Molecular Pathogenesis of Genetic and Inherited Diseases

Emerin-Lacking Mice Show Minimal Motor and Cardiac Dysfunctions with Nuclear-Associated Vacuoles

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Emery-Dreifuss muscular dystrophy is an inherited muscular disorder clinically characterized by slowly progressive weakness affecting humero-peroneal muscles, early joint contractures, and cardiomyopathy with conduction block. The X-linked recessive form is caused by mutation in the *EMD* gene encoding an integral protein of the inner nuclear membrane, emerin. In this study, mutant mice lacking emerin were produced by insertion of a neomycin resistance gene into exon 6 of the coding gene. Tissues taken from mutant mice lacked emerin. The mutant mice displayed a normal growth rate indistinguishable from their littermates and were fertile. No marked muscle weakness or joint abnormalities were observed; however, rotarod test revealed altered motor coordination. Electrocardiography showed mild prolongation of atrioventricular conduction time in emerin-lacking male mice older than 40 weeks of age. Electron microscopic analysis of skeletal and cardiac muscles from emerin-lacking mice revealed small vacuoles, which mostly bordered the myonuclei. Our results suggest that emerin deficiency causes minimal motor and cardiac dysfunctions in mice with a structural fragility of myonuclei. (*Am J Pathol* 2006, 168:907–917; DOI: 10.2353/ajpath.2006.050564)

Emery-Dreifuss muscular dystrophy (EDMD) is an inherited disorder characterized clinically by the triad of 1) slowly progressive weakness and wasting that affects the humero-peroneal muscles in the early stages; 2) early contractures of the elbows, Achilles tendons, and post-cervical muscles; and 3) cardiomyopathy with conduc-

tion defects that would result in high-risk sudden death.^{1,2} Three forms of inheritance are known, X-linked recessive (X-EDMD), autosomal dominant (AD-EDMD), and autosomal recessive (AR-EDMD) forms.

X-EDMD is caused by mutations in the *EMD* gene on chromosome Xq28, which encodes the inner nuclear membrane protein emerin.^{3–5} Emerin has an N-terminal LEM domain and a single transmembrane domain at the C-terminus. The LEM domain is defined by an ~40-residue folded domain, which is also observed in the other inner nuclear membrane proteins of lamina-associated polypeptide 2 (LAP2) and MAN antigen 1 (MAN1). Emerin has been shown to bind several nuclear proteins including lamins,^{6–9} MAN1,¹⁰ a conserved chromatin protein of barrier-to-autointegration factor,¹¹ transcription repressors of germ cell-less¹² and Bcl-2-associated transcription factor,¹³ a splicing factor of YT521-B,¹⁴ giant actin binding proteins of nesprins,^{15,16} and nuclear actin.¹⁷ These interactions imply multiple functions of emerin including gene expression, nuclear assembly, cell cycles, and stabilization of nuclear envelope. Emerin is expressed in most human tissues, but it has yet to be elucidated why only limited tissues are affected in X-EDMD.

Autosomal forms of EDMD are caused by mutations in the *LMNA* gene encoding nuclear lamins A and C¹⁸ and are clinically indistinguishable from X-EDMD. Mutations in *LMNA* cause not only EDMD but a wide variety of human diseases including limb girdle muscular dystrophy type 1B,¹⁹ dilated cardiomyopathy with conduction defect,²⁰ lipodystrophy syndromes,^{21–23} hereditary neuropathy,²⁴ and progeria syndrome.^{25–28} It is still a mystery as to why mutations in *LMNA* can cause such a wide spectrum of disorders.²⁹

There have been four mouse models for lamin A/C diseases: a *LMNA* knockout (*Lmna*^{−/−}) and three knockin

