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Which skeletal myoblasts and how to be transplanted for cardiac repair? ☆

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Abstract

Clinical efficacy of skeletal myoblast (skMb) transplantation is controversial whether this treatment produces beneficial outcome in patients with dilated cardiomyopathy (DCM). Based on immunological tolerance between wild-type and DCM hamsters with the deletion of δ-sarcoglycan (SG) gene, skMb engraftment in TO-2 myocardium (3×10^5 cells in ~100 mg heart) was verified by the donor-specific expression of δ-SG transgene constitutively produced throughout myogenesis. At 5 weeks after the transplantation, the cell rates expressing fast-myosin heavy chain (MHC) exceeded slow-MHC in δ-SG^+ cells. Fifteen weeks after (corresponding to ~12 years in humans), fast MHC^+ cells nullified, but the δ-SG^+ and slow MHC^+ cell number remained unaltered. These skMbs fused with host cardiomyocytes *via* connexin-43 and intercalated disc, modestly improving the hemodynamics without arrhythmia, when engrafted skMbs were sparsely disseminated in autopsied myocardium. These results provide us evidence that disseminating delivery of slow-MHC^+ myoblasts is promising for repairing DCM heart using histocompatible skeletal myoblasts in future.

Keywords: Cell transplantation; Skeletal myoblast; Fast-twitch muscle; Slow-twitch muscle; Dilated cardiomyopathy; δ-Sarcoglycan; Myosin heavy chain; Connexin-43; Intercalated disc; Fusion

DCM accounts for approximately one-half of clinical cases with advanced heart failure (AdHF). In spite of the steady progress in both basic and clinical researches, the prognosis of patients with DCM is still poor [1]. Cardiac transplantation is so far the most life-saving treatment of AdHF, though various sociomedical problems exist intrinsic to this treatment. Skeletal myoblast (skMb) transplantation has now reached a novel strategy for improving cardiac dysfunction secondary to ischemic injury [2–5], leaving clinical argument of arrhythmogenic substrate [6] or equivocal outcome in randomized and placebo-controlled study in MAGIC trial [7].

When a responsible gene causing DCM is identified, gene-targeted therapy using a long-lasting and non-harmful vector is one of the most promising options for preventing the progression from moderate cardiac dysfunction to AdHF [8]. This treatment might be less effective for restoring the necrotic cardiomyocytes. Though DCM is non-coronarogenic, the final pathway to AdHF would be common to ischemic heart disease [9–11]. SkMb transplantation

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after the gene normalization *ex vivo* would have the potential for supplementing adaptable cells in DCM heart where the original cardiomyocytes are already lost. TO-2 strain hamster is suitable for the study of human DCM, because the δ-SG gene is commonly deleted in both species with DCM [12–14] and similar clinical features are progressively shown including AdHF and sudden death [8,9,14].

Most studies on the cell-transplantation have employed immunosuppression procedures or immunodeficient animals to avoid rejection provoked in the recipients. As presented by δ-SG gene knockout mice, X-ray irradiation increased the number of donor-derived nuclei without advantage for the transgene expression [15]. Present study was focused following fundamental problems; (1) identification of promising cells among skMbs, (2) their efficient delivery and engraftment, using immunologically tolerant allografts from normal control to DCM hearts and (3) evaluation of physiological features of the engrafted heart after the short- and long-term follow-up.

Materials and methods

Because the space for this publication is limited, we shifted all sections for Experimental procedures to Supplementary Material. All statistical values are expressed by means ± SEM. Multiple groups were compared by one-way ANOVA followed by Bonferroni's post hoc test. *P* value below 0.05 was considered statistically significant.

Results and discussion

As a source of progenitor cells for cardiac muscle repair, a wide variety of stem cells have been reported, including skMb, cardiomyoblast, adipose tissue, and bone marrow cells, but it is still controversial whether the non-myogenic cells actually transdifferentiate to cardiomyocytes [16–18]. Considering clinical setting in the treatment of AdHF, all of these cells should be evaluated with great care to assure both their safety and efficacy. Selection of soleus muscle in the pioneer work by Taylor et al. was very appropriate for the skMb transplantation, because slow-twitch muscle fibers are preferentially present in this muscle [2]. When the life-threatening arrhythmias are avoided [5,6], skMb might be the most promising for the clinical application among these candidate tissues, because human studies have already shown a clear improvement of cardiac failure after myocardial infarction [3] and successful engraftment within a scar [4]. Other progenitor cells are attractive but still remain at an experimental stage [19]. The cause of discrepancy between two phase I studies [20,21] on intracoronary injection trial of bone marrow-derived progenitor cells in acute myocardial infarction may be explained by the heterogeneity of administered cells.

