Functional Rescue of the Sarcoglycan Complex in the BIO 14.6 Hamster Using δ-Sarcoglycan Gene Transfer

Kathleen H. Holt,*§ Leland E. Lim,*§ Volker Straub,* David P. Venzke,* Franck Duclos,* Richard D. Anderson,‡ Beverly L. Davidson,¹ and Kevin P. Campbell*‡
*Howard Hughes Medical Institute
Department of Physiology and Biophysics and Department of Neurology
University of Iowa College of Medicine
Iowa City, Iowa 52242
†Department of Internal Medicine
University of Iowa College of Medicine
Iowa City, Iowa 52242

Summary

Four types of limb-girdle muscular dystrophy (LGMD) are known to be caused by mutations in distinct sarcoglycan genes. The BIO 14.6 hamster is a model for sarcoglycan-deficient LGMD with a deletion in the δ-sarcoglycan (δ-SG) gene. We investigated the function of the sarcoglycan complex and the feasibility of sarcoglycan gene transfer for LGMD using a recombinant δ-SG adenovirus in the BIO 14.6 hamster. We demonstrate extensive long-term expression of δ-sarcoglycan and rescue of the entire sarcoglycan complex, as well as restored stable association of α-dystroglycan with the sarcolemma. Importantly, muscle fibers expressing δ-sarcoglycan lack morphological markers of muscular dystrophy and exhibit restored plasma membrane integrity. In summary, the sarcoglycan complex is requisite for the maintenance of sarcolemmal integrity, and primary mutations in individual sarcoglycan components can be corrected in vivo.

Introduction

The dystrophin–glycoprotein complex (DGC) is a large oligomeric complex of sarcolemmal proteins and glycoproteins in skeletal and cardiac muscle (reviewed in Campbell, 1995; Ozawa et al., 1995). This complex consists of dystrophin, a large cytoskeletal protein that binds F-actin; α- and β-dystroglycan, which bind laminin-2 and the cysteine-rich region of dystrophin, respectively; α-, β-, γ-, and δ-sarcoglycan (δ-SG), which form a distinct subcomplex; and the syntrophins, which bind the C-terminal domain of dystrophin. Sarcospan (25 kDa) is the most recently identified member of the DGC and is unique in that it is predicted to span the membrane four times (Crosbie et al., 1997). The DGC is believed to play an essential role in maintaining the normal architecture of the muscle sarcolemma by constituting a link between the subsarcolemmal cytoskeleton and the extracellular matrix.

Components of the DGC have been implicated in several human muscular dystrophies (reviewed in Straub and Campbell, 1997). Mutations in dystrophin cause Duchenne (DMD) and Becker muscular dystrophy (Hoffman et al., 1987). Two forms of congenital muscular dystrophy are caused by mutations in the extracellular matrix protein laminin-2 (Helbling-Leclerc et al., 1995; Allamand et al., 1997). Mutations in each of α-, β-, γ-, and δ-SG cause autosomal recessive LGMD types 2D, 2E, 2C, and 2F, respectively (Roberds et al., 1994; Bönnemann et al., 1995; Lim et al., 1995; Noguchi et al., 1995; Piccolo et al., 1995; Jung et al., 1996a; Nigro et al., 1996a, 1996b; Passos-Bueno et al., 1996). In the sarcoglycan-deficient LGMDs, a mutation in one sarcoglycan gene leads to the loss or dramatic reduction of all four sarcoglycan proteins from the sarcolemma.

The BIO 14.6 hamster is a widely studied animal model of muscular dystrophy and congenital myopathy (Homburger et al., 1962). In this animal, the sarcoglycan complex is missing and dystrophic features including central nucleation and necrosis of muscle fibers are evident. Previous studies have documented a disruption in the integrity of the DGC in skeletal and cardiac muscle of this animal, with a dramatic decrease in the amount of α-dystroglycan (Iwata et al., 1993; Roberds et al., 1993b). Recently, it was shown that a mutation in the δ-SG gene leads to sarcoglycan complex disruption and dystrophic changes (Okazaki et al., 1996; Nigro et al., 1997; Sakamoto et al., 1997). These findings make the BIO 14.6 hamster an excellent model for the study of human sarcoglycan-deficient LGMD.