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Figure 1. A: Targeting strategy to disrupt *Emd*. The schema of structure of the wild-type *Emd* (Wild) and that of the targeting vector (*Emd*⁻) is shown at the top. In the mutant allele, bovine growth hormone polyadenylation signals (bGHpA) and PGK-neo cassette (Neo^r) replace with exon 6. The schema of wild-type (Wild) and mutant (*Emd*⁻) proteins are described at the bottom. **B:** Southern blotting analysis of the *XhoI*- and *EcoRV*-digested genomic DNA from the wild-type and the chimeric mouse (Ch). Using a 5' probe, 17.7-kb and 11.5-kb hybridization bands correspond in size to homologous recombinant and the wild-type allele, respectively. The 3' probe hybridized ~13.5-kb wild-type and ~9.8-kb recombinant alleles.

mutants carrying a human AD-EDMD mutation (L530P, H222P, or N195K). *Lmna*^{-/-} mice display no overt abnormality at birth, but their growth rate is retarded. They develop skeletal and cardiac myopathies and die by 8 weeks of age.³⁰ Homozygous mutant mice carrying the L530P mutation have a reduction in their growth rate with symptoms that are consistent with progeria. These mice die by 4 weeks of age.³¹ Recently, mice with the H222P or N195K mutation have been reported to have muscular dystrophy and cardiomyopathy with conduction defects.^{32,33} Heterozygous mice with either mutation, however, are indistinguishable from wild-type littermates.

On the other hand, there has been no vertebrate animal model for emerin deficiency. It was therefore the main aim of this study to produce mice lacking emerin. The mouse emerin gene (*Emd*) is composed of six exons and encodes a serine-rich protein comprising 259 amino acids and is 73% identical to human emerin.³⁴ Mice were engineered to lack most of exon 6 of the gene, including the part encoding the transmembrane domain at the C-terminal end of emerin. The resulting emerin-lacking mice showed minimal motor and cardiac dysfunctions. Electron microscopic analysis of skeletal and cardiac muscles showed vacuoles mostly associated with myonuclei.

Materials and Methods

Production of Emerin-Deficient Mutant Mice

Genomic clones of *Emd* were isolated from a 129/SvJ library from Genome Systems (St. Louis, MO). Identities

of the isolated clones were confirmed by DNA sequencing. The targeting vector was designed to carry a deletion of 84 amino acids, including the transmembrane domain of emerin, by replacing 702 bp (3167 to 3868) with a bovine growth hormone polyadenylation (bGHpA) sequence and neomycin resistance gene (Neo) obtained from Genome Systems (Figure 1A). The targeting vector was transfected into 129/SvJ embryonic stem cells, and clones carrying the specific targeted locus were confirmed by Southern blotting. A total of 20 µg of genomic DNA from ES cell clones was digested with *XhoI* (for 5' and Neo probes) and *EcoRV* (for 3' probe) and processed for Southern blot analysis (Figure 1B). Using the 5' probe, hybridization bands of ~11.5 kb, which corresponded in size to wild-type allele, and >17.7-kb band, which corresponded to homologous recombinant allele, were detected. The 3' probe hybridized ~13.5-kb wild-type and ~9.8-kb recombinant alleles. Two recombinant embryonic stem cell lines were confirmed by 5', 3' and Neo probes and injected into C57/BL6 blastocysts. Five chimeras were obtained and crossed with C57/BL6 wild-type mice to obtain heterozygous mice. The genotypes of the mice were verified by polymerase chain reaction using primer sets for *Emd* (forward: 5'-CCTAATTATTCTGCAGGTGCG-3', reverse: 5'-AGGAAGAGTAACAGCTGGCC-3') and *Neo* (forward: 5'-GCTTGGGTGGAGAGGCTATTC-3', reverse: 5'-CAAGGTGAGATGACAGGAGATC-3'). The mutant mice were backcrossed with C57/BL6 wild-type mice for 10 generations. Wild-type littermates were used as controls. Animals were housed and all experimental procedures were performed in accor-

dance with the guidelines for the care and use of experimental animals at the National Institute of Neuroscience, National Center of Neurology and Psychiatry.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from skeletal muscles of emerin-lacking, heterozygous, and wild-type littermates at 5 and 46 weeks of age, by using a standard technique. Total RNA was also extracted from cardiac muscles from 5-week female littermates with each genotype. Quantitative RT-PCR was performed using iCycler (Bio-Rad Laboratories Inc., Richmond, CA) according to the manufacturing protocols. Primer sets for emerin (forward: 5'-GTTATTGACCACCAAGACATACGGG-3', reverse: 5'-GGTGATGGAAGGTATCAGCATCTACA-3') and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (forward: 5'-CTGGAGAAACCTGCCAAGTATG-3', reverse: 5'-TTGAAGTCGCAGGAGACAACCTG-3') were used. The values of mRNA for emerin were normalized to that of G3PDH.

