Deficiency of triad formation in developing skeletal muscle cells lacking junctophilin type 1

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Abstract **Junctophilins (JP-1, JP-2, and JP-3) are transmembrane proteins expressed in the junctional membrane complexes in excitable cells. Both JP-1 and JP-2 are co-expressed in the triads of skeletal muscle, but only JP-2 is expressed in cardiac muscle. We analyzed the roles played by JP-1 and JP-2 in triad formation in skeletal muscle by comparing developing skeletal muscles in wild-type and JP-1-knockout (KO) mice (both before and after birth). In the skeletal muscles of embryos, most of the couplings between sarcoplasmic reticulum (SR) and transverse tubule (T-tubule) were diads, with triads being very scarce. The number of triads increased markedly after birth in wild-type mice. However, there was no increase in the number of triads in the neonates of JP-1-KO mice, and they died within 1 day after birth. JP-2 expression was constant before and after birth, while expression of JP-1 increased with birth. Quantitative and morphological differences were not seen between wild-type and JP-1-KO mice in the formation of diads in the period just before the JP-1-KO mice died. The SR swelled and developed large vacuoles in skeletal muscle cells just before the JP-1-KO mice died. The present results strongly suggest that JP-1 and JP-2 play important roles in the formation of triads and diads, respectively, during the development of skeletal muscle in mouse.** **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Skeletal muscle; Development; Triad; Junctophilin; Knockout mouse

1. Introduction

The triad, a coupling between the sarcoplasmic reticulum (SR) and transverse tubule (T-tubule), is formed surrounding the myofibrils of skeletal muscle cells. Two types of calcium channels, the ryanodine receptor and the dihydropyridine receptor, are incorporated into the membranes of the SR and T-tubule, respectively, and both are involved in functional coupling at the triad. When depolarizations in the muscle cell membrane are transmitted to the ryanodine receptor via the dihydropyridine receptor, Ca^{2+} ions stored in the SR are discharged into the cytoplasm through the ryanodine receptor and trigger a contraction in muscle cells. Many previous studies have reviewed the details of the physiological functions of the triad in the regulation of the intracellular Ca^{2+} ion concentration and in excitation–contraction coupling in muscle cells (e.g. [1–6]). However, the molecular architecture of the triad and the mechanisms involved in triad formation are still unclear. To help clarify the latter in mammalian skeletal muscle cells, we previously isolated some membrane proteins (such as mitsugumin 29 and junctophilin) expressed in the triad and analyzed the functions performed by these proteins in triad formation and in excitation–contraction coupling [7–12]. Our previous studies suggested that mitsugumin 29 participates in the organization of the SR networks and T-tubules surrounding the myofibrils [8,13,14]. We have also suggested that junctophilin is involved in the formation of peripheral couplings in developing cardiac muscle cells [11] and in triad formation in developing skeletal muscle cells [12].

There are three tissue-specific junctophilin subtypes, designated JP-1, -2 and -3, and these are derived from distinct genes in mammalian genomes [11,15]. Both JP-1 and JP-2 are expressed in skeletal muscle, but only JP-2 is expressed in cardiac muscle. Mutant mice lacking either JP-1 (JP-1-KO) or JP-2 die without forming triads in their skeletal muscle cells or peripheral coupling structures in their cardiac muscle cells, respectively [11,12]. The junctophilin gene has been identified in nematodes as well as in mammalian muscle cells, and has been shown to play important roles in nematode muscle function [16].

In this study, we analyzed the roles played by JP-1 and JP-2 in the formation of the triad in developing skeletal muscle cells. The formation of triads and the expressions of JP-1 and JP-2 in developing skeletal muscle were compared between wild-type and JP-1-KO mice.

2. Materials and methods

2.1. Animals

JP-1-KO mice were generated as described in our previous paper [12]. JP-1-KO and wild-type mice were obtained by crossing the heterozygous mutants.