Furthermore, endogenous δ-SG at the sarcolemma (SL) might be reliable for identifying the engrafted skMb and its maturation, because of the three reasons listed below; (1) δ-SG exclusively exists in muscle cells, but not in other contaminant cells [22,23], (2) Among all SGs, δ-SG is exceptionally expressed throughout myogenesis from myoblast to myocyte [24] and (3) δ-SG is the most resistant to hydrolysis by an endogenous protease, m-calpain, which degrades other SGs as well as dystrophin *in vitro* [10]. Unique characters of m-calpain are compatible with recent scheme that the loss of component of SG proteins may be caused by the activated m-calpain secondary to pathogenic conditions [11,12,23,25]. All of these settings are associated with the disruption of dystrophin and increment of the SL permeability, resulting in the vicious cycle of cardiac muscle degeneration in failing hearts in various animal models and humans [9].

Time-dependent expression of fast- and slow-twitch MHCs in DCM heart

Preliminary study with skin grafting indicated an immunological tolerance between the control and TO-2 strain hamsters (Tezuka et al., in press). At 5 weeks after the skMb transplantation, double fluorescence microscopy revealed that ~90% of the engrafted cells identified by δ-SG expression on the SL (Fig. 1A) coexhibited prelabeled $DAPI^+$ nuclei beneath the SL (pink arrows in Fig. 1B) or at the center of myoplasm (pink arrowheads in Fig. 1B). They showed an alternative staining for fast-twitch MHC (red arrowheads in Fig. 1C) or slow-twitch MHC (blue arrows in Fig. 1D) in the adjacent serial sections. Because amino acid sequence of slow-twitch MHC is the same as β cardiac MHC (26), immunological identification using MHC antibodies was difficult to discriminate transfected slow-muscle fibers from the recipient cardiomyocytes.

We employed another marker, δ-SGs as donor-derived cells. Those cells presenting fast-twitch isoform of MHC were more abundant than those presenting slow-twitch MHC (blue arrows in Fig. 1D). Some cells (~10%) were stained for both the slow and fast isoforms of MHC (yellow arrows in Figs. 1C, D). Quadriceps femoris muscle from which the skMb allografts were prepared showed δ-SG on the SL (data not shown) and demonstrated an alternative expression of fast- or slow- twitch MHC. No cells were co-stained with both fast- and slow-twitch MHC antibodies. The predominant expression of fast-twitch MHC after the engraftment was similar to original constituent of MHC isoform in normal donor muscle.

At 15 weeks, the population of fast- and slow-twitch MHC^+ cells was reversed. Most cells (~80%) that were intensely stained with anti-δ-SG antibody (yellow arrows in Fig. 1E) or weakly stained (yellow arrowheads in Fig. 1E) matched with pre-labeled $DAPI^+$ nuclei of the engrafted cells beneath the SL (pink arrows in Fig. 1F) or at the center of myoplasm (pink arrowheads in Fig. 1F). The cells with an intense staining for δ-SG (yellow arrowheads in Fig. 1E) expressed slow- twitch MHC (blue arrows in Fig. 1H) and the rest shrunk cells with a weak stain for δ-SG (yellow arrowheads in Fig. 1E) revealed co-expression of both fast- and slow MHCs (blue arrowheads in Fig. 1G, Fig. 2H). The cells exclusively demonstrating fast-twitch MHC were not detected.

