RECS1 deficiency in mice induces susceptibility to cystic medial degeneration

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RECS1 is a novel shear stress-responsive gene that encodes a protein putatively forming seven-span transmembrane domains. We reports here that mouse RECS1 (mRECS1) transcripts is detected in most tissues except for thymus, spleen and testis. The putative cytoplasmic N-terminus of mRECS1 has a high content of proline (23%) and glycine (12%) residues, contains one PPXY motif, multiple PXXP motifs and one overlapping P(T/S)AP and PPXY motif (P(T/S)APPXY). The PPXY motif lies within one potential PEST sequence (PEST score: +7.65). We prepared anti-RECS1 polyclonal antibody and found by western blot analysis that the mRECS1 protein in the lung and aorta was detected as a 34.4 kDa band. However, one shifted 58 kDa band or three shifted bands (48, 69, 82 kDa) were detected in the heart or the liver, respectively. Since northern blot detected only one species of mRECS1 mRNA in heart and liver tissues, as well as other tissues (~2.2 kb), these differences in molecular weight seem to be due to posttranslational modification. Biochemical fractionation and RECS1-GFP fusion protein revealed that RECS1 localizes at the endosomal/lysosomal membranes in the cytoplasm. To understand the function of RECS1 in the body, we made RECS1 knockout (KO) mice and found that RECS1 KO mice (older than 14 months) are prone to cystic medial degeneration (CMD). Taken together, we conclude that RECS1 is an endosomal/lysosomal membrane protein which plays protective roles in vascular remodeling.

Key words: aging, cystic medial degeneration, knockout, RECS1, shear stress

INTRODUCTION

Cystic medial degeneration (CMD) is a frequently encountered pathological finding in aortic aneurysms that was first described by Erdheim (Erdheim, 1929; Erdheim, 1930; Schlatmann and Becker, 1977; Larson and Edwards, 1984). It refers to mucoid degeneration (accumulation of basophilic ground substance) of the aortic media that is often accompanied by smooth muscle loss or elastic fragmentation (Schlatmann and Becker, 1977). Study of normal human aging aortas by Schlatmann and Becker revealed that CMD also presents in the normal aging aorta, leading them to declare that CMD is not the cause of dissecting aneurysms (Schlatmann and Becker, 1977). While Schlatmann and Becker have proposed that CMD results from repetitive aortic injury and aging, and that hemodynamic impact may be the initiating cause (Schlatmann and Becker, 1977), the molecular mechanisms underlying CMD remain largely unknown.

Previously, we used cDNA-subtractive hybridization to comprehensively isolate the RECS genes from endothelial cells, the expression of some of which, including RECS1, is up-regulated by steady laminar shear stress (Yoshisue et al, 2002). Here we report that RECS1 knockout mice (older than 14 months) are prone to cystic medial degeneration.
eration (CMD). Intensive investigations of RECS1 knockout mice will help us understand the molecular mechanisms underlying CMD.

MATERIALS AND METHODS

Cell culture. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in air. Human umbilical vein endothelial cells (HUVEC), human aortic endothelial cells (HAEC), human aortic smooth muscle cells (HASMC) were purchased from Clonetics (CC-2517, CC-2535, CC-2571). Endothelial cells were cultured in gelatin coated 10 cm dishes using Endothelial Cell Growth Medium BulletKit® (EGM® BulletKit®, CC-3124, Clonetics), smooth muscle cells were cultured using SmGM-2 Bullet-kit (CC-3182, Clonetics). HelaS3 and HT1080 (human fibrosarcoma) cells were maintained in DMEM (Sigma) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Transfection was carried out using Lipofectamine Plus reagent (Invitrogen, Corp) with transfection medium Opti-MEM® I (Invitrogen), according to the manufacturer's instructions. 70–80% confluent cells in 35 mm dishes were transfected with 2 µg of the mammalian expression vectors pEGFP-N1 (BD Biosciences), pRe/CMV (Invitrogen), pCMV6myc and these vectors containing the in-frame RECS1 ORF. For transient expression, cells were harvested for analysis 48 hours after introduction. For stable transfection, cells were selected for 21 days with growth medium containing 1 mg/mL G418 with pooling and passing every 3~4 days before use in experiments.