Clinical Examinations

Each mouse was weighed every 2 weeks from 3 weeks of age. Muscle strength was evaluated by using the hanging basket test. Mice were hung on a 20 cm × 20 cm upsetting wire basket for 10 minutes, and the time to drop was noted. Rotarod balance test was performed to evaluate motor coordination under load using Rotarod Treadmill (Ugo Basile Biological). After practice, each mouse was forced to walk on the rotating rod maintained at a constant speed of 50 rpm/minute for 10 minutes. Each mouse was given three trials and the longest latency to fall down from the rod was recorded. Statistical analyses were performed using an unpaired Student's *t*-test. To determine the involvement of both the skeletal and cardiac muscles, mice were placed into a 50 cm × 40 cm × 20 cm plastic tank filled with water for 5 minutes and monitored for their swimming and recovery process.

Electrocardiogram

The Softron ECG Processor SP2000 (Softron Co., Japan) was used for electrocardiogram data analysis. Under ether anesthesia, mice underwent digitalized electrocardiogram measurement of lead I and lead II using subcutaneous needle electrodes. To avoid the possible influence of anesthesia, recording was started just before recovery, wherein the heart rate had returned to near normal level (more than 500 bpm). Electrocardiogram recordings were performed three times with 2-second durations each. The P-wave duration, PR interval, QRS complex duration, QT interval, and RR interval were calculated and averaged. Long lead recording for 1 minute was also performed and repeated three times to detect arrhythmias.

Histopathological Analysis of Muscle Samples

Muscles were taken from bilateral triceps, quadriceps femoris, hamstrings, anterior tibialis, gastrocnemius, paravertebral, diaphragm, and heart. Muscle samples were flash-frozen in isopentane and chilled with liquid nitrogen. Serial frozen sections (6 μ m) of skeletal and cardiac muscles were stained with a battery of histochemical reagents and immunostained with the following antibodies: anti-emerin (Novocastra Laboratory, Newcastle-upon-Tyne, UK), anti-lamin A and anti-lamin C.⁸ Sections of cardiac muscle were also stained with anti-connexin 40 (Zymed Laboratories Inc., South San Francisco, CA) and anti-connexin 43 (Zymed Laboratories Inc.). Alexa 488- or 568-labeled goat anti-rabbit or anti-mouse IgG (Molecular Probes, Eugene, OR) was used as secondary antibody.

Immunoblotting Analysis

Immunoblotting analysis was performed as previously described.³⁵ The density of the band corresponding to myosin heavy chain on postblotted sodium dodecyl sulfate-polyacrylamide gel was estimated, and the amount of skeletal muscle protein was normalized. Two emerin antibodies were used: a monoclonal anti-emerin (Novocastra) that was raised against N-terminal 222 amino acids for human emerin and polyclonal anti-emerin (FL-254; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) that was raised against human emerin corresponding to 3 to 254 amino acids. Rabbit antisera for lamin A and lamin C⁸ were also used. Histofine Simple Stain Max PO (Multi) (Nichirei Co., Japan) was used as secondary antibody. The immunoreactive bands on the membranes were visualized using the POD Immunostain Set (Wako Co., Japan). Similar amount of myosin heavy chain was detected in each lane.