The goal of our study was to explore the function of the sarcoglycan complex and the feasibility of sarcoglycan gene transfer for LGMD. Here, we report that a recombinant δ-SG adenovirus restores the entire sarcoglycan complex to the sarcolemma in BIO 14.6 skeletal muscle. Biochemical studies reveal a coincident stabilization of α-dystroglycan, the extracellular laminin-binding component of the DGC. Furthermore, fibers with persistent sarcoglycan expression lack two characteristic features of disease progression. First, myofibers expressing δ-SG exhibit a dramatic decrease in centralized nuclei, suggesting that the sarcoglycan complex provides protective effects against degeneration of muscle fibers and subsequent rounds of regeneration. Secondly, the integrity of the sarcolemma has been restored in myofibers expressing δ-SG. Our results suggest that one role of the sarcoglycan complex is to stabilize the plasma membrane by preserving the link between the extracellular matrix and the cytoskeleton. This constitutes the first example of sarcoglycan gene replacement and demonstrates that primary mutations in individual sarcoglycan components can be corrected in vivo.

Results

Injection of δ-SG Plasmid DNA Rescues the Sarcoglycan Complex

To test the hypothesis that δ-SG gene transfer could restore the sarcoglycan complex in the BIO 14.6 hamster, we performed direct plasmid DNA injections into
Figure 1. Injection of δ-SG Plasmid DNA in BIO 14.6 Skeletal Muscle
The α-, β-, γ-, δ-, and δδ-SG expression constructs were coinjected with a β-galactosidase reporter plasmid into the quadriceps femoris muscle of 3-week-old BIO 14.6 hamsters. 7 days later, sections (7 μm) were analyzed by immunofluorescence using antibodies for individual sarcoglycans as shown. Serial sections were analyzed for β-galactosidase activity to identify fibers that had taken up DNA. Bars = 50 μm.

BIO 14.6 skeletal muscle. Previous studies have shown that de novo expression of dystrophin in a small percentage of fibers can be achieved by direct injection of plasmid DNA expression vectors into mdx mouse skeletal muscle (Acsadi et al., 1991; Danko et al., 1993; Fritz et al., 1995). Expression of dystrophin in these experiments leads to the restoration of the entire dystrophin-glycoprotein complex. We adapted this methodology for the BIO 14.6 hamster to test our hypothesis that introduction of normal copies of δ-SG cDNA would lead to ordered sarcoglycan protein complex assembly. Due to the overall structural similarity of the sarcoglycan proteins to each other, we decided to test each of the individual sarcoglycans in this in vivo reconstitution assay.

We engineered expression vectors encoding myc epitope-tagged versions of human α-, β-, γ-, and δ-SG, as well as an extracellular deletion construct (δδ-SG) that recapitulates a mutation found in LGMD2F (Nigro et al., 1996b). The sarcoglycans share a similar topology, with a single putative transmembrane domain, a short intracellular domain, and a larger extracellular domain. The extracellular domains contain clusters of cysteine residues, which may form intra- and intermolecular disulfide bonds, as well as consensus sequence recognition sites for asparagine-linked glycosylation. These constructs were transiently expressed in Chinese hamster ovary (CHO) cells to examine the integrity of the expressed proteins. Anti-myc immunoblot analysis of cell lysates revealed that α-, β-, γ-, δ-, and δδ-SG expression vector constructs directed synthesis of proteins that fractionate by SDS-PAGE according to their predicted molecular weights (data not shown).

All sarcoglycan expression constructs were coinjected with a β-galactosidase reporter construct to identify fibers that harbored the DNA constructs. Expression of δ-SG restored the sarcoglycan complex to the sarcolemma based on sarcoglycan-specific antibody detection by immunofluorescence of serial cryosections (Figure 1 and data not shown). In all δ-SG cDNA-injected animals, expression of β-galactosidase in muscle fibers was coincident with sarcoglycan expression. In contrast, fibers expressing α-, β-, γ-, and δδ-SG did not have detectable sarcoglycan proteins at the sarcolemma (Figure 1). It is of interest to note that even overexpression of γ-SG, which is highly related at the amino acid sequence level (approximately 60% identity) to δ-SG, cannot compensate for the loss of δ-SG. Furthermore, deletion of the extracellular cysteine-rich region of δ-SG, analogous to one mutation identified for LGMD2F (δδ-SG) (Nigro et al., 1996b), blocked the ability of δ-SG to rescue the sarcoglycan complex. Although this technique is limited because of the low efficiency of gene transfer (typically 1%-2% of the fibers take up and express the plasmid), the data suggest that δ-SG is required for sarcoglycan
complex assembly in the BIO 14.6 hamster. Finally, the results indicate that key structural determinants of δ-SG likely reside in the extracellular cysteine-rich region of the protein.