2.2. Electron microscopy

Hindlimb, digastric, and diaphragm muscles obtained from embryos at embryonic day 14 (E14) through to neonates at day 3 after birth (P3) were examined. Skeletal muscles of the femoral region were examined in the hindlimb. Only hindlimb muscle was examined in the wild-type mice at E14. The skeletal muscles from embryos and neonates of JP-1-KO and wild-type mice were fixed in 3% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M cacodylate buffer (pH 7.4). After washing with the buffer solution, they were post-fixed in 1% OsO_4 and 0.1 M cacodylate buffer (pH 7.4). Then, they were washed with the buffer solution, dehydrated using alcohol and acetone, and embedded in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate. These sections were examined under the electron microscope (JEM-200CX or JEM-1010; JEOL, Japan).

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2.3. Immunoblot analysis

Total microsomal proteins were prepared from the skeletal muscles of hindlimb, and subjected to immunoblot analysis as described previously [11,12]. As shown in a previous report [12], expression of JP-1 was at a low level from E14 to neonates at P7. Therefore, we enhanced the sensitivity of the immunoblot to show expressed JP-1.

2.4. Immunohistochemistry

Skeletal muscle of the femoral region was dissected from the hindlimb of embryos at E14 and neonates just after birth (P0) and at P1. The tissues were fixed in cold acetone for 1 h, embedded in OCT compound (Tissue Tek, CA, USA), and quick-frozen in 2-methylbutane, which was cooled in liquid nitrogen. Cryosections (10–20 μm) were first labeled with antibodies for JP-1 and JP-2 [11], and then subjected to secondary labeling with FITC- or rhodamine-conjugated antibodies. The non-immune serum was used as a primary antibody for control staining. The sections were examined using a photomicroscope (BX60; Olympus, Tokyo, Japan) equipped with a BX-FLA, and photographed using a cooled CCD camera (SV-16E; Koheisha, Saitama, Japan).

2.5. Measurements and statistical analysis

The numbers of A-I junctions, diads, and triads were counted in electron micrographs (×27 000). The significance of differences between groups was determined using analysis of variance and Student's *t*-test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Electron microscopy

In hindlimb muscle from E14 wild-type mice, SR was seen around the myofibrils, and it was attached to the myofibrils at the Z bands. A small number of diads was identified near the myofibrils. The observed couplings between SR and T-tubule involved only diads, no triad being identified at this stage (data not shown).

In wild-type mice at E17, many couplings between SR and T-tubule were observed around the myofibrils, and these were seen equally in the hindlimb, digastric, and diaphragm muscles (Fig. 1A). Most of the couplings between SR and T-tubule were still diads, and they were distributed at regular intervals in the surroundings of the myofibrils. However, the locations of the diads and triads did not always correspond to the region of the A-I junction. The SR and T-tubule were of large diameter in the region of the diads and triads. In the hindlimb, digastric, and diaphragm muscles, a clear morphological difference was not seen in the intracellular organelles (including diads and triads) between wild-type and JP-1-KO mice (Fig. 1B).

In wild-type neonates at P0, many diads and triads were observed at the positions of the A-I junctions in the myofibrils. The SR and T-tubules of both diads and triads were smaller in diameter than those seen at E17. A clear morphological difference was not seen in intracellular organelles between wild-type and JP-1-KO mice.

In wild-type neonates at P1, the SR and T-tubules were of small diameter, and looked like those seen in adult mice. Most