Electron Microscopic Analysis

Muscle specimens were fixed in 2% glutaraldehyde in phosphate buffer and were kept in 0.1 mol/L calcium cacodylate at 4°C. After incubation in a mixture of 4% osmium tetroxide, 1.5% lanthanum nitrate, and 0.2 mol/L s-collidine buffer for 2 to 3 hours, samples were embedded in epoxy resin. Ultrathin sections (50 nm thick) were double-stained with uranyl acetate and lead citrate (Raynolds) and examined using a Hitachi H-7100 electron microscope. Cardiac muscles at 1, 21, 87, and 97 weeks of age and skeletal muscle at 78 weeks of age from emerin-lacking male mice were examined. Muscles from wild-type mice at 1 and 81 weeks of age were also examined.

Cell Culture

Skeletal and cardiac myocytes and skin fibroblasts from mice at 3 weeks of age were prepared by standard technique with minor modifications. Cells were incubated in Dulbecco's modified Eagle's medium-F12 supple-

Figure 2. **A:** Quantitative RT-PCR. The values of mRNA for emerin are normalized to that of G3PDH. Expression level of emerin mRNA from cardiac and skeletal muscles from hemizygous mutant male mouse (*Emd*^{-/-}) at 5 weeks of age are normal compared to wild-type male (wild) and heterozygous (*Emd*^{+/-}) female littermates. **B:** Double immunostaining of emerin (**a, b**) and lamin C (**c, d**) in muscles from wild-type (**a, c**) and emerin-lacking mice (**b, d**). Emerin is observed at the nuclear membrane in the wild-type mouse (**a**) but not detected in the mutant mouse (**b**). **C:** Immunoblotting analysis of emerin and lamin C in muscles from wild-type, heterozygous (*Emd*^{+/-}) and emerin-lacking male (*Emd*^{-/-}) and female (*Emd*^{-/-}) mice. A 38-kd emerin band is not detected in the mutant mice, but similar amounts of lamin C protein are noted. Scale bar, 50 μm.

mented with 20% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Immunocytochemistry was performed using anti-emerin (Santa Cruz Biotechnology, Inc.), anti-lamin A, and anti-lamin C,⁸ anti-lamin B2 (Novocastra Laboratories Ltd.) and anti-LAP2 (BD Transduction Laboratories, Lexington, KY) antibodies.

Results

Emd mutant mice were produced with a deletion of emerin's C-terminal 84 amino acids, which include the transmembrane domain. Male (*Emd*^{-/-}), and female (*Emd*^{+/-} and *Emd*^{-/-}) offspring with the mutant allele and wild-type littermates (*Emd*^{+/-} and *Emd*^{+/+}) were obtained and analyzed. Quantitative RT-PCR revealed normal expression levels of mRNA for emerin in both skeletal and cardiac muscles from mutant mice at 5 weeks of age (Figure 2A) and skeletal muscle at 46 weeks of age (data not shown).

Immunohistochemical analysis was performed using skeletal and cardiac muscles and brain. Emerin was present at nuclear membrane in tissues from wild-type mice; however, the immunoreaction was completely neg-

ative in *Emd*^{-/-} and *Emd*^{-/-} mice (Figure 2B). Positive/negative mosaic expression in heterozygous female mice was seen (data not shown). The expression and distribution of lamin A and lamin C showed no alteration in both skeletal and cardiac muscles and brain of the mutant mice.

On immunoblotting analysis, the two different anti-emerin antibodies detected a protein with an apparent molecular mass of 38 kd in muscles from wild-type mice, a protein that is larger than human emerin. This protein of 38 kd, which had been recognized by two different anti-emerin antibodies, was completely absent from muscles of *Emd*^{-/-} and *Emd*^{-/-} mice and noted to be reduced in muscles of *Emd*^{+/-} mice (Figure 2C). The amounts of lamin A (data not shown) and lamin C (Figure 2C) were the same in the wild-type and mutant mice.

