the epidermis during gestation. Bves expression is seen in the epidermis throughout development, as observed by colocalization with cytokeratin, ZO-1, and E-cadherin markers at various developmental stages. Colocalization of Bves (Fig. 3B) and cytokeratin (Fig. 3A) at E12.5 shows expression of Bves in keratin producing cells, which continues throughout gestation. Expression of Bves in subdermal blood vessels is not observed (arrowheads, Fig. 3G–3I). Near the end of gestation, expression of Bves (Fig. 3K) is seen in the epidermis at a position apical to that of E-cadherin (Fig. 3J). Polyclonal anti-Bves antibody (B846, Fig. 3D) and monoclonal anti-Bves antibody (SB1, Fig. 3E) show a similar distribution in the epidermis during development. DAPI counterstain used to visulalize nuclei in blue (Fig. 3A–L).

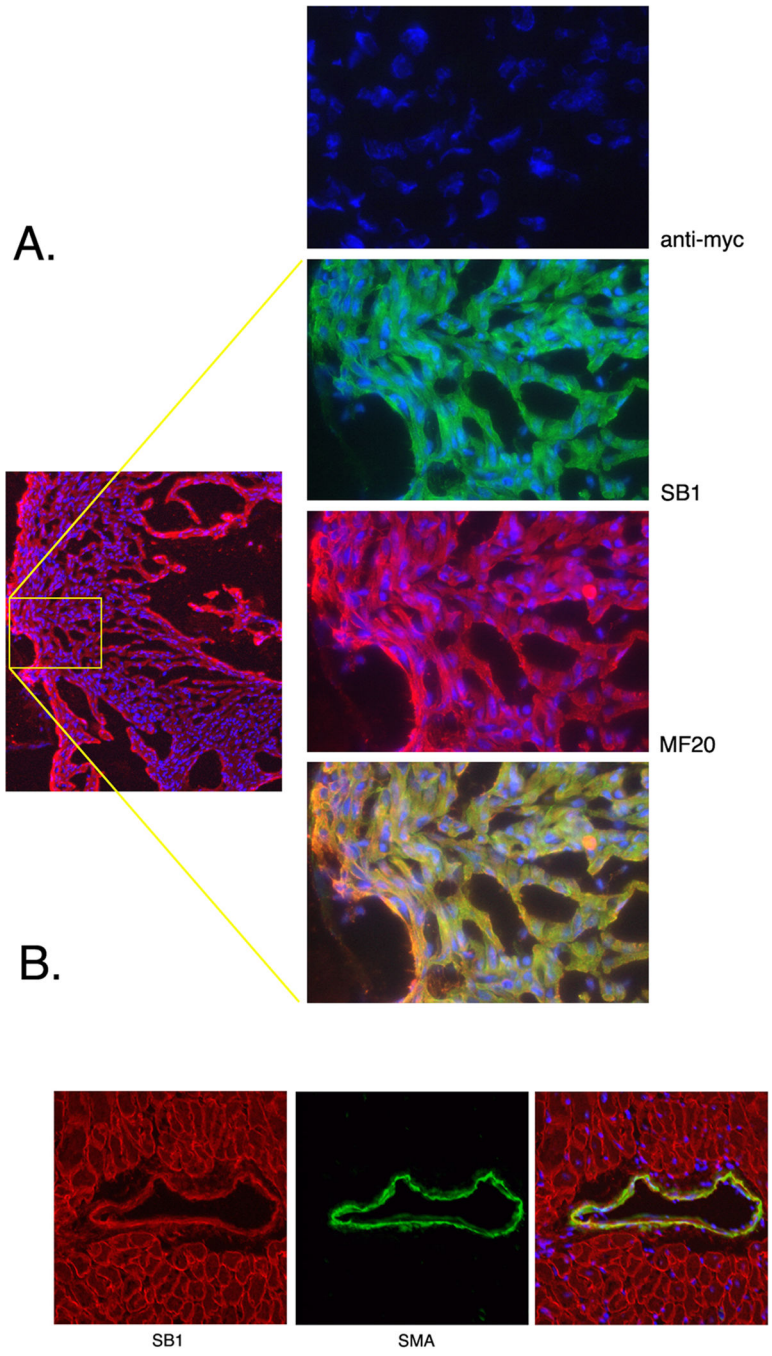

Figure 4.