Northern blot analysis. Total RNA from murine tissues was isolated by the acid guanidinium thiocyanate-phenol chloroform (AGPC) extraction method. Total RNA (5~10 µg) was subjected to Northern hybridization as previously described (Yoshisue et al., 2002). The mouse ORF cDNA was labeled and used as a probe.

Anti-RECS1 antibodies. Two affinity-purified polyclonal antibodies specific for recombinant RECS1 fusion proteins that were N-terminally linked to GST were raised in rabbits according to standard procedures. One of the polyclonal antibodies used at a 1:100 dilution, followed by incubation with an HRP-conjugated anti-rabbit Ig secondary antibody at a dilution of 1:1000 (NA934V, Amersham Biosciences Corp.). All of the procedures were performed at room temperature.

Biochemical fractionation and Microscopy. Stably transfected HT1080 cells that overexpress hRECS1 were subjected to biochemical fractionation. The Subcellular Proteome Extraction Kit (Calbiochem Corp.) was used to sequentially extract the cell contents. Alternatively, fractionation by differential centrifugation was performed as follows. Cells were scraped into a 0.25 M sucrose solution (containing 1 mM MgCl₂, 10 mM Tris-HCl pH 7.5, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 0.7 µg/ml pepstatin A and 0.2 mM PMSF) and homogenized with a dounce homogenizer. After centrifugation at 700 × g for 5 min, the postnuclear supernatant was subjected to sequential centrifugation steps at 4°C, as follows. First, a 5 min 7000 × g centrifugation yielded the mitochondria and lysosome fraction. This was followed by a 90 min 105,000 × g centrifugation to obtain the ER, Golgi, endosome and plasma membrane fraction. The supernatant contained the cytoplasmic fraction. The fractions were dissolved in UreaTD solution or adjusted by adding a urea and 10% Triton-100/5% DTT (wt/vol) solution.

To analyze the subcellular localization of hRECS1, HelaS3 cells transfected with pEGFP-N1-hRECS1 or the pEGFP-N1 vector were grown on glass slides in growth medium containing 75 nM LysoTracker Red DND-99 (L7528, Molecular Probes Inc.) for 40–50 min. They were then observed by using a Zeiss LSM-510 Confocal Microscope. The signals from GFP and LysoTracker were then merged.

Genomic cloning and generation of RECS1-null mice. Using primers derived from the mouse RECS1 cDNA, sense/K3: 5'-GGTTACCCGTGTTCCTGAGCTTCAG-3' and antisense/AK5: 5'-GGTAAAGGGAAGGAGATA-ACC-3', a genomic bacterial artificial chromosome (BAC) clone was obtained by PCR screening of a mouse 129 BAC library (Genome Systems, St. Louis, MO, USA) and mapped by restriction enzyme analysis. An 18 kb EcoRI fragment and a 6.4 kb XbaI fragment were subcloned into pBluescriptII SK+. The 18 kb EcoRI fragment was sequenced after performing Transposon Random Cloning using Ez::TN™ TRANSPOSON TOOLS Ez::TN™<KAN-2> Insertion Kit (Cat. No. EZ1052K, Epicycle Technologies Corporation, Madison, WI, USA). This fragment contains the entire mRECS1 gene, comprising 11 exons and spanning approximately 7.2 kb of genomic sequence. Before constructing the targeting vector, an AsCl site was generated between the KpnI-XbaI
sites of the pPNT targeting vector. A 2.5 kb SpeI-5’-XbaI-3’-SpeI fragment from the 6.4 kb XbaI fragment in pBluescriptII SK+ was subclone into the SpeI site of pBluescriptII SK+ to generate a 2.6 kb 5’-XhoI-3’-NcoI fragment. This fragment was then inserted into the XhoI-NcoI site of the pPNT targeting vector. An AscI site was created within the XbaI-NcoI site of the pET28a+ vector, a 7.6 kb 5’-EcoRI-3’-BspHI fragment was subclone into the EcoRI-NcoI site of this vector, and then the 7.6 kb EcoRI-AscI fragment was inserted into the EcoRI-AscI site of the pPNT vector. The resulting plasmids were linearized by NotI digestion before being introduced into D3 embryonic stem (ES) cells by electroporation.