Mice lacking emerin displayed similar growth rate and survival curve pattern when compared to their heterozygous and wild-type littermates (Figure 3). They were able to maintain sexual fertility and breed 10 generations of offsprings, as of the preparation of this article. Emerin-lacking mice were not observed to have waddling gait, scoliosis/kyphosis, or contractures as described in *Lmna*^{-/-} mice.³⁰ They showed no obvious muscle weak-

Body weight (g)

Figure 3. Growth curves of wild-type and emerlin-lacking mice. Time is given on the x axis and mean body weight on the y axis. No significant difference is observed between wild-type ($n = 13$) and mutant ($Emd^{-/-}$) ($n = 23$) male or between wild-type ($n = 12$), heterozygous ($Emd^{+/-}$) ($n = 17$), and mutant ($Emd^{-/-}$) ($n = 10$) female mice.

ness, and except for a few overweight mice, could suspend on a wire basket for more than 10 minutes, as observed in wild-type mice. Mice lacking emerlin could swim for more than 5 minutes in a water pool and recovered to an active state within 30 seconds. Although no apparent muscle weakness was seen, balance testing using rotarod revealed significant differences in motor function between emerlin-lacking, heterozygous, and wild-type mice. There was no difference between male and female mice. Only 43% of emerlin-lacking male mice, 30% of emerlin-lacking female mice, and 58% of heterozygous female mice were able to remain on the rotating rod for 10 minutes, whereas all wild-type littermates younger than 20 weeks of age could remain on the rotating rod for more than 10 minutes (Figure 4A). A greater number of younger (4 to 10 weeks) mutant mice was able to remain on the rotating rod longer than older (11 to 20 weeks) mutant mice. The time that the mutant mice could keep on the rod was quite variable from less than 1 minute to more than 10 minutes. The mean latency to drop down in the wild-type male, wild-type female, heterozygous female, emerlin-lacking male, and emerlin-lacking female mice were 600, 600, 394, 301, and 243 seconds, respectively.

Cardiomyopathy with conduction block is one of the major clinical features in human patients with EDMD. To investigate cardiac involvement in mice lacking emerlin, limb-lead electrocardiograms were recorded on 122 emerlin-lacking male, 75 emerlin-lacking female, 58 heterozygous female, 66 wild-type male, and 82 wild-type

female littermates from 3 to 100 weeks of age. The PR intervals in emerlin-lacking male mice tend to prolong with aging (Figure 5). The mean PR interval in emerlin-lacking male mice from 41 to 100 weeks of age was 42.5 ± 3.1 ($n = 44$), whereas that in wild-type littermates was 41.0 ± 2.1 ($n = 21$) ($P < 0.05$). No significant differences in PR intervals were seen between mice with any genotype younger than 40 weeks of age, and the mean PR intervals were 39.1 ± 3.4 and 38.8 ± 4.0 in wild-type and mutant mice, respectively. Interestingly in female mice, no significant difference in the mean PR interval from 41 to 100 weeks of age was observed between wild-type (41.7 ± 3.0 , $n = 26$), heterozygous (41.3 ± 0.4 , $n = 2$), and homozygous emerlin-lacking mice (40.0 ± 3.6 , $n = 26$). The PR:RR ratio was evaluated to avoid the influence of heart rate. Again, male mice were observed to have prolonged PR:RR intervals with aging (data not shown). The mean PR:RR ratio of wild-type male, emerlin-lacking male, wild-type female, heterozygous female, and emerlin-lacking female mice from 41 to 100 weeks of age were 0.37 ± 0.07 , 0.40 ± 0.05 , 0.41 ± 0.07 , 0.42 ± 0.05 , and 0.39 ± 0.05 , respectively. The P waves, QRS complexes, QT intervals, and RR intervals were comparable among the five groups. No arrhythmia was observed during the 3-minute recording.