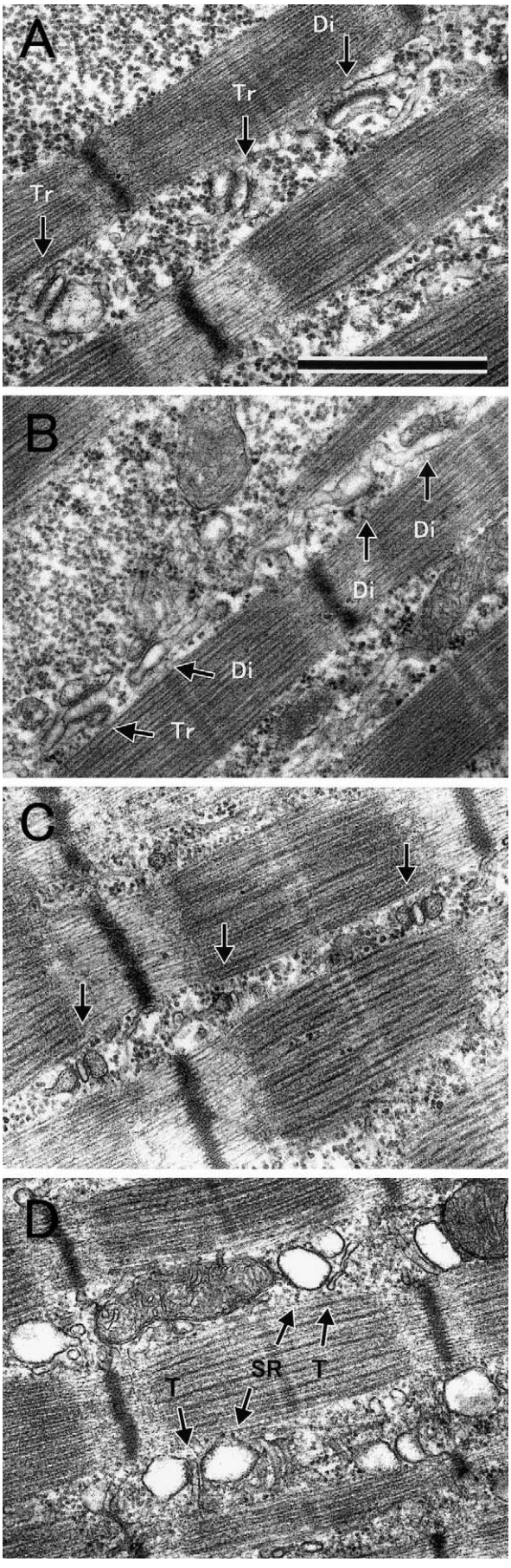

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Fig. 1. Electron micrographs showing development of diads and triads in the skeletal muscle of the hindlimb. A,B: Muscle cells of a wild-type and a JP-1-KO embryo, respectively, at E17. Arrows indicate diad (Di) and triad (Tr) among the myofibrils. Bar indicates 1 μm. C: Muscle cell of a wild-type neonate at P1. Arrows indicate triads adjacent to the A-I junction regions of myofibrils. D: Muscle cell of a JP-1-KO neonate just before death. Arrows indicated vacuolated SR (SR) and T-tubule (T).

of the diads and triads were distributed at the positions occupied by the A-I junctions (Fig. 1C). The JP-1-KO mice died within 1 day after birth. Therefore, for our neonatal data we examined the skeletal muscles of JP-1-KO mice just before death (neonates at 12–17 h after birth) as P1 in this study. In the JP-1-KO mice at P1, the SR was swollen in all skeletal muscles examined (Fig. 1D). The swelling of the SR was especially marked in the region of the terminal cisterna in both the triads and diads. However, such swelling was not observed in the T-tubules.