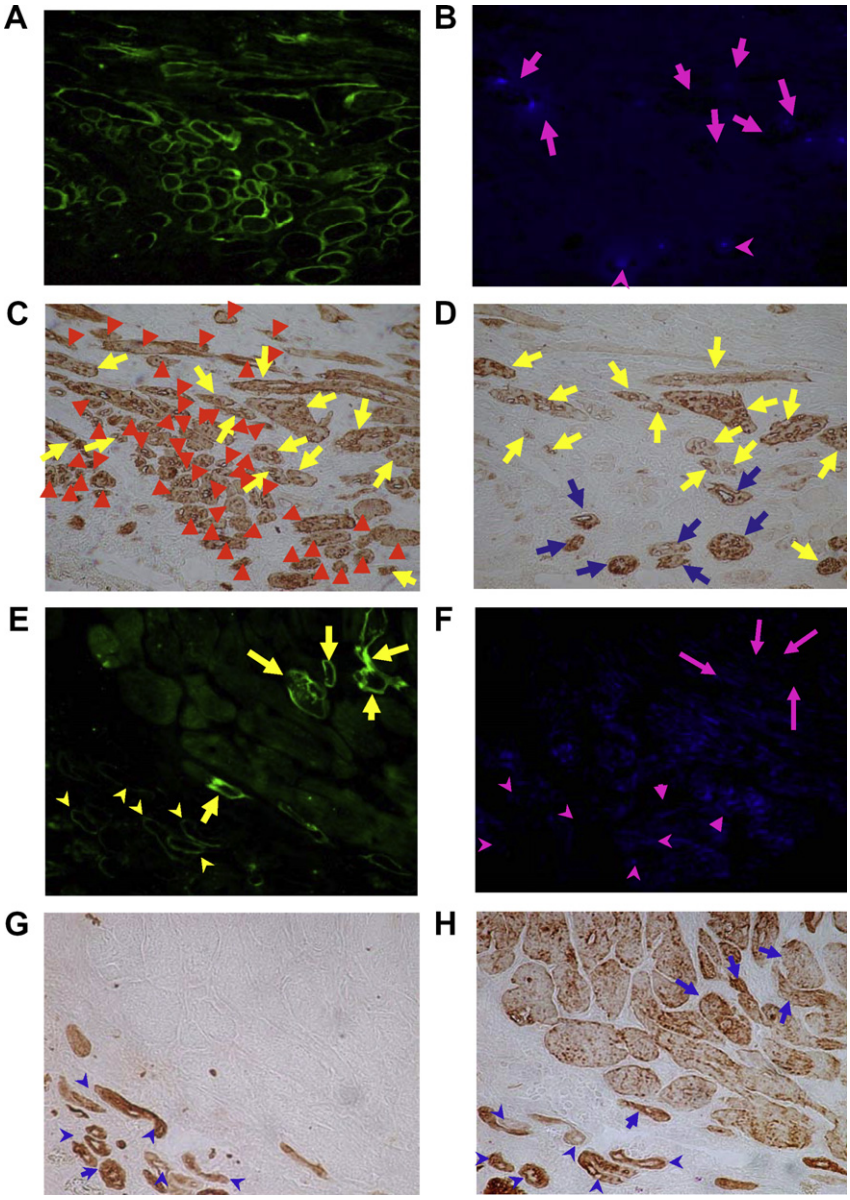

Fig. 1. Histological evaluation of engrafted skMbs in TO-2 heart at 5 weeks (A–D) or 15 weeks (E–H) after the cell transplantation. Double fluorescence microscopy of FITC-labeled antibody to δ-SG (A) or DAPI-prelabeled nuclei (B) of the donor cells shows the predominant expression of fast-twitch MHC (orange arrowheads in C, G), compared with slow-twitch MHC (D,H). Note that some cells expressed both fast-twitch and slow-twitch MHC (yellow arrows in C and D). Nuclei of engrafted skMbs with intense staining along SL (yellow arrows in E) and weak staining (yellow arrowheads in E) were situated at the center (pink arrows in F) and the subsarcolemma (pink arrowheads in F) of engrafted cells, respectively. Blue arrows and arrowheads in G or H denote the engrafted skMbs expressing slow-twitch MHC alone and both fast- and slow-twitch MHC, respectively. Original magnification 100×, and bar length indicates 100 μm.