Bves expression in the heart. Broad expression of Bves is observed in the heart at E17.5 (Fig. 4A) as observed by labeling with SB1 (anti-Bves, green) and MF20 (anti-sarcomeric myosin, red). Negative control using anti-myc antibody shows no background reactivity of secondary antibody (Fig. 1A). Nuclei are visualized using DAPI (Fig. 4A, 4B) In the adult heart, subcelllular localiaztion of protein becomes more restricted (Fig. 4B). Bves (red) is observed to localize to periphery of cardiac myocytes in definitive myocardium. Expression

of Bves in vascular smooth muscle of the coronary arteries is also observed using SB1 (anti-Bves, red) and α-smooth muscle actin (green).

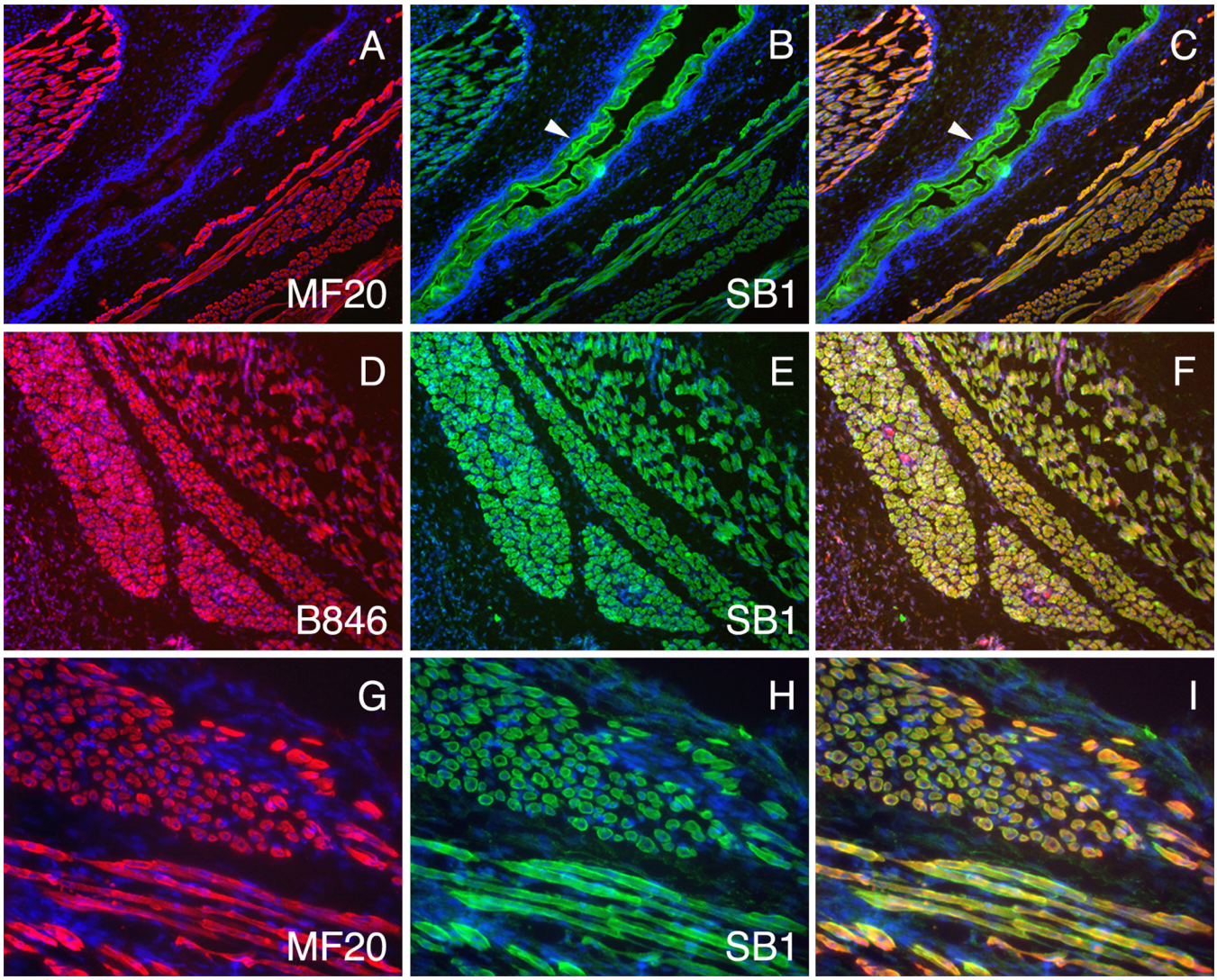

Figure 5.

Bves expression in developing skeletal muscle at E17.5. Bves expression is visualized using SB1 antibody (Fig.5B and 5H, green) and MF20 antibody is used to visualize sarcomeric myosin (Fig.5A and 5G, red). Co-labeling with MF20 demonstrates expression in skeletal muscle. Note that the epithelial epidermis (arrowheads) is positive for SB1 while MF20 labeling is negative; while both antibodies are positive for adjacent skeletal muscle. Polyclonal antiserum (B846, Fig. 5D) also recognizes Bves expression in skeletal muscle in a similar pattern to anti-Bves monoclonal antibody (SB1, Fig. 5E). DAPI used to visualize nuclei in blue (Fig. 5A–I).