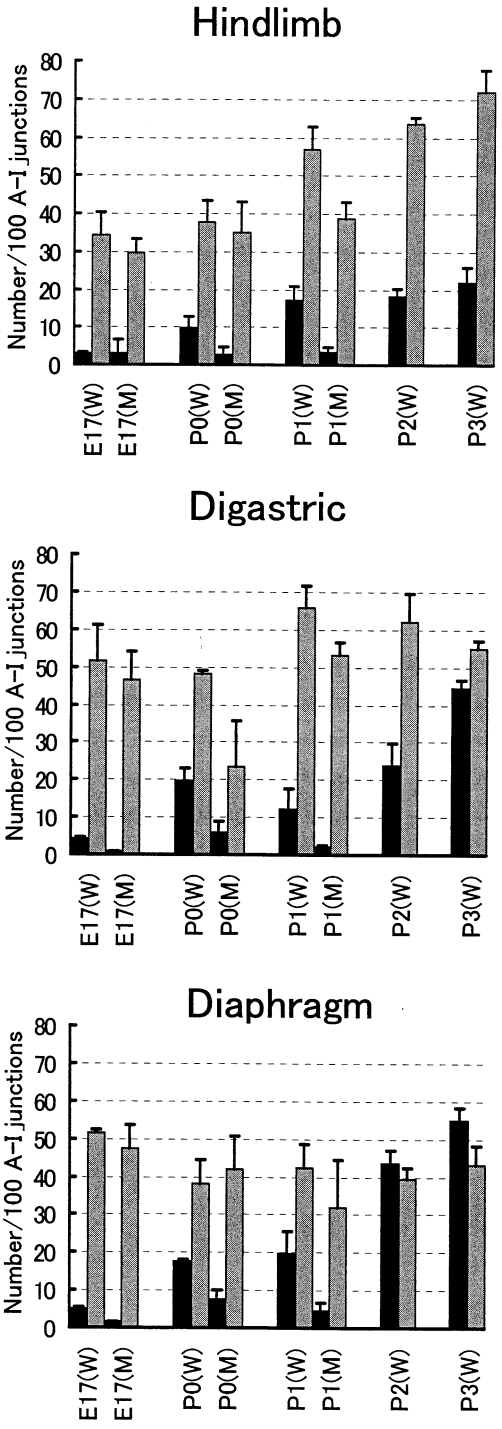

Fig. 2. Appearance of diads and triads in skeletal muscle cells before and after birth. Three mice were examined for both wild-type (W) and JP-1-KO (M) mice at each stage (except digastric muscle of P1 (W) and P0 (M), for which six neonates were examined because the individual variation was larger in these cases). An area including 1239 A-I junctions (on average) was examined in each mouse, and the numbers of diads and triads were counted in this area. Gray and black bars show, respectively, the numbers of diads and triads per 100 A-I junctions. Data are presented as mean ± S.D.

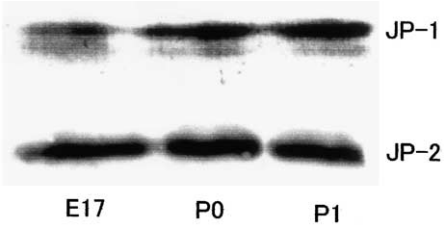

Fig. 3. Western blotting showing expressions of JP-1 and JP-2. Total microsomes prepared from hindlimb muscle of wild-type mice were analyzed using antibodies specific to JP-1 or JP-2.

The developmental changes in the numbers of diads and triads are shown in Fig. 2. There was no clear difference in the number of diads between wild-type and JP-1-KO mice. Although the number of triads increased markedly with birth in wild-type mice, such an increase in the number of triads was not seen in JP-1-KO mice.

3.2. *Western blot analysis of JP-1 and JP-2 in developing skeletal muscle in wild-type mice*

A large amount of JP-2 was expressed fairly constantly in hindlimb muscle from E17 to P1. In contrast, the expression of JP-1 was at a very low level compared to JP-2 during these stages. The results after sensitization show the amount of JP-1 increased gradually from E17 to P1 (Fig. 3).