**Recombinant δ-SG Adenovirus-Mediated Gene Transfer into the Quadriceps Femoris Muscle of BIO 14.6 Hamster**

To achieve high efficiency gene transfer, an adenovirus construct encoding human δ-SG was prepared for injection into hamster skeletal muscle. In the δ-SG adenovirus-injected BIO 14.6 muscle, the majority of the fibers were converted to δ-SG-positive (Figure 2A). The extent of δ-SG localized to the sarcolemma was similar to that of unaffected control F1B hamster (Figure 2A), and in addition some cytoplasmic staining was evident, possibly due to high levels of expression generated by the CMV promoter. An adenovirus construct encoding human α-SG was also analyzed as a control. In uninjected BIO 14.6 quadriceps muscle and in BIO 14.6 quadriceps muscle injected with the α-SG adenovirus, δ-SG was not detected by immunofluorescence (Figure 2A). Likewise, α-, β-, and γ-SG proteins were not present in serial sections (data not shown). Renewed expression of δ-SG at the sarcolemma was coincident with the rescue of the entire sarcoglycan complex to the sarcolemma based on α-, β-, and γ-SG-specific immunofluorescence of serial sections (Figure 2B). In addition, α-DG was restored to the sarcolemma in fibers expressing δ-SG (data not shown). We routinely observed that 80% or more of the fibers were converted to sarcoglycan-positive. These results confirm the ability of δ-SG to restore the entire sarcoglycan complex to the sarcolemma in the BIO 14.6 hamster.

To assess the persistence of δ-SG expression over time, hamsters were injected with the δ-SG adenovirus and examined at various time points (Figure 3). Remarkably, δ-SG expression was evident at all time points up to 198 days postinjection, without the use of immunosuppressive agents. Expression at 198 days was observed in hamsters injected at 11 days of age into the hamstring. Because of the small size of the 11-day-old muscle at injection and its tremendous growth over the next 6 months, similar levels of expression as in the 3-week-old injected hamsters were not seen in the 11-day-old injection hamsters. Nevertheless, long-term expression of δ-SG following a single injection of the adenovirus was achieved.

**Correction of the Structural Defects in the DGC Following Rescue of the Sarcoglycan Complex in the BIO 14.6 Hamster**

We next performed biochemical studies to determine if the restored sarcoglycan complex associated correctly with other components of the DGC. Glycoprotein preparations were examined by immunoblot analysis using antibodies raised against δ-SG, the DGC, and the 156 kDa α-dystroglycan (α-DG), as indicated. The upper band in the DGC blot is nonspecific. To demonstrate equal loading of protein samples, an identically loaded gel was silver stained (Silver).

Molecular weights in kDa are indicated at left. The results indicate that key structural determinants of δ-SG likely reside in the extracellular cysteine-rich region of the protein.
Figure 5. Correction of the Structural Defects in the DGC in the BIO 14.6 Hamster
Glycoprotein preparations were separated using sucrose-gradient fractionation. The sedimentation profiles (lanes 1–13 represent fractions 2–14) of F1B, BIO 14.6, and BIO 14.6 injected with 10⁹ particles of δ-SG adenovirus fractions were analyzed by immunoblotting using antibodies against α-dystroglycan (α-DG) (top panels), δ-SG (middle panels), or α-, β-, and γ-SG (bottom panel). Peaks 8–12 correspond to the known 18S sedimentation size of the DGC. Molecular weights in kDa are indicated at left.