The fast-twitch MHC in δ-SG$^+$ cells occupied $66.0 \pm 7.1\%$ at 5 weeks and decreased to $1.3 \pm 1.0\%$ at 15 weeks (Fig. 2A, $p < 0.01$). In contrast, the expression level of the slow-twitch MHC shared $23.0 \pm 1.1\%$ of δ-SG$^+$ cells at 5 weeks and enhanced to $80.0 \pm 9.0\%$ at 15 weeks ($p < 0.01$). Within 10-week interval, the estimated cell number of fast-MHC$^+$ cells drastically reduced in the whole heart (Fig. 2B), while Cx-43$^+$ cells slightly but significantly increased ($p < 0.05$). It should be intensified that the number of slow-twitch MHC$^+$ and δ-SG$^+$ cells remained unchanged between 5 and 15 weeks after the cell transplantation ($27.3 \pm 9.7 \times 10^3$ *vs.* $18.2 \pm 5.0 \times 10^3$, respectively).

The time-dependency of cell population was confirmed by the Western blotting of fast-twitch MHC protein. Compared with the trace expression in cultured skMbs (Fig. 2C, lane s), the protein density corresponding to the fast-twitch MHC increased 5.99 ± 1.58-fold (Fig. 2C, lanes a–d, $n = 4$) at 5 weeks after the cell transplantation, suggesting the differentiation from skMbs to matured fast-twitch myocytes and their proliferation in the host hearts. Considering the total amount of protein in whole left ventricular muscle and skMbs, enormous amount of fast-twitch MHC was estimated to be synthesized after the cell-transplantation.

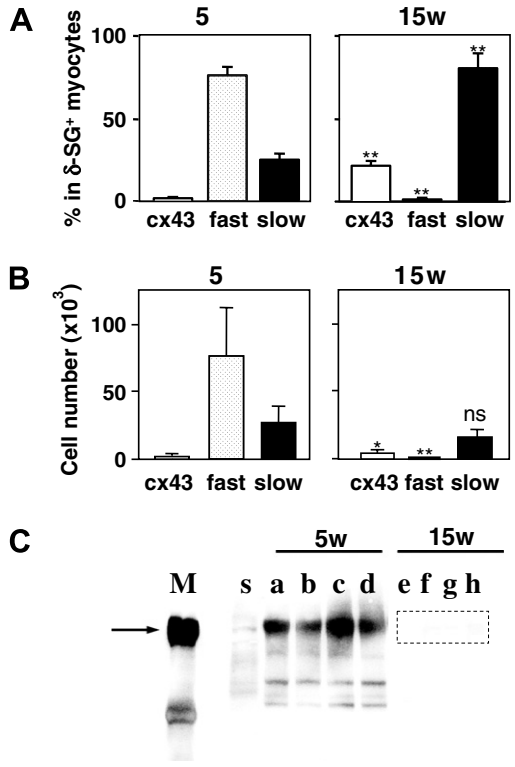

Fig. 2. Time-dependent alteration of positive cell-rates for Cx-43, fast- or slow-twitch MHC in δ-SG$^+$ cells at 5 and 15 weeks after the skMb-transplantation (A) or estimated cell-number in whole heart (B). Western blotting of fast-twitch MHC (arrow in upper picture) in the hearts of TO-2 hamsters at 5 (a–d) or 15 weeks (e–h) after the skMb-transplantation (C). M, T, and s denote the positive control of fast-twitch MHC, TO-2 heart without the cell-transplantation, and whole protein sample in skMbs applied, respectively. Note the huge production of fast-twitch MHC at 5 weeks, but the trace expression at 15 weeks (within a rectangle under lane e–h).

At 15 weeks after the cell transplantation, however, the amount of fast-twitch MHC dramatically reduced (0.36 ± 0.04-fold, $p < 0.01$), suggesting the trace expression (Fig. 2C, within a dotted rectangle in lanes e–h, $n = 4$). The interval between 5 and 15 weeks in hamsters corresponded to ~12 years in humans, as it would be sufficiently long to evaluate therapeutic efficacy of the cell transplantation.

Morphological alterations of engrafted myoblasts

The connexin (Cx)-43 expression in facet between the grafted and host cells was also dependent on the duration after the engraftment. At 5 weeks after the cell-transplantation, most of engrafted cells did not show Cx-43 between the donor and the host cells (data not shown). At 15 weeks after, however, more than 10% of the transplanted cells carrying δ-SG demonstrated positive staining for Cx-43 (Figs. 3A and B), suggesting that Cx-43 synthesized *de novo* in the engrafted cells formed a gap junction (Figs. 3B and F) and might serve for an electrical conduction, as is the case between cardiac muscle cells [21]. Double fluorescence microscopy of δ-SG immunostaining and Dil as the SL marker to identify the donor cells clearly demonstrated that not all transplanted skMbs, but the δ-SG$^+$ and DiI$^+$ cells, selectively presented Cx-43 (Fig. 3C). Between 5 and 15 weeks after the cell-transplantation, the cell population co-expressing both Cx-43 and δ-SG on the same cells increased 4-fold (from $2.56 \pm 0.55\%$ to $10.4 \pm 3.7\%$, $p < 0.01$, Fig. 2A).