3.3. *Immunohistochemical analysis of JP-1 and JP-2 in developing skeletal muscle*

To show the deficiency of JP-1, skeletal muscle cells of JP-1-KO mice at P0 were double-immunolabeled with antibodies against JP-1 and JP-2. No labeling for JP-1 was shown though labeling for JP-2 was clearly shown as periodically arranged stripes across muscle cells (Fig. 4A,B). Appearance and distribution of JP-1 and JP-2 in developing skeletal muscle cells were examined in the hindlimb of wild-type mice. In muscle cells of E14 mice, only weak labeling was seen for JP-1 antibody (Fig. 4C). On the other hand, periodic patches (or straps) across muscle cells were clearly labeled for JP-2 antibody (Fig. 4D). In muscle cells of E17 mice, labeling of clear stripes for JP-1 and JP-2 was seen, and they crossed the muscle cells at regular intervals (Fig. 4E,F). In muscle cells of P1 mice, stripes in pairs crossing the muscle cells were clearly observed for both JP-1 and JP-2 (Fig. 4G,H), a pattern of labeling for JP-1 and JP-2 the same as that seen in the mature skeletal muscle of adult mice.

4. Discussion

In the developing skeletal muscle of wild-type mice, the formation of the T-tubules and coupling between SR and T-tubule begins at E15–E16 [17]. A spontaneous twitching of skeletal muscle appears at E15, coincidental with the formation of couplings between SR and T-tubule [18]. The present electron microscopy showed that most of the couplings between SR and T-tubule involved diads in the skeletal