Six ES cell lines out of 48 G418-gancyclovir-resistant clones were verified to be homologous recombinants by PCR screening. Using the sense/KSP3' : 5’-CGACGG-TATCGATAAGCTTG-3'; and antisense/KO12 : 5’-ACAGAGTTTGGTGCATACTCGTC-3' primers, amplification of a ~2.5 kb band (95°C 3 min followed by 40 cycles of 3-step reaction: 95°C 30 sec, 55°C 30 sec for annealing, 72°C 160 sec) identified those ES cell clones which were homologous recombinants. This was confirmed by standard Southern blot analysis as follows: 20 µg of genomic DNA was digested with EcoRI and separated on 1% agarose gels. After transfer to nylon membranes and baking for 2 h at 80°C, blots were hybridized with a [α-32P]dCTP radiolabeled 5’-external probe (+9000~+8341EcoRI-PvuII, 659 bp) as described above, or with a 3’-external probe (+6328~+6983 SacI-KpnI, 655 bp) labeled according to the manufacturer’s protocol (Gene Images AlkPhos Direct Labelling and Detection System, Amersham Pharmacia).

Three out of the six correctly targeted embryonic stem cell lines were injected into C57BL/6J blastocysts, two lines of male chimeric mice (>90% derived from ES cells as judged by agouti coat color) were obtained, bred to C57Bl/6J females (Jackson Laboratories, Bar Harbor, ME, USA) and produced heterozygous F1 offspring. Tail genomic DNA was isolated for the screening of heterozygous mice by PCR analysis. Female heterozygous mice
(RECS1+/–) were bred to C57Bl/6J males six times before generating homozygous animals (RECS1–/–) with a >98% background of C57Bl/6J. WT littermates served as controls for all lines studied. Final genotyping of knockout animals was performed by PCR with DNA isolated from tail tissue (sense/S8: 5'-CGATTCCCATGGAACATCAT-3', for wild type allele, and sense/KSP3' for targeted allele; antisense/AS89: 5'-GATGGTGACAGAGATGGATA-3'). Reaction conditions were the same as above except for an extension time of 40 sec. Genotypes were further confirmed by Southern blot analysis, verified by detection of the fragments that differ in size between the wild type and the targeted locus. For detection of RECS1 expression in the mutants, total RNA was isolated from mouse tissues, and Northern blotting was performed using the mouse ORF cDNA as a probe.

Mice were housed under a 12/12 h day/night cycle at a temperature of 25°C and fed a standard diet. Young (2-6 months) and old (14–17 months) male mice were used for the experiments. These had a >98% and >87.5% C57Bl/6 background, respectively. All experiments complied with the Guidelines on Animal Experiments of Osaka University.

Histology. Mice were killed by cervical dislocation and descending thoracic aortas (together with surrounding tissues) were harvested carefully. Isolated aortas were then directly embedded in Tissue-Tek OCT compound to generate frozen cross-sections (10–12 µm), or fixed with 4% paraformaldehyde to generate paraffin-embedded cross-sections (5 µm). The sections were subjected to Elastic Van Gieson and Alcian Blue/Periodic Acid Schiff staining.

Plasma isolation. Mice were fasted for 16 hours, anaesthetized and heparinized by an intraperitoneal injection of Pentobarbital Sodium (20 µg/g body weight) containing heparin sodium (1 unit/g body weight). Blood was obtained by right ventricle puncture and centrifuged
Cystic medial degeneration in aged RECS1 knockout mice

Statistical analysis. The significance of mucoid deposition was analyzed by Mann-Whitney’s U test. Other data were expressed as means ± SD and analyzed by ANOVA and Bonferroni-type multiple t-test. A P value of < 0.05 was considered to indicate statistical significance.