Furthermore, serial adjacent sections (Figs. 3D–F) using triple or quadra-staining with Dil (red), DAPI (blue), δ-SG (green), and fast-twitch (Fig. 3D) or slow-twitch MHC (Fig. 3E) distinctly indicated that the engrafted skMbs present cardiac muscle-like features at 15 weeks after the cell-engrafting for the following four reasons; (1) the expression of Cx-43 specific to myocardial cell (Figs. 3A and B), (2) selective production of not fast-twitch (Fig. 3D), but slow-twitch MHC (Fig. 3E) of which the amino acid sequence completely matched with cardiac MHC [26], and (3) single nucleus at the center of myoplasm of the engrafted cells like cardiomyocytes (pink arrow in Fig. 3F), being totally different from skeletal muscle cells that show multiple nuclei beneath the SL.

Cell density-dependent degeneration of grafted skMbs

The survival of engrafted cells was dependent on the cell density for the transplantation. In sham operation without the cell-transplantation, no cells showed Dil (Fig. 4A) in TO-2 heart and numerous TUNEL$^+$ nuclei (Fig. 4B) at the locus corresponding to the degraded tissue (Fig. 4C). When skMbs were transplanted at the locally dense condition (Fig. 4D), TUNEL$^+$ cells became scarcely detected except the non-specific staining at the site corresponding to the densely located DiI$^+$ cells (Fig. 4E) and degraded myocardium (Fig. 4F).

In contrast, when prelabeled DiI$^+$ skMbs were sparsely disseminated in TO-2 heart (Fig. 4G), no cells demonstrated TUNEL$^+$ staining (Fig. 4H) and showed neatly arranged myocardium without degradation (Fig. 4I). In the progression of heart failure from mild to advanced stage, not only apoptosis but also autophagy would contribute to myocardial cell death in DCM hearts [27]. Because TUNEL staining uses end-labeling of fragmented DNA, it was difficult to precisely discriminate these two types of cell-death. Present results, however, clearly demonstrate that sparse and diffuse delivery was preferable for the efficient cell-engrafting.

The exact reason why not all DiI$^+$ cells present δ-SG was unclear (Fig. 3C). It would be conceivable that fast-twitch myocytes with highly energy-consuming and requiring large amount of oxygen and nutrients might be eradicated by the natural selection secondary to apoptosis (Fig. 4E) in TO-2 heart, leaving slow-twitch muscles under the limited myocardial slow. In general, the amount of constituent protein is dependent on the dynamic equilibrium of biosynthesis and the proteolysis *in vivo*. We have shown that dystrophin and its related proteins remained constant at the early phase of ischemic cardiomyopathy without