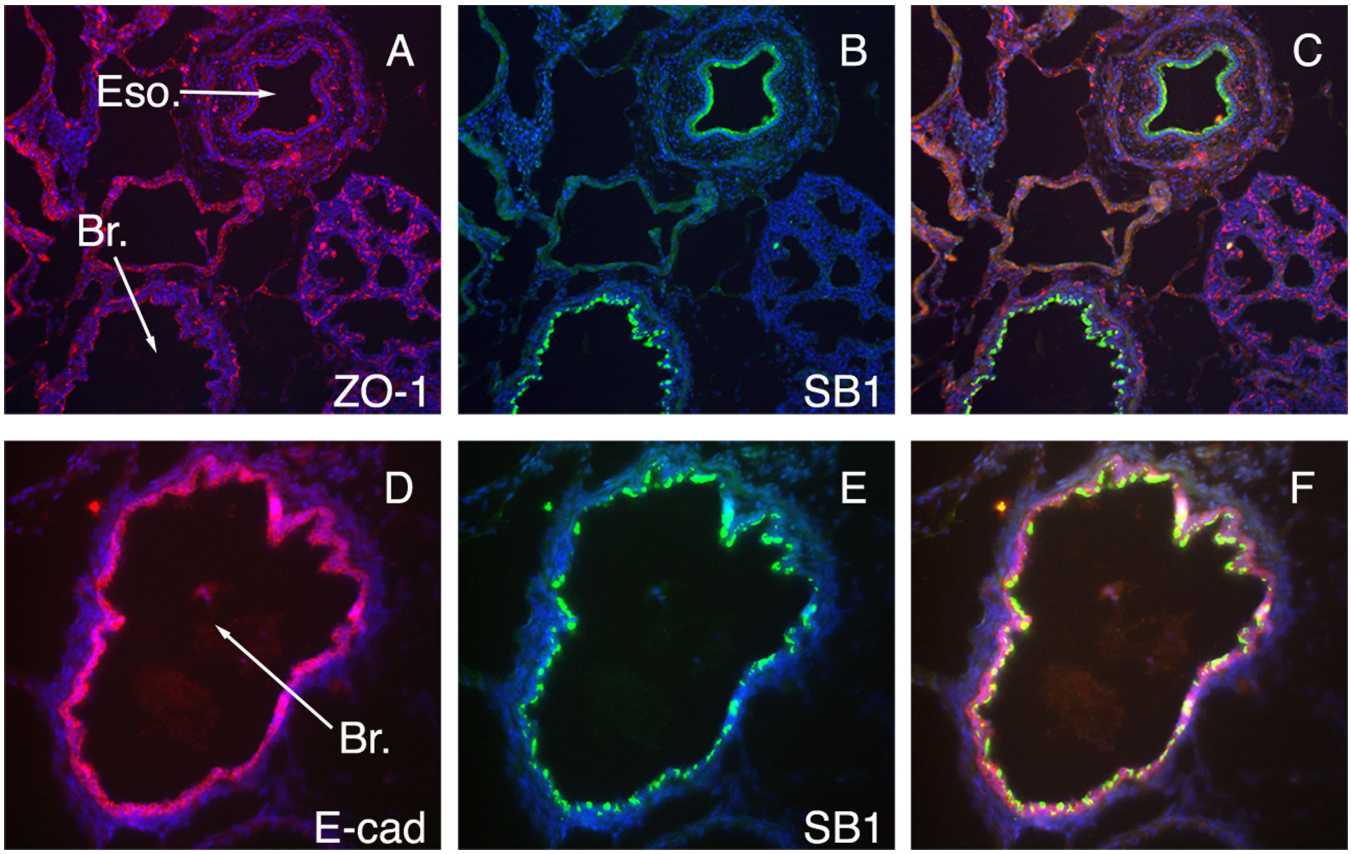

Fig 6.