RESULTS

RECS1 is ubiquitously expressed in the body and is located in the endosome/lysosome compartment. Human RECS1 encodes a 311 amino acid protein (hRECS1, GenBank Accession no. BD094043, calculated Mr 34.6 kDa), mouse RECS1 encodes a 309 amino acid protein (mRECS1, Gen Bank Accession No. AB097685, calculated Mr 34.4 kDa). A homology search in the databases revealed two homologous proteins (Fig. 1): rat neural membrane protein 35 (NMP35) or human Lifeguard (LFG), and rat glutamate binding protein (GBP) (Schweitzer et al., 1998; Somia et al., 1999; Kumar et al., 1991). Rat NMP35 was described as a postsynaptic membrane protein (Schweitzer et al., 2002). Human LFG was cloned as a factor conferring resistance to Fas-mediated apoptosis (Somia et al., 1999). GBP is a component of a chemically purified NMDA receptor-like complex from rat brain synaptic membranes, containing GlyBP, CPP-binding protein and another 36 kDa protein (Michaelis et al., 1998). This complex may form NMDA receptor-like channels, but the neuronal function of the complex remains unknown. Orthologs of GBP are a putative MAPK activating protein (Q7Z429) (Matsuda et al., 2003), mouse LAG (AF182040), and bovine OTMP (Szuchet et al., 2001), but no further functional experiments have been done.

Several transmembrane topology prediction programs (SOSUI, TMHMM 2.0, and Tmpred) indicate that RECS1 is a putative seven-span transmembrane (7-TM) protein with a 100 amino-acid-long cytoplasmic N-terminal region (Fig. 1). This cytoplasmic N-terminus has a high content of proline (24% hRECS1, 23% mRECS1) and glycine (16% hRECS1, 12% mRECS1) residues, contains one PPXY motif and several PXXP motifs. Interestingly, one overlapping P(T/S)AP and PPXY motif, P(T/S)APPXY (Strack et al. 2000), was found in the N-terminal cytoplasmic region of both RECS1 (PSAPPPYE) and LFG (PSAPPSYE). The PPXY motif lies within one potential PEST sequence, which targets proteins for rapid degradation (Rechsteiner et al., 1996), in the N-terminal region of

Fig. 4. Subcellular localization of RECS1. (A) Analysis of intracellular hRECS1 expression by biochemical fractionation using HT1080 cells stably transfected with hRECS1 cDNA. Western blot was performed with antibodies for hRECS1 and intracellular compartment-specific markers. Upper: the cells were biochemically fractionated by using the Subcellular Proteome Extraction Kit. This revealed that RECS1 is a resident of organelle membranes. Lower: the postnuclear supernatant was fractionated by sequential centrifugation. The 7000 × g pellet contains the mitochondria and lysosomes, the 105,000 × g pellet contains the ER, Golgi, endosomes and plasma membrane, while the 105,000 × g supernatant contains the cytosol. (B) Analysis of intracellular hRECS1 expression in HelaS3 cells expressing the hRECS1-GFP fusion protein by confocal microscopy. Green: the fusion protein; Red: LysoTracker Red DND-99 signal; Yellow: the merged image; Gray: differential interference contrast (DIC). Scale bar = 10 μm.
Fig. 5. Targeted disruption of the mRECS1 gene in mice. (A) Restriction maps of the WT gene, the targeting construct and the targeted locus. Filled and open boxes represent coding and non-coding exons, respectively. PGK-neo, neomycin-resistance gene linked to the phosphoglycerate kinase promoter; HSV-tk, thymidine kinase gene derived from the Herpes simplex virus linked to the HSV promoter (the orientation is indicated by arrows). Restriction sites: As, AscI; Bs, BspHI; E, EcoRI; K, KpnI; Spe, SpeI; Xb, XbaI; Xh, Xhol. m stands for methylation. (B) Southern blot of the 3'-external probe-hybridized EcoRI-digested tail genomic DNA. (C) Northern blot of the heart and liver (10 µg/lane) using the mRECS1 ORF as the probe. The mRECS1 mRNA signal in WT was detected as a single band at ~2.2 kb. It was undetectable in the KO mouse tissues, which indicates they are null mice. (D) Western blot analysis. The WT mice show one 58 kDa band in the heart (arrowhead) and three bands in the liver of 48, 69, and 82 kDa (arrowheads). The mRECS1 protein was undetectable in the KO mouse tissues.