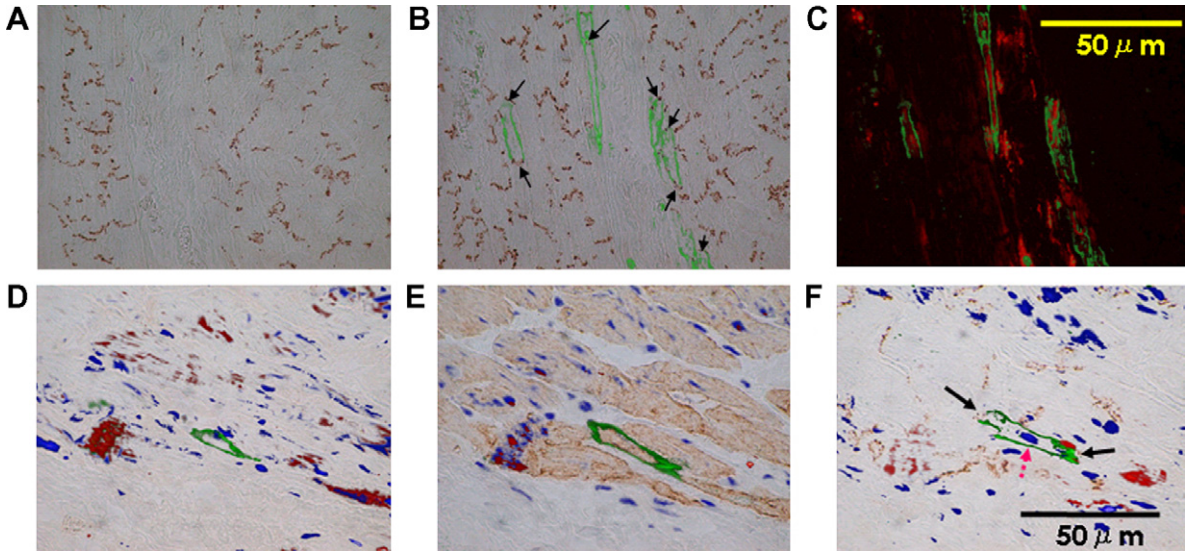

Fig. 3. Connexin (Cx)-43 expression between the engrafted skMbs and host cardiomyocytes. Immunostaining of Cx-43 (A), merged picture with δ-SG along SL (B), and double fluorescence picture of δ-SG and DiI-prelabeled skMbs (C). Quadra-staining of serial three sections of engrafted cells in TO-2 heart at 15 weeks (D–F). Red, blue, and green indicate florescence of DiI for the SL, DAPI for the nucleus, and FITC-labeled antibody to δ-SG, respectively. Note that the engrafted skMbs expressed δ-SG along the SL (D–F), not fast-twitch (D), but slow-twitch MHC (E), Cx-43 at both ends (black arrows in F), and nucleus at the center of the myoplasm (pink dashed arrow in F). Original magnification 100×, and bar length indicates 100 μm.