We further characterized the reconstituted DGC by sucrose gradient sedimentation. In Figure 5, α-dystroglycan from the F1B muscle sedimented as two peaks (lanes 2–4 and 8–11), and the sarcoglycans cosedimented with α-dystroglycan in the later peak fractions (lanes 8–11). This latter peak corresponds to the known 18S sedimentation size of the DGC (Ervasti et al., 1990). In contrast, α-dystroglycan from the BIO 14.6 muscle sedimented as a single peak (lanes 2–4) and no sarcoglycans were present, as expected. We believe the earlier sedimenting dystroglycan reflects a species of dystroglycan that does not associate with the sarcoglycans, possibly the dystroglycan at the myotendinous and/or neuromuscular junctions (V. S. and K. P. C., unpublished data). Strikingly, expression of δ-SG in the BIO 14.6 muscle not only restored the sarcoglycan complex to the sarcolemma, but also reestablished the association between the sarcoglycan complex and α-dystroglycan, as evidenced by the cofractionation of these proteins (Figure 5, lanes 8–11). Together, these results provide strong evidence that the integrity of the DGC has been restored in the BIO 14.6 muscle expressing the δ-SG adenovirus.

Dystrophic Features Are Greatly Reduced in Muscle Expressing δ-SG
Histological features of the dystrophic muscle in the BIO 14.6 hamster include cellular infiltration, variation in myofiber size, and central nucleation (Homburger et al., 1962). Central nucleation is assumed to be a sign of continued cycles of degeneration and regeneration, with the earliest onset at 2–3 weeks of age in the BIO 14.6 hamster (data not shown). However, genetic intervention at age 3 weeks resulted in a marked reduction of the number of centrally located nuclei in the injected BIO 14.6 hamster compared to uninjected BIO 14.6 littermates (Figure 6A). The percentage of fibers with centrally located nuclei was calculated from multiple fields of age-matched samples of F1B (1.4%), BIO 14.6 (94%), and BIO 14.6 hamsters injected with the δ-SG adenovirus (7.2%) (Figure 6B). Clearly, restoration of the sarco-glycan complex significantly prevents the degenerative process in the δ-SG adenovirus-injected muscle.

Sarcolemmal Membrane Integrity Is Normal in Muscle Fibers Expressing δ-SG
An intact plasma membrane is a critical component of the highly regulated transfer of molecules into and out of muscle fibers. The stresses of contraction can induce tears in the sarcolemma, allowing for release of muscle enzymes and growth factors as well as the influx of calcium. These transient breaks are thought to be one mechanism by which skeletal muscle can sense and respond to incremental changes in external load. In the case of dystrophic muscle, chronic membrane disruptions are thought to contribute to fiber necrosis (reviewed by McNeil and Steinhardt, 1997). Evidence from muscular dystrophies that arise from defects in the DGC clearly demonstrates the importance of intact molecular
be used as a tracer molecule to monitor sarcolemmal membrane integrity in various dystrophic mouse models involving the absence of dystrophin and/or utrophin (Matsuda et al., 1995; Grady et al., 1997; Straub et al., 1997). In the present study, EBD was injected intravenously into F1B, BIO 14.6, and BIO 14.6 hamsters that had expressed the δ-SG adenovirus for 12 weeks, and dye uptake into muscle fibers was examined after 72 hr. Macroscopically, EBD incorporation into skeletal muscle was clearly visible in various muscle groups in all BIO 14.6 hamsters, whereas no dye incorporation into muscle could be detected in the F1B hamsters (data not shown). The quadriceps femoris muscles of these animals were cryosectioned and analyzed by immunofluorescence (Figure 7). Autofluorescence of EBD was not detected in fibers from the F1B hamster. In contrast, EBD was found within groups of fibers in the BIO 14.6 hamster. Strikingly, EBD was excluded from fibers that had been converted to sarcoglycan-positive by δ-SG adenovirus in the BIO 14.6 hamster (Figure 7). These results demonstrate that sarcolemmal membrane integrity is compromised in the BIO 14.6 hamster, but can be reversed by restoration of the sarcoglycan complex.

Discussion

The pace of discovery in the field of muscular dystrophy research has been rapid since the discovery of the DMD gene in 1986 (Monaco et al., 1986). The DMD gene encodes dystrophin, a large cytoskeletal protein (Hoffman et al., 1987). Dystrophin functions together with other components of the DGC to span the sarcolemma, forming a strong link between the actin cytoskeleton and laminin in the extracellular matrix. This structural linkage is thought to protect muscle fibers from the mechanical stress of contraction. Loss of dystrophin due to mutations in the gene destroys the link, and leads to muscle fiber degeneration. The fact that similar dystrophic features occur due to autosomal recessive mutations in the sarcoglycans (LGMD) and laminin-2 (congenital muscular dystrophy) provides support for the hypothesis that the function of the DGC as a whole is dependent on intact molecular interactions between its individual subunits.