Histological analysis of skeletal and cardiac muscles from emerlin-lacking mice and heterozygous female mice from 3 to 127 weeks of age did not reveal notable pathological changes compared to wild-type controls (Figure 6). The structural integrity of the myofibers was well pre-

(A)

	Wild ♂	<i>Emd</i> ^{-/-} ♂	Wild ♀	<i>Emd</i> ^{+/-} ♀	<i>Emd</i> ^{-/-} ♀
4-10w	10/10 100%	7/13 54%	7/7 100%	5/7 71%	5/16 31%
11-20w	10/10 100%	3/10 30%	9/9 100%	6/12 50%	6/21 29%
Total	20/20 100%	10/23 43%	16/16 100%	11/19 58%	11/37 30%

Figure 4. Result of rotarod test. **A:** The table shows number and ratio of mice that could walk on the rod for 10 minutes. All wild-type mice could complete the task but only 43% of emerlin-lacking male mice, 30% of emerlin-lacking female mice, and 58% of heterozygous female mice could complete the task. **B:** Average time that mice were able to remain on the rod was calculated from the same experiment as in **A**. Although some mutant mice could remain on the rod more than 10 minutes, others dropped within a minute. The mean time that mutant mice could remain on the rotating rod is significantly shorter than wild-type littermates (** $P < 0.01$, * $P < 0.05$). Older mutant mice were observed to have worse motor coordination, whereas no difference was seen among the wild-type mice regardless of age.

served with no necrotic or regenerating fibers noted. Skeletal muscles from the emerlin-lacking male mice had many tubular aggregates at 24 weeks of age or older, and the number and size of tubular aggregates increased with age (Figure 6B, arrow). However, wild-type male littermates were also noted to have tubular aggregates of similar number and size (Figure 6A, arrow). No tubular aggregates were seen in female mice even at 64 weeks of age (Figure 6, E and F). Tubular aggregates in muscles from both mutant and wild-type mice did not immunostain for anti-emerin antibodies (data not shown). Intramuscular peripheral nerves were well myelinated and showed no notable abnormality.

To investigate the cardiac conduction system, the expression and localization of connexin 40 and connexin 43 were examined by immunohistochemistry. A strong immunostaining reaction to connexin 40 had been observed in cells associated with the conduction system from the atrioventricular node to the Purkinje cells in hearts of both wild-type and mutant mice. Connexin 43 was located at the intercalated disks of the myocardium. The staining patterns and intensity for both connexin 40 and connexin 43 were indistinguishable in mutant and wild-type mice. Amounts of connexin 40 and connexin 43 detected on immunoblots were identical in cardiac mus-

cles from both wild-type and mutant mice (data not shown).

Toluidine blue staining of 0.5- μ m semithin sections of cardiac muscles from mice lacking emerlin revealed abundant vacuoles mostly bordering the myonuclei of both atrium and ventricle. The vacuoles were observed in the cardiac myocytes of the emerlin-deficient mice at 21, 87, and 97 weeks of age, but not in a 1-week-old mouse. The number of vacuoles was increased along with age. Electron microscopic observation revealed that some vacuoles were suggested to locate between the inner and outer nuclear membrane (Figure 7A). Other vacuoles, however, were close to the nucleus with distinct membrane from outer nuclear membranes (Figure 7B). Irregular shaped heterochromatin was also observed in some myonuclei that suggested nucleoplasm extrusion associated with disrupted nuclear membrane (Figure 7B, arrow). Further, a few nuclei contained unique circularly outlined structures of ~40 nm in diameter (Figure 7C). Many vacuoles were also observed in the transverse sections of skeletal muscle from an emerlin-lacking 78-week-old male mouse, especially at both edges of the myonuclei (Figure 7D, arrows). These vacuoles were barely detectable in the nuclei of intramuscular blood vessels. No vacuoles

Figure 5. The PR intervals of male (A, C) and female (B, D) mice showing dot blots (A, B) and bar graph (C, D). Emerin-lacking male mice (gray circles and gray bar) show prolonged PR time after 41 weeks of age, but female mice show no marked difference. The error bar shows the SD.

associated with myonuclei were seen in cardiac or skeletal muscles from 1- and 81-week-old male wild-type mice.