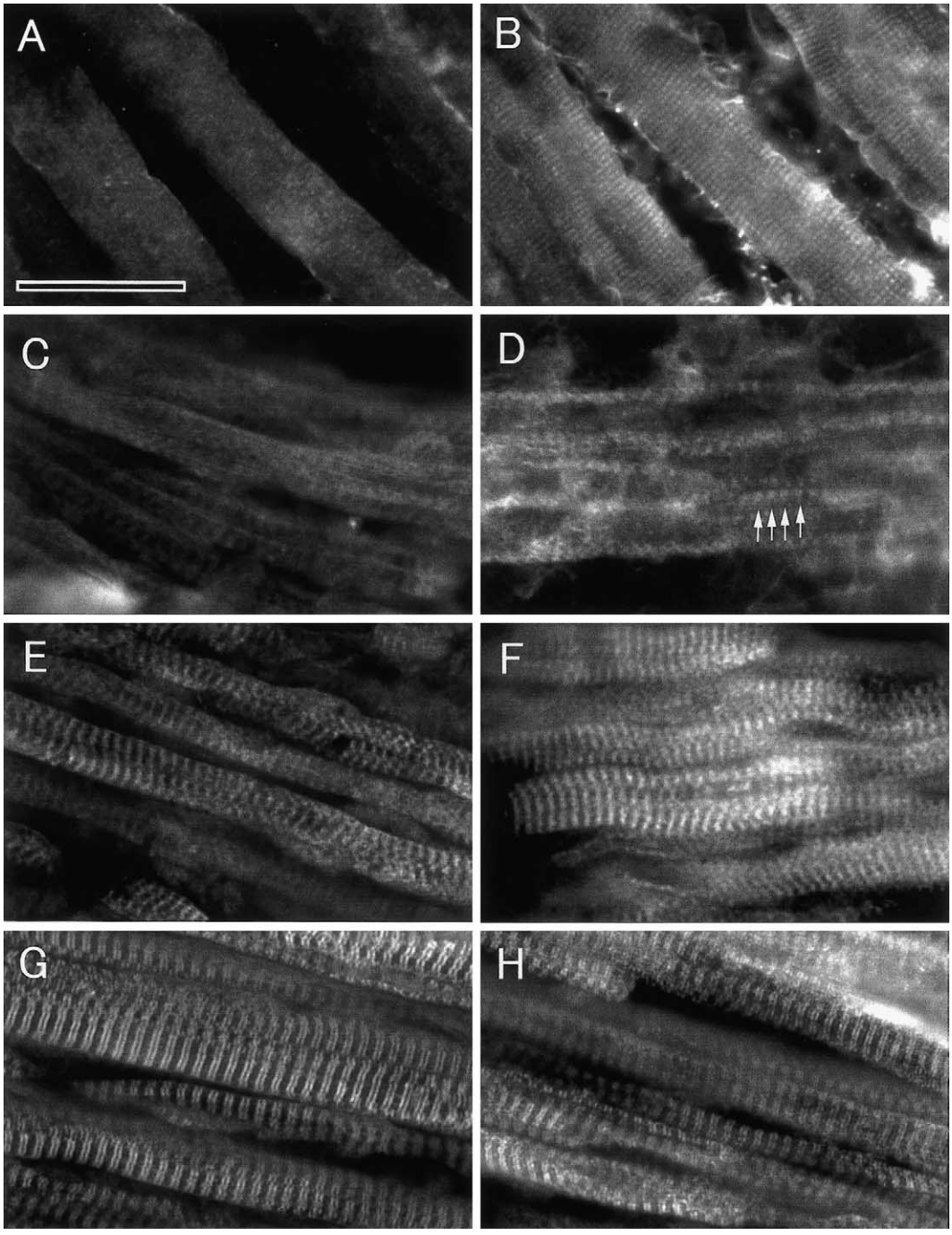

Fig. 4. Appearance and distribution of JP-1 and JP-2 in developing skeletal muscle of wild-type mice. Immunofluorescence labeling for JP-1 and JP-2 is shown in hindlimb muscle. A,B: Results of double immunofluorescence labeling with antibodies to JP-1 and JP-2, respectively, in muscle cells of JP-1-KO mouse at P0. No labeling for JP-1 (A) was observed though labeling for JP-2 (B) is clearly shown as periodically arranged transverse bands across muscle cells. C,E,G: Labeling for JP-1. D,F,H: Labeling for JP-2. C,D: Hindlimb muscles at E14. Labeling for JP-2 (D) appears clearly as patches (arrows) seen periodically across muscle cells, but labeling for JP-1 (C) could not be observed clearly. E,F: Hindlimb muscles at E17. Labeling for JP-1 (E) and JP-2 (F) is clearly shown as periodically arranged transverse bands across muscle cells. G,H: Hindlimb muscles at P1. Two lines in pairs were clearly observed to form the cross-striation in muscle cells in the case of labeling for both JP-1 (G) and JP-2 (H) at P1. Bar indicates 30 μm.

muscle cells of embryos and neonates. There was no clear difference in the number of diads between wild-type and JP-1-KO mice before and after birth. The number of triads in the skeletal muscle cells of wild-type increased rapidly with birth. However, increase in the number of triads after birth was deficient in JP-1-KO mice. In our previous study, we found that expression of JP-1 could be clearly detected in skeletal muscle of mice 14 days after birth, although expression of JP-2 was clearly detectable at E17 [12]. The present detailed examination makes clear that the expression of JP-1 increases with birth. Immunohistochemistry showed that clear labeling for JP-2 antibody could be seen in the muscle of hindlimb at E14, when the formation of diads begins. On the other hand, clear labeling was seen for JP-1 antibody at E17, when the

formation of triads begins. These facts strongly suggest that JP-2 and JP-1 play important parts in the formation of diads and triads, respectively, during the early development of skeletal muscle.

In cardiac muscle cells, only JP-2 is expressed, and the coupling between SR and T-tubule involves diads. The present results show that JP-1-KO mice are as able to form diads as wild-type mice. This implies that formation of diads in skeletal muscle can be accomplished when only JP-2 is expressed. On the other hand, the JP-1-KO mice lacked the increased number of triads after birth seen in wild-type mice. Possibly, expression of JP-1 in skeletal muscle is necessary and indispensable for triad formation after birth. From the present results, however, we cannot tell whether triads can be formed with only a contribution from JP-1.