Much has been learned recently regarding the autosomal recessive mutations in α-, β-, γ-, and δ-SG that cause LGMD2D, LGMD2E, LGMD2C, and LGMD2F (Roberts et al., 1994; Bönneman et al., 1995; Lim et al., 1995; Noguchi et al., 1995; Piccolo et al., 1995; Jung et al., 1996a; Nigro et al., 1996a, 1996b; Passos-Bueno et al., 1996). Unfortunately, functional characterization of the sarcoglycan complex has proven to be elusive. Our results demonstrate that the sarcoglycan complex is requisite for the maintenance of sarcolemmal integrity. Our experiments also suggest that obligatory steps in the biosynthetic pathway for sarcoglycan complex assembly cannot occur if individual sarcoglycan proteins are aberrant or missing. This makes the sarcoglycan-deficient LGMDs attractive candidates for gene transfer therapy.

In the case of the BIO 14.6 hamster, providing a normal copy of δ-SG cDNA is necessary and sufficient to correct the defect in sarcoglycan complex assembly and allow
for stable anchorage of α-dystroglycan to the extracellular face of the sarcolemma. We demonstrate that renewed expression of the sarcoglycan complex greatly reduces central nucleation, one hallmark of myopathic progression in this animal. Furthermore, we provide the first direct evidence that the absence of the sarcoglycan complex results in altered membrane permeability, which can be corrected by δ-SG gene transfer. These data demonstrate for the first time that the sarcoglycan complex plays an important role in the maintenance of sarcolemmal stability and the preservation of muscle cell integrity.

One remarkable result of our study was the ability to achieve long-term gene expression from a single injection of adenovirus. By RT-PCR, we have confirmed the persistence of transcription for up to six months from the adenovirus construct (data not shown). Previous studies of adenoviral-mediated gene transfer have been plagued by rapid loss of recombinant gene expression, since the protein product of the transgene is often recognized as a neoantigen and is targeted by the host’s immune system. This is consistent with our preliminary observations that the β-galactosidase adenovirus expression diminished over time (data not shown). One possible explanation for the persistence of expression is that δ-SG is very similar to γ-SG, which is predicted to be translated normally, but then degraded in the absence of a stable sarcoglycan complex. On the other hand, there may be low levels of expression of at least part of the coding sequence of δ-SG from the remaining genomic sequence. In either case, the adenovirus-encoded δ-SG may not be identified as a foreign antigen.

Based on these observations, we remain cautiously optimistic that low levels of protein may be expressed in patients with missense mutations, and thus, adenoviral-mediated sarcoglycan gene therapy may not necessarily result in immunological destruction of the target tissue.

Another reason that the prospects of LGMD gene therapy are so encouraging lies within the relatively small size of the sarcoglycan cDNAs. Since all sarcoglycan cDNAs are less than 2 kb, well within the limits of adenoviral vectors, adenovirus-encoded vectors for each of the sarcoglycans can be easily produced. This is in contrast to the current studies of DMD gene therapy, which have been hampered by the large size of the dystrophin cDNA. This, together with the potential lack of a robust inflammatory response, makes LGMD adenoviral-mediated gene therapy a very real possibility.

Our results demonstrate that adenovirus-mediated gene transfer for the treatment of limb-girdle muscular dystrophy is obtainable with high efficiency, long-term expression, and functional benefit. It would be of interest to further explore the functional effect of sarcoglycan complex restoration, possibly by electromyography or force generation measurements. Our work reveals novel aspects of the pathogenesis of human muscular dystrophy. Importantly, these new insights help to build a solid foundation for the development of future strategies aimed at the treatment of human muscular dystrophy.

**Experimental Procedures**

**Animals**

Male F1B and BIO 14.6 cardiomyopathic hamsters were obtained from BioBreeders (Fitchburg, MA).
**Antibodies**
Monoclonal antibodies were produced in collaboration with Louise Anderson: IIH6 against α-DG (Ervasti and Campbell, 1991). 20A6 against γ-SG (Piccolo et al., 1995); SB1 against α 89-152 of human β-SG; 218B against α 167-178 of rabbit γ-SG; and 312C1 against α 1-15 of human γ-SG. Rabbit 98 polyclonal antibody against γ-SG has been described previously (Roberds et al., 1993b). An affinity-purified rabbit antibody (Rabbit 208) was produced against a C-terminal fusion protein of rabbit γ-SG. An affinity-purified rabbit antibody (Rabbit 215) was produced against an amine-terminal peptide of human δ-SG (MPMQEQYTHHRSTMPGAA). Rabbit 229 recognizes the carboxy terminus of human δ-SG. Goat 20 serum was previously described (Jung et al., 1996b).