Primary cultured myocytes and fibroblasts from mice lacking emerin had similar growth rates and shapes

compared to cells from wild-type mice. Immunocytochemical analysis revealed that emerin was absent from the cells of mutant mice. Lamin A, lamin C, lamin B2, and LAP2 were present in emerin-lacking cells with no alteration in the distribution (data not shown). Nu-

Figure 6. H&E staining of skeletal (A, B, E, F) and cardiac (C, D, G, H) muscles from wild-type (A, C, E, G) and emerin-lacking mice (B, D, F, H) at the age of 46 weeks. No dystrophic changes were observed in the mutant mice muscles. Numerous tubular aggregates were seen in both wild-type (A) and emerin-lacking (B) male muscles (arrows). Scale bars, 50 μ m.

Figure 7. Electron microscopic observation of atrium from 87-week male mutant mouse (**A–C**), and skeletal muscle from 78-week male mutant mouse (**D**). **A:** A vacuole is located between inner and outer nuclear membranes. Higher magnification of the edge of the vacuole is shown in the **inset**. **B:** Another vacuole bordering on the nucleus has distinct membrane from outer nuclear membrane. Higher magnification is shown in the **inset**. Irregularly shaped heterochromatin suggests extrusion of nucleoplasm from disrupted nuclear membrane (**arrow**). **C:** Circular structures (**arrows**) are observed in a myonucleus from mutant mice. **D:** Toluidine blue staining of a semithin section of skeletal muscle from 78-week-old emerlin-lacking male mouse revealed abundant vacuoles mainly observed in both edges of myonuclei (**black arrows**). A **white arrow** shows tubular aggregates. Scale bars, 5 μm.

clear herniations that had been observed in fibroblasts from *Lmna*^{−/−} mice³⁰ were not seen in fibroblasts from mice lacking emerlin.

Discussion

Emerin is an integral protein of the inner nuclear membrane. Together with other inner nuclear membrane proteins such as LAP1, LAP2, lamin B receptor, and MAN1, emerlin is suggested to provide structural integrity to the nuclear envelope in addition to mediating attachment between heterochromatin and the inner nuclear membrane. However, the biological function of emerlin remains to be elucidated.

Mouse emerlin contains 259 amino acids and is slightly larger than its human ortholog, with four additional amino acids at the C-terminus.³⁴ Using anti-emerlin antibodies, a protein with an apparent molecular mass of ~38 kD, which is larger than 34-kD human emerlin, was detected in wild-type mouse muscles by immunoblotting of proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular mass of emerlin of both mouse and human is larger than its predicted size

from amino acid sequence, possibly attributable to post-translational modification because emerlin contains multiple possible phosphorylation sites and one glycosylation site.

Mutant mice were produced by creating a deletion of most of exon 6 in *Emd*. In the mutant mice, although mRNA levels were normal, a truncated protein was not detected by immunoblotting analysis by two different antibodies that can recognize N-terminal region of emerlin. In humans, several mutations were reported that cause deleted C-terminal transmembrane domain, and those mutations resulted in absence of the emerlin protein.^{3,36} Maniatis and colleagues³⁷ also reported a patient with an in-frame deletion of six amino acids from the C-terminal transmembrane region that caused almost complete absence of emerlin from muscle with no localization to the nuclear membrane, although the mRNA level was normal. Further, even if a truncated emerlin is expressed as observed in *in vitro* transfection studies, a lack of the transmembrane domain precludes the protein from being located at the nuclear envelope and thus, degraded rapidly, loses its biological function.^{6,38,39}

The emerlin-lacking mice showed mild clinical phenotypic expression. They did not have prominent muscle

weakness or joint contractures. However these mutant mice were shown to have motor impairment detected by using a complex locomotor test and were unable to remain on a rotating rod for a longer period of time when compared to the wild-type littermates. The reason for the diminished motor coordination among the mutant mice is unclear. The skeletal muscles did not show notable dystrophic changes, and intramuscular peripheral nerves were well myelinated. The role of abundant nuclear-associated vacuoles in motor impairment is unknown. Numerous tubular aggregates were observed in emerin-lacking male mice but also in wild-type male littermates. Tubular aggregates are thought to derive from the sarcoplasmic reticulum and have been observed in various human diseases including periodic paralysis.⁴⁰ Recently, emerin expression in tubular aggregates has been reported in human muscles.⁴¹ In mice, however, tubular aggregates can be seen in the type IIB glycolytic muscle fibers of normal male inbred mice,⁴² and emerin was not detected in the tubular aggregates in wild-type mice when muscles had been subjected to immunohistochemistry using anti-emerin antibodies. To clarify the result of the rotarod test, detailed analysis including nervous system is needed because emerin is ubiquitously expressed, including central and peripheral nervous system, and may have some roles for proper motor coordination in mouse.