Table 1 Blood pressure, heart rate measurement and plasma biochemical examination

<table>
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<tr>
<th>Age (months)</th>
<th>HR (bpm)</th>
<th>SBP (mmHg)</th>
<th>MBP (mmHg)</th>
<th>DBP (mmHg)</th>
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<tbody>
<tr>
<td>young WT (5–6)</td>
<td>606 ± 60</td>
<td>108 ± 4</td>
<td>82 ± 5</td>
<td>69 ± 6</td>
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<tr>
<td>Young KO (5–6)</td>
<td>586 ± 55</td>
<td>110 ± 7</td>
<td>82 ± 4</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>old WT (16–17)</td>
<td>617 ± 66</td>
<td>108 ± 8</td>
<td>84 ± 5</td>
<td>71 ± 5</td>
</tr>
<tr>
<td>old KO (16–17)</td>
<td>614 ± 49</td>
<td>104 ± 7</td>
<td>80 ± 5</td>
<td>69 ± 6</td>
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<tr>
<th>Age (months)</th>
<th>Glucose (mmol/L)</th>
<th>TC (mmol/L)</th>
<th>TG (mmol/L)</th>
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<td>young WT (3)</td>
<td>6.33 ± 0.77</td>
<td>2.19 ± 0.17</td>
<td>0.92 ± 0.25</td>
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<tr>
<td>young KO (3)</td>
<td>6.09 ± 0.76</td>
<td>2.39 ± 0.28</td>
<td>0.93 ± 0.25</td>
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<tr>
<td>old WT (15–17)</td>
<td>6.33 ± 1.53</td>
<td>2.91 ± 0.71</td>
<td>1.18 ± 0.31</td>
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<tr>
<td>old KO (15–17)</td>
<td>5.52 ± 1.04</td>
<td>2.78 ± 0.47</td>
<td>0.86 ± 0.14</td>
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Blood pressure and heart rate (HR) were measured by a noninvasive computerized tail-cuff method (BP-98A, Softron Corp.). SBP, MBP and DBP stand for systolic, mean and diastolic BP (mmHg). Glucose (Glu), total cholesterol (TC) and triglyceride (TG) levels in the plasma were measured by using the Glucose CII-test Wako, Cholesterol E-Test Wako and triglyceride E-test Wako kits (Wako Pure Chemical Industries). Data were expressed as means ± SD.
RECS1 or LFG. PEST sequence can be found as an element existing in SUMO1 targets (Kim et al., 1999; Melchior et al., 2000). One KXD/E minimal consensus SUMO acceptor site exists in RECS1, and two are found in LFG. While no high score PEST sequence or KXD/E motif exists in LAG, it is also proline-rich in its N-terminus. The remaining two thirds of the C-terminal domain of RECS1, LFG (NMP35), and LAG are highly hydrophobic and share a high degree of similarity, forming the seven transmembrane domains and harboring only 5–18-residue-long loops, and a conserved sequence of SPED/EY in the relatively long loop between TM6 and TM7, which may potentially be a functional element for these proteins.

Unlike NMP35 (Schweitzer et al., 1998) whose distribution is predominantly in the central nervous system, RECS1 is expressed in most tissues except for thymus, spleen and testis, as revealed by northern blot analysis (Fig. 2). RECS1 mRNA was also detected in fibroblasts and white blood cells (WBC) by RT-PCR (not shown).

To detect RECS1 protein expression, we made polyclonal antibodies which specifically recognize hRECS1 and mRECS1 as detected by Western blot analysis (Fig. 3A). The hRECS1 protein could be detected as a 30 kDa band in cultured vascular endothelial cells (HUVECs, HAECs) and smooth muscle cells (HASMCs) by western blot (Fig. 3B). RECS1 protein can be dissolved in lysis buffer only in the presence of = 8M Urea, and it became more successful when using UreaDT. This is due to the high hydrophobicity of the RECS1 protein that drives protein aggregation in the absence of Urea.