In the skeletal muscle cells of JP-1-KO mice just before death, the SR of the terminal cisterna was very swollen. Such vacuolated SR has been demonstrated in the skeletal muscle cells of mutant mice lacking both ryanodine receptor type 1 and type 3 [19], and in cardiac muscle cells of mutant mice lacking ryanodine receptor type 2 [20]. In these mice, an abnormality in Ca^{2+} regulation in the SR has been suggested as a cause of the swelling. In the present JP-1-KO mice, the vacuolation of the SR may conceivably have been induced by an abnormal Ca^{2+} regulation in the SR due to a deficiency in triad formation.

The number of triads increased after birth more markedly in the digastric and diaphragm muscles than in the hindlimb muscles. This may be a reflection of the relative importance of the first two muscles in life soon after birth (for instance, in breathing and sucking). Therefore, if triad formation is obstructed after birth due to a lack of expression of JP-1, the function of these important skeletal muscles may be defective. As a result, breathing and sucking may be deficient or absent, and individuals will die soon after birth.

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References

[1] Schneider, M.F. (1994) Annu. Rev. Physiol. 56, 463–484.
[2] Sutko, J.L. and Airey, J.A. (1996) Physiol. Rev. 76, 1027–1071.
[3] Franzini-Armstrong, C. and Protasi, F. (1997) Physiol. Rev. 77, 699–729.
[4] Shoshan-Barmatz, V. and Ashley, R.H. (1998) Rev. Cytol. 183, 185–270.
[5] Mackrill, J.J. (1999) Biochem. J. 337, 345–361.
[6] Berchtold, M.W., Brinkmeier, H. and Muntener, M. (2000) Physiol. Rev. 80, 1215–1265.
[7] Nishi, M., Komazaki, S., Iino, M., Nakagawa, K. and Takeshima, H. (1998) FEBS Lett. 432, 191–196.
[8] Nishi, M., Komazaki, S., Kurebayashi, N., Ogawa, Y., Noda, T., Iino, M. and Takeshima, H. (1999) J. Cell Biol. 147, 1473–1480.
[9] Shimuta, M., Komazaki, S., Nishi, M., Iino, M., Nakagawara, K. and Takeshima, H. (1998) FEBS Lett. 431, 263–267.
[10] Takeshima, H., Shimuta, M., Komazaki, S., Ohmi, K., Nishi, M., Iino, M. and Kangawa, K. (1998) Biochem. J. 331, 317–322.
[11] Takeshima, H., Komazaki, S., Nishi, M., Iino, M. and Kangawa, K. (2000) Mol. Cell 6, 317–322.
[12] Ito, K., Komazaki, S., Sasamoto, K., Yoshida, M., Nishi, M., Kitamura, K. and Takeshima, H. (2001) J. Cell Biol. 154, 1059–1067.
[13] Komazaki, S., Nishi, M., Kangawa, K. and Takeshima, H. (1999) Dev. Dyn. 215, 87–95.
[14] Komazaki, S., Nishi, N., Takeshima, H. and Nakamura, H. (2001) Dev. Growth Differ. 43, 717–723.
[15] Nishi, M., Mizushima, A., Nakagawara, K. and Takeshima, H. (2000) Biochem. Biophys. Res. Commun. 273, 920–927.
[16] Yoshida, M., Sugimoto, A., Ohshima, Y. and Takeshima, H. (2001) Biochem. Biophys. Res. Commun. 289, 234–239.
[17] Takekura, H., Flucher, B.E. and Franzini-Armstrong, C. (2001) Dev. Biol. 239, 204–214.
[18] Chaudhari, N. and Beam, K.G. mRNA for cardiac calcium channel is expressed during development of skeletal muscle, (1993) Dev. Biol. 155, 507–515.
[19] Ikemoto, T., Komazaki, S., Takeshima, H., Nishi, M., Noda, T., Iino, M. and Endo, M. (1997) J. Physiol. 501, 305–312.
[20] Takeshima, H., Komazaki, S., Hirose, K., Nishi, M., Noda, T. and Iino, M. (1998) EMBO J. 17, 3306–3316.