Bves expression in epithelial components of the digestive and respiratory tracts. Bves labeled using SB1 antibody (Fig. 6B, green) is observed in epithelia of the esophagus as seen by colocalization with ZO1 (Fig. 6A, red). Expression of Bves (Fig. 6D, green, SB1 antibody) in a subset of epithelial cells that line bronchi is demonstrated through colocalization with E-cadherin (Fig. 6C, red). Nuclei are visualized in blue using DAPI (Fig. 6A–F). Note that not all epithelial cells in respiratory passageway are Bves positive, and that Bves expression is absent in smaller airways. (Eso. = esophagus, Br. = bronchi)

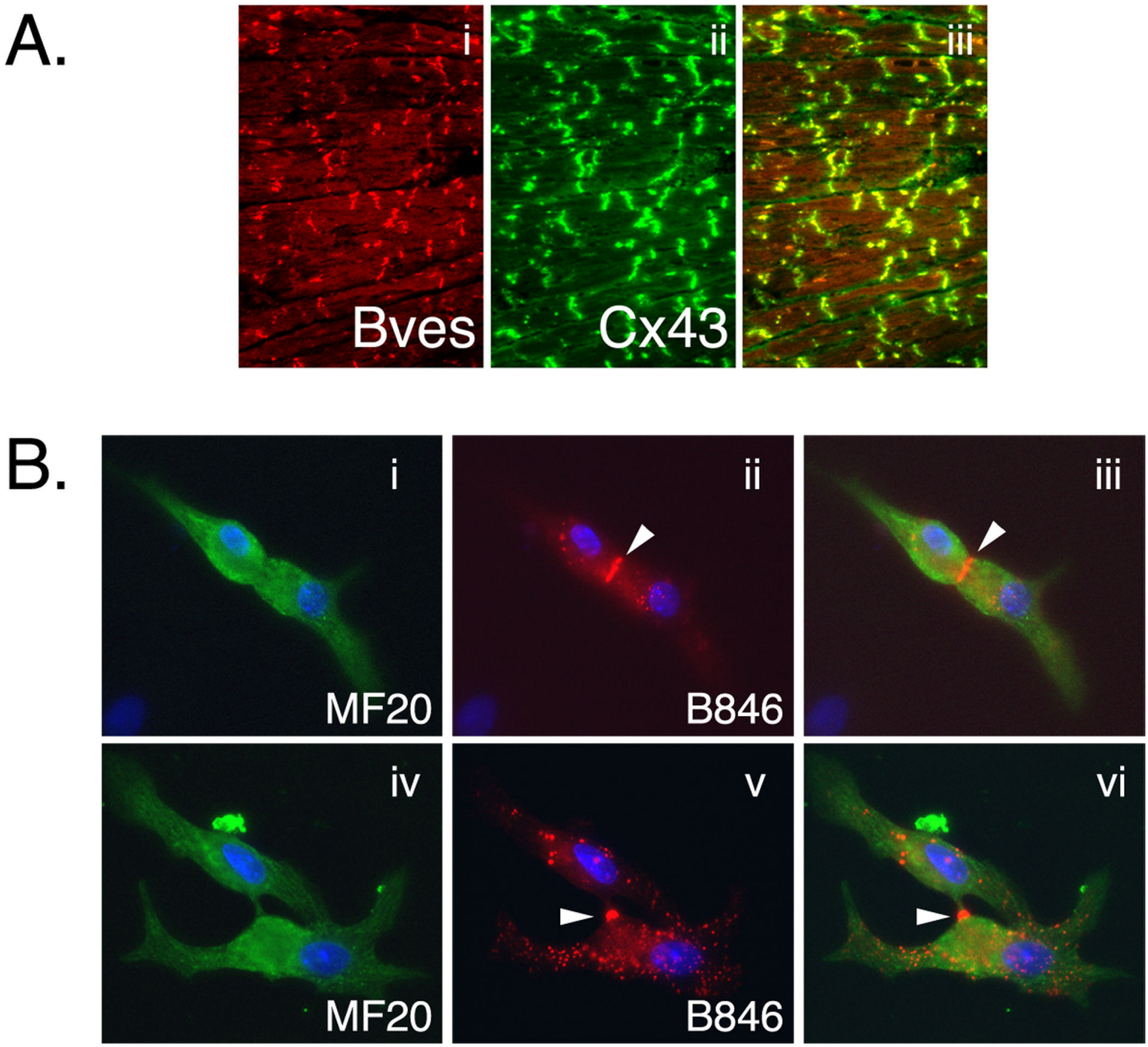

Fig 7.

Bves localizes to points of myocyte-myocyte contact *in vivo* and *in vitro*. Anti-bves antibody B846 (Fig. 7A, red) shows Bves colocalizes with Cx43 (Fig. 7B, green) to the intercalated disc structure in adult mouse heart. Bves (Fig 7B ii and Fig 7B v, red) also localizes to myocyte-myocyte contact points in primary cultures of mouse N2 myocytes. DAPI used to visualize nuclei in all panels, and anti-sarcomeric myosin antibody MF20 used to label myocytes (Fig. 7B i and iv, green).