To determine the intracellular localization of RECS1, HT1080 cells were stably transfected with hRECS1 cDNA and subjected to biochemical fractionation (Fig. 4A). This revealed hRECS1 was located in organelle membranes. Furthermore, when HelaS3 cells were transiently transfected with hRECS1-GFP, confocal microscopy revealed the GFP signal in intracellular and membrane-associated vesicles (Fig. 4B). Its co-localization with the LysoTracker signal indicates that RECS1 is a membrane protein. To detect RECS1 protein expression, we made monoclonal antibodies which specifically recognize hRECS1 and mRECS1 as detected by Western blot analysis (Fig. 3A). The hRECS1 protein could be detected as a 30 kDa band in cultured vascular endothelial cells (HUVECs, HAECs) and smooth muscle cells (HASMCs) by western blot (Fig. 3B). RECS1 protein can be dissolved in lysis buffer only in the presence of = 8M Urea, and it became more successful when using UreaDT. This is due to the high hydrophobicity of the RECS1 protein that drives protein aggregation in the absence of Urea.

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Cystic medial degeneration in aged \textit{RECS1} knockout mice

![Fig. 6. Characterization of RECS1 KO mice.](image)
protein located in endosomal/lysosomal compartments.

**Targeted disruption of RECS1 in mice.** To investigate the function of RECS1, we generated RECS1 KO mice. The entire exon 1–6 region of the RECS1 gene plus its 5′-untranslated sequence (~3.8 kb) was targeted and replaced by a neomycin resistance cassette (PGK-neo) through homologous recombination (Fig. 5A). The disruption of this region in the KO mice was verified by PCR (data not shown) and Southern blot analysis (Fig. 5B) of genomic DNA. Moreover, Northern blot analysis of the heart and liver showed the RECS1 gene is inactivated in the KO mice (Fig. 5C). The mRECS1 proteins in the KO mice were also undetectable when evaluated by Western blot analysis (Fig. 5D). The mRECS1 protein in lung of WT mice was expressed as a 34.4 kDa protein by Western blot analysis (which is the predicted size, data not shown). Although a single ~2.2 kb mRECS1 mRNA was detected in multiple tissues of WT mice by northern blot analysis (Fig. 2 and 5C), one shifted 58 kDa band was detected in the heart and three shifted bands (48, 69, 82 kDa) were detected in the liver (Fig. 5D). Since northern blot revealed only one species of mRNA in heart and liver tissues, as well as other tissues, these differences in molecular weight seem to be due to posttranslational modification.

The RECS1 KO mice were fertile with an expected Mendelian frequency and did not exhibit any obvious defects at the embryonic or postnatal stages or in adulthood. Significant alterations in body weight (data not shown), heart rate, total plasma cholesterol levels and triglyceride or glucose levels were not observed (Table

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**Fig. 7.** Inflammatory changes in association with CMD (cystic medial degeneration) were excluded as follows. (A) Serial paraffin-embedded cross sections were subjected to Alcian blue and H&E staining. Chronic inflammatory changes (such as lymphocyte accumulation) associated with mucoid deposition were not detected as judged by the H&E stain. The magnification is 320 ×. (B) The absence of lymphocytes and macrophages was confirmed by immunostaining of aortic frozen cross-sections with anti-CD3e and anti-Mac3 antibodies, respectively. The effectiveness of these antibodies in immunohistochemical analysis was confirmed by simultaneously staining murine thymus and lung specimens, respectively (insets). Arrowheads indicate large foci of CMD in a background of spread mucoid deposition as judged by the AB/PAS stain (not shown). The magnification is 400 ×. The photos are of one of 4–5 independent experiments.
Blood pressure measurements of the KO and WT mice did not differ when measured at 5–6 or 16–17 months of age by a noninvasive computerized tail-cuff method (Table 1).

We then made a thorough observation of the aortic tissues. The nRECS1 protein in the aorta of WT mice was also detected as a 34.4 kDa band, which is the predicted size (Fig. 6A). Overt thoracic aortic media degeneration was observed in aged (>14 months) KO mice compared with WT mice at the same age (Fig. 6B and 6C), as judged by Alcian Blue/Periodic Acid Schiff (AB/PAS) staining, which indicates acidic mucopolysaccharide deposition. These features have been called CMN (cystic medial necrosis) or cystic medial degeneration (Erdheim, 1929; Erdheim, 1930; Schlatmann and Becker, 1977). When the degree of mucoid deposition in the descending thoracic aorta of 3–4- and 14–17-month-old mice was evaluated, the aged KO mice showed statistically significant higher mucoid deposition compared with WT mice at the same age (Figure. 6B, P<0.05). However, no mucoid deposition was detected in the young KO and WT mice. In the aged KO mice, there was no obvious elastic fragmentation as judged by Elastic Van Gieson (EVG) staining (not shown). In some severely affected regions of the thoracic aortas of the aged KO mice, “cysts” were observed (not shown). These cysts are now believed to be artifacts caused by shrinkage during the preparation of the sections; consequently, only the mucoid degeneration of the media is commonly used to define CMD (Schlatmann and Becker, 1977).