**Human Sarcoglycan Expression Constructs**
Human α-, β-, γ-, and δ-SG expression constructs were prepared by PCR amplification of cDNA using primers containing appropriate restriction sites for subcloning into pcDNA3 (Pharmacia). For the δ-SG construct, a stop codon was introduced immediately following a restriction site for subcloning into pcDNA3 (Pharmacia). For the δ-SG construct, a stop codon was introduced immediately following the stop codon.

**Recombinant Adenovirus**
The human δ-SG sequence was amplified by PCR and subcloned into the pAdCMVpA adenovirus shuttle vector. The δ-SG construct was then incorporated into an adenovirus vector through standard methods of homologous recombination with Ad5 backbone dI309 by the University of Iowa Gene Transfer Vector Core. Recombinant viruses were purified using established methods (Graham and van der Eb, 1973; Davidson et al., 1994). Lysates from the infected cells were collected and tested for the expression of δ-sarcoglycan protein using a δ-sarcoglycan-specific polyclonal antibody. Recombinant virus was plaque purified, amplified, and purified by CsCl gradient centrifugation. A human δ-sarcoglycan adenovirus was made and tested as a control.

**Plasmid DNA and Recombinant Adenovirus Injections**
Hamsters were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, Abbott Laboratories) at a calculated dose of 75 mg/kg. The skin overlying the quadriceps femoris muscle was disinfected and a 1 cm vertical incision was made. Plasmid DNA (100 μg of sarcoglycan plasmid DNA and 25 μg of β-galactosidase reporter plasmid DNA) in a total volume of 100 μl in normal saline (0.9% NaCl w/v) was injected into the quadriceps femoris muscle (Acsadi et al., 1991; Danko et al., 1993; Fritz et al., 1995). The incision was closed with 3-4 sutures. For 11-day-old hamsters, the adenovirus was injected directly through the skin into the hamstring. Hamsters recovered with continual supervision and were housed postoperatively at the University of Iowa Animal Care Facility. Hamsters were housed in BL2 containment with isolator cages. 7-198 days after injection, hamsters were euthanized with CO2. Asphyxiation was performed using a CO2 mask. Frozen tissue was homogenized in 4 ml of buffer A (Iwata et al., 1993) with a polytron for 30 s (3×), followed by Dounce homogenization. Samples were centrifuged at 50,000 rpm for 3 min. The pellet was resuspended in 4 ml of buffer A and the homogenization steps were repeated. The two supernatants were pooled and incubated with WGA-Sepharose at 4°C overnight with rotation. The resin was washed and proteins were eluted with 0.4 M α-n-acetyl glucosamine. Eluted proteins were fractionated by centrifugation (4 hr at 30,000 rpm) through a 5%–30% sucrose gradient. The eluted proteins and fractions from the sucrose gradient were analyzed by immunoblotting using specific antibodies and enhanced chemiluminescence.

**In Vivo Membrane Permeability Assay**
Evans blue dye injections and microscopic analysis were performed as described previously (Straub et al., 1997), with some modifications. Evans blue dye was dissolved in phosphate-buffered saline (10 mM) and sterilized by passage through membrane filters with a pore size of 0.2 μm. Hamsters were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, Abbott Laboratories) at a calculated dose of 75 mg/kg. Subsequently 0.25 μl/10 g body weight of the dye solution was injected intravenously through the front limb vein. Animals were euthanized 72 hr after injection by asphyxiation with gaseous CO2. All dye-injected hamsters were skinned and inspected for dye uptake in the skeletal muscles, by the appearance of blue staining. Muscle sections for microscopic Evans blue detection were incubated in ice-cold acetone at −20°C for 10 min, washed in PBS, and mounted with Vectashield (Vector Laboratories). All sections were examined and photographed under a Zeiss Axiosplan fluorescence microscope.

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