Delayed conduction time is a notable clinical feature that had been detected in the emerin-lacking male mice. In human patients with EDMD, cardiomyopathy with conduction system defects is described to range from sinus bradycardia to prolongation of the PR interval to complete atrioventricular block, all of which poses major clinical problems. Cardiac pacemaker implantation is often needed to avoid life-threatening conduction block.⁴³ Our male mice lacking emerin did not have malignant cardiac arrhythmias but presented with mild prolongation of the PR intervals with aging, although these mice had no clinical symptoms and the life span was not different from wild-type littermates. Interestingly, female homozygous emerin-lacking mice did not have significant PR prolongation, despite the absence of emerin protein expression. Several mouse models have displayed sex-specific differences in phenotype, and male mice generally have a higher incidence of cardiac dysfunction than female mice. Mice carrying H222P-*Lmna* mutation also have marked sex-specific differences in dilated cardiomyopathy and abnormalities of the conduction system.³² The PR interval reflects the time required for excitation to transverse the atrium, atrioventricular node, and bundle of His. Components of the murine conduction system, including peripheral Purkinje fibers, are morphologically indistinguishable from surrounding cardiomyocytes, making routine histological analysis impossible. However, connexin 40 is a limited molecular marker mainly found in the atrium and conduction system. Connexin 40-null mice have first-degree atrioventricular block with associated bundle branch block.^{44,45} Connexin 43 is located in the atrial and ventricular myocardium and the distal parts of the conduction

system.⁴⁶ It is essential for maintaining the heart rhythm in postnatal life based on studies of null mutant mice.⁴⁷ Therefore the hearts of the present emerin-lacking mice were examined for expression of connexin 40 and connexin 43, but no detectable abnormalities were noted by immunohistochemical and immunoblotting analyses. Detailed pathological analysis is needed although it is difficult to identify myofibers associated with the conduction system by electron microscope. Although the mechanism remains unclear, the results in this study conclusively showed that emerin is important for normal function of the cardiac system.

Using electron microscope, we identified vacuoles mostly bordering the myonuclei in both atrium and ventricles from emerin-lacking mice but not in the wild-type littermates. The vacuoles were increased in number with age. Vacuoles were also observed in the emerin-lacking skeletal muscles and located mainly at both edges of myonuclei on the longitudinal sections. Interestingly, abundant vacuoles were surrounded by distinct membrane from nuclear membrane, although other vacuoles existed between the inner and outer nuclear membranes. Fragile nuclear envelope in emerin-deficient cells was also suggested from nuclei with possible extrusion of heterochromatin from disrupted nuclear membrane. Further we found unique circular structures contained in some myonuclei. These structures were similar to those reported in myonuclei in human X-EDMD patients.^{48,49} Detailed origin of the vacuolar membrane together with circular structures should be clarified; defect of emerin may cause a fragile membrane system associated with myonuclei.

In contrast to the human patients with X-EDMD, the emerin-lacking mice in this study show only mild symptoms with no dystrophic changes of muscles. This may be a result of interspecies differences in possible overlapping functions of inner nuclear membrane proteins. In *Caenorhabditis elegans*, down-regulation of emerin expression using RNA interference does not produce a detectable phenotype throughout development.⁵⁰ Therefore, in mice as well as *C. elegans*, other inner nuclear membrane protein(s) may compensate for the loss of function of emerin. This premise is strengthened by the observation in *C. elegans* showing that emerin and MAN1 have at least partially overlapping functions.⁵¹ Emerin-lacking mice produced in this study could provide precious information to elucidate the functional role of emerin and the pathophysiology in X-EDMD.

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