The CMD lesions were not associated with inflammatory changes since there was no lymphocyte infiltration around the foci as judged by H&E staining or anti-lymphocyte immunostaining using anti-CD3e antibody (Fig. 7A and 7B). Moreover, macrophages were absent as determined by anti-macrophage immunostaining using an anti-Mac3 antibody (Fig. 7B).

**DISCUSSION**

The unique finding of the present study is that RECS1 KO mice are prone to cystic medial degeneration (CMD). The molecular mechanisms underlying CMD are currently largely unknown. Segura et al. have investigated the possible relationship between MMPs (matrix metalloproteinases) and CMD by analyzing thoracic aortic aneurysmal tissues from patients with Marfan’s Syndrome (Segura et al., 1998). They demonstrated that smooth muscle cells (SMCs) at the border of the CMD areas show stronger immunoreactivity for MMP-1, -2, -3 and -9 (particularly MMP-2 and -9) than in other regions. The authors also showed medial SMCs are the predominant sources of the elevated MMP levels in the aortic aneurysms and hypothesized that these upregulated MMPs progressively destroy the connective tissue, thereby contributing to the development of the CMD and aortic aneurysms that characterize Marfan’s Syndrome (Segura et al., 1998). Thus, further investigation of possible alterations of MMPs in RECS1 KO mice is particular intriguing.

As shown in Fig. 1, multiple conserved motifs were found in RECS1. The PPXY motif mediates protein-protein interactions between proline-rich peptide stretches and protein interaction modules, such as WW domains (Kay et al., 2000), while the PXXP motif represents potential binding sites for SH3 domain-containing proteins. Strack et al. (2000) looked for proteins harboring the P(T)/S/APPXY sequence through a SwissProt database search and identified the gap junction protein connexin 45, Gag proteins of rodent intracisternal A-type particles, and the NS3 protein of Broadhaven virus. Connexin 43 also harbors adjacent P(T)/S/AP and PPXY motifs in the cytoplasmic domain. In addition to RECS1 and LFG, we further found that the small integral membrane protein of the lysosome/late endosome (SIMPLE), also named LITAF/PIG7 (LPS-induced TNF-alpha factor/p53-induced gene 7) or EET-1 (estrogen inducible gene) (Moriwaki et al., 2001; Myokai et al., 1999; Polyak et al., 1997; Everett et al., 1997;), contains this motif. LITAF was found to be LPS inducible and plays a role in the activation of the TNF-α gene. Similar to RECS1 or LFG, in connexin 43, connexin 45 and the NS3 proteins, the P(T)/S/AP and PPXY overlapping motif or the adjacent P(T)/S/AP and PPXY motifs are located in the cytoplasmic domain. SIMPLE contains two PPXY motifs in its NH2-terminus and interacts with the WW domains of WWOX through the first PPXY motif (PY1) and the second motif (PY2) enhances the PY1-WW domain interaction (Ludes-Meyers et al., 2004). This PY1 motif, but not the PY2, also interacts with the WW domain of the ubiquitin-protein ligase NEDD4 (Jolliffe et al., 1996). The PY1 lies within the P(T)/S/APP(S/T)/YE region. Thus the P(T)/S/APP(S/T)/YE motif, which is conserved in RECS1, LFG, and SIMPLE (LITAF/PIG7/EET-1) may be an important element in protein-protein interactions.

In conclusion, we show here that knocking out RECS1 in mice induces susceptibility to cystic medial degeneration (CMD). RECS1 is thus an important molecule that plays protective roles in vascular remodeling. Why CMD lesions only observed in aged RECS1 KO mice and the underlying mechanisms need further investigation.

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