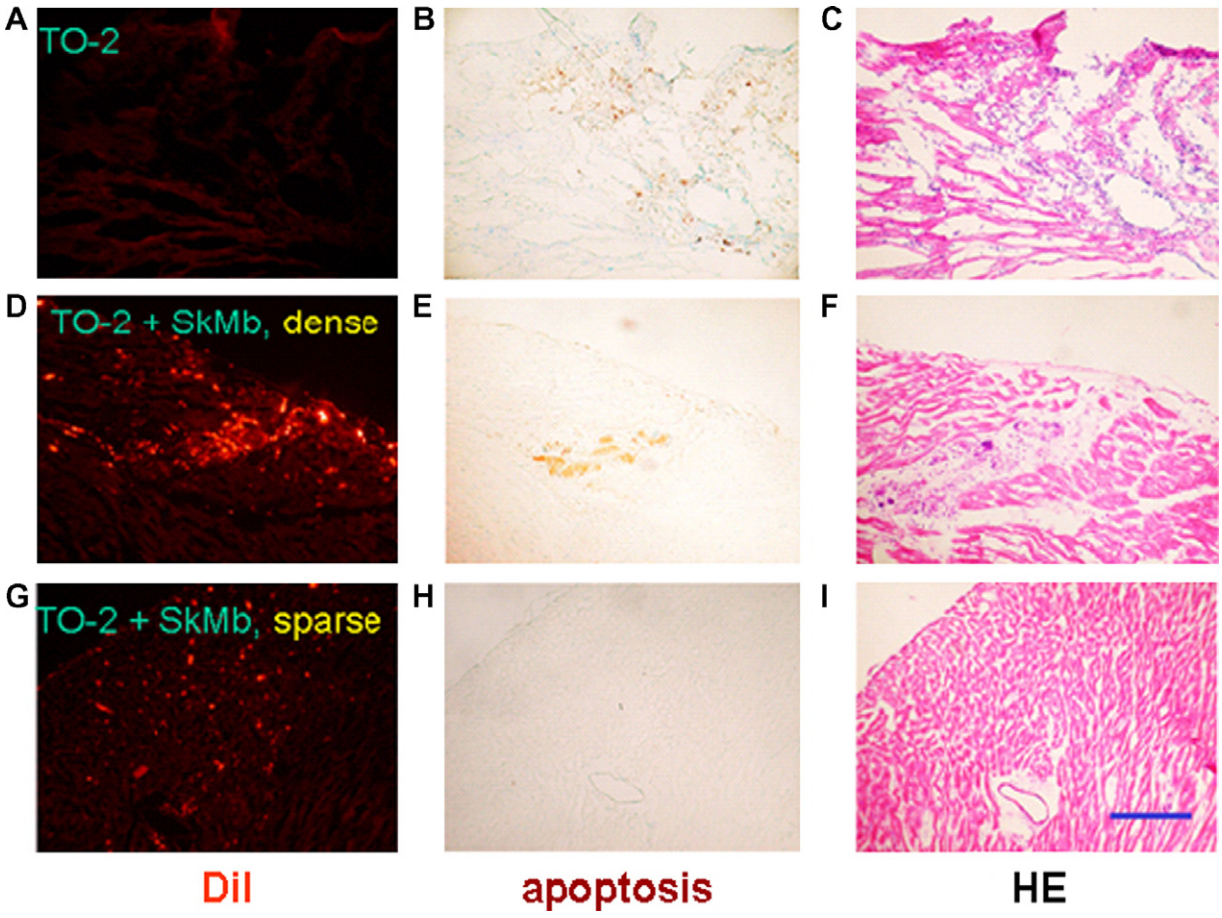

Fig. 4. Cell density dependent apoptosis and/or autophagy of engrafted cells detected by TUNEL staining in TO-2 hearts. Without cell-transplantation, most cells showed no DiI (A) and numerous TUNEL$^+$ nuclei (B) at the loci corresponding to degraded myocardium (HE staining in C). When the skMbs were transplanted at locally dense condition (upper DiI$^+$ portion near to epicardium, D), TUNEL$^+$ cells were scarcely detected except the non-specific staining at the densely transplanted site (E) and degraded myocardium (F). In contrast, when DiI$^+$ skMbs were sparsely disseminated in TO-2 heart (G), no cells showed TUNEL$^+$ staining (H) in neatly arranged myocardium (I). Original magnification 100×, and bar length indicates 100 μm.

heart failure, accompanying the compensatory overexpression of each mRNA. In contrast, at the end stage of heart failure, no compensatory expression of both the transcript and transgene caused loss of these proteins, being exceeded by their increased proteolysis [11]. The cell subgroup with less intense δ-SG$^+$ at 15 weeks after the skMb-transplantation (Fig. 2A) might represent the degrading cells during the selection of transplanted cells.

Pouly et al. have reported functional efficacy of skeletal myoblast transplantation, using CHF147 strain hamsters which genetically lack δ-SG gene [28]. At 4 weeks after the intramural administration of skMb, 2D echo study revealed functional improvement, myotube formation, and positive staining for fast-twitch MHC of skeletal muscle cells. The histological study also demonstrated multinuclear fiber oriented parallel to the surrounding cardiac cells. These findings are in agreement with our data at 5 weeks in TO-2 strain after the cell-transplantation, though the expression of slow-twitch MHC was not examined. As was shown by Attar et al. [29], long-term follow-up to 1 year in rat myocardial infarction model demonstrated the functional benefits of autologous skMb transplantation. However, they reported that both the fast- and slow-twitch MHC isoforms were expressed. The exact reason of discrepancy is unknown, but might caused by the difference between the clinical stetting *i.e.,* DCM of genetic origin and myocardial infarction.

Effect of skMb-transplantation on hemodynamics and arrhythmia

When hemodynamic study was restricted to the disseminated engrafted cases ($n = 6$) in the autopsy, the skMb transplantation on the hemodynamics was shown to be beneficial for improving several indices. The available space to discuss the effect on physiological action is limited and will be presented in the supplementary section.

Current results would imply the therapeutic significance in cell-transplantation employing not the mixture of fast- and slow-twitch cells that may cause either substrate for serious arrhythmias or heterogeneous contraction, but selected slow-twitch muscle cells carrying the similar characteristics as cardiac muscle fibers with enduring mechanical performance, metabolic activities [30] as well as homogeneous electric conduction *via* Cx-43 and intercalated disc synthesized *de novo*. Future cardiac repair for the regenerative medicine should target the progenitor cells with similar properties to cardiac muscle cells and how to select the slow-twitch myoblasts *ex vivo*.

Two abstracts [31,32] have been reported to show our priority concerning of this article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.11.084.

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