In Vivo Gene Transfer of PPARγ Is Insufficient to Induce Adipogenesis in Skeletal Muscle

Ayako BAN1), Keitaro YAMANOUCHI1)*, Takashi MATSUWAKI1) and Masugi NISHIHARA1)

1)Department of Veterinary Physiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan

(Received 5 March 2008/Accepted 19 March 2008)

**CORRESPONDENCE TO: YAMANOUCHI, K., Department of Veterinary Physiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan.**

**ABSTRACT.** Skeletal muscle contains several progenitor/stem cells with myogenicity as well as adipogenicity such as satellite cells. Our previous study demonstrated that forced expression of PPARγ is sufficient to induce transdifferentiation of predetermined myoblasts in vitro. In the present study, we examined whether introduction of PPARγ gene could induce adipogenesis of satellite cells in vivo. A plasmid vector containing enhanced green fluorescent protein (EGFP) or PPARγ gene was introduced into rat tibialis anterior muscle by electroporation. Histological analyses revealed that electroporation induces degenerative/regenerative response in skeletal muscle, including activation of satellite cells. When EGFP gene was introduced, newly formed myotubes resulted from fusion of activated satellite cells, showed EGFP expression, indicating that electroporation could transfect satellite cells with exogenously introduced gene. Gene transfer of PPARγ resulted in an increase of PPARγ-positive mononucleated cells on day 3 after electroporation but failed to induce adipogenesis thereafter. These results suggested that, in addition to an expression of PPARγ, niches that support adipogenesis are required for satellite cells to enter adipogenesis in vivo.

**KEY WORDS:** adipogenesis, electroporation, PPARγ, satellite cells, skeletal muscle.

---

Adult skeletal muscle contains several progenitor/stem cells that are capable of differentiating to cells of mesenchymal lineages [4]. Amongst these cells, satellite cells are the cells that contribute to postnatal muscle growth and regeneration due to their myogenicity [4, 10]. Satellite cells are quiescent in adult skeletal muscle, and located between basal lamina and plasma membrane of muscle fibres [4, 10]. Upon injury or mechanical load to muscle fibres, they are activated, proliferate, and eventually generate a pool of predetermined myoblasts, expressing myogenic transcription factors such as MyoD and myogenin, that terminally differentiate to myotubes by fusion [4]. Recent evidence indicates that satellite cells can also differentiate to adipocytes or osteocytes under some circumstances in vitro [3, 12, 21, 22], and suggested that satellite cells may be one of the cellular origins of adipose tissue developed in skeletal muscle that is seen under some pathological conditions such as muscular dystrophy [19] and sarcopenia [14, 16]. In addition to satellite cells, even predetermined myoblasts can be driven to transdifferentiate to adipocytes by forced expression of adipogenic transcription factor, peroxisome proliferated-activated receptor-γ (PPARγ) in vitro [8, 23]. However, whether satellite cells can differentiate or be transdifferentiated to adipocytes in vivo is unknown.

Electroporation is the efficient method to introduce exogenous gene into skeletal muscle. Introduction of genes of osteogenic growth factor, bone morphogenetic protein (BMP)-2 and -7, into skeletal muscle by electroporation has been shown to induce ectopic bone formation [9], suggesting that the presence of cells with osteogenicity and the appropriate niches are required for them to terminally differentiate to osteocytes. Recent study indicated that electroporation induces degenerative/regenerative response of skeletal muscle, thus leading to an activation of satellite cells, and can be used not only to introduce genes of interest into skeletal muscle fibres but also to transfect satellite cells in vivo [11].

In the present study, electroporation was used to examine whether satellite cells can be transdifferentiated to adipocytes by forced expression of PPARγ in vivo. For this purpose, PPARγ gene was introduced into rat tibialis anterior muscle and histological evaluation was performed.

**MATERIALS AND METHODS**

**Animals:** Adult (9 to 14 weeks old) male Wistar Imamichi rats purchased from Institute for Animal Reproduction (Ibaraki, Japan) were used throughout this study. They were housed in a room at 23°C with a lighting condition of 12 hr-light and 12 hr-darkness (lights on at 0700 hr). Food and water were provided ad libitum. All animal experiments performed in this study were according to the Guideline for the Care and Use of Laboratory Animals, The University of Tokyo.

**Plasmids:** Enhanced green fluorescent protein (EGFP)-expressing plasmid (pCXN2-EGFP) is described in a previous report [24]. The entire open reading frame region of rat PPARγ cDNA (1518 bp) was amplified by polymerase chain reaction (PCR) using plasmid vector, pBABE-PPARγ [8], as a template. The primer set for PCR was 5′-CTC-GAGGTGTTATGGGTGAAACTCTGGGAGAA-3′ (for-
Electroporation: Rats were anesthetized by ethyl ether, and their skin over the tibialis anterior (TA) muscle was incised. A pair of stainless electrode needles was stuck into TA muscle, and 100 µl of plasmid solution (1 mg/ml in saline) was injected between the needles. Immediately after the injection, three square pulses of 50 V for 50 msec were applied at 100-msec intervals, followed by three more pulses of the opposite polarity using an electroporator (CUY21, NEPA GENE Co., Ltd., Chiba, Japan). On the indicated days after electroporation, the rats were killed by inhalation of carbon dioxide gas and their TA muscles were obtained. The tissue samples were snap-frozen in isopentane cooled with liquid nitrogen, and stored at −85°C until use.

Histological analyses: Frozen tissue sections (7–8 µm thickness) of the rat TA muscles were prepared transversely, and nearly the broadest sections were subjected to histological analyses. For hematoxylin-eosin (HE) staining, sections were fixed with 4% paraformaldehyde in phosphate buffered saline (4% PFA/PBS). After washing with running tap water for 5 min, the sections were stained with hematoxylin. After washing with running tap water for 20 min, the sections were immersed in eosin solution for 20 min, then dehydrated and mounted with Diatex (Matsunami Glass Ind., Ltd., Osaka, Japan).

For immunohistochemistry, the sections were fixed with 4% PFA/PBS for 15 min at RT and washed with PBS. Then the sections were blocked with 5% normal goat serum (NGS) in PBS (0.6% hydrogen peroxide (H₂O₂) was included when horse radish peroxidase (HRP)-conjugated secondary antibody was used) for 30 min. After blocking, the sections were incubated with primary antibody for 2 hr at RT. The following primary antibodies were used: anti-desmin (clone DE-U-10 from Sigma, St. Louis, MO, U.S.A., 1:400 dilution), anti-MyoD (clone 5.8A from Novocastra, Newcastle upon Tyne, UK, 1:100 dilution), anti-myogenin (clone F5D from Developmental Studies Hybridoma Bank, Iowa City, IA, U.S.A., 1:400 dilution) and anti-PPARγ (clone A3409A, Perseus Proteomics Inc., Tokyo, Japan, 1:200). The specificity of the anti-PPARγ antibody has been shown in manufacturer’s instruction sheet. The sections were washed with PBS and incubated with secondary antibody for 1 hr at RT. The following secondary antibodies were used: simple stain MAX-PO (M) (HRP-conjugated secondary antibody) and AlexaFluor594 labeled anti mouse IgG (Invitrogen, Carlsbad, CA, U.S.A., 1:100 dilution). All the antibodies were diluted with 5% NGS/PBS. Cell nuclei were counterstained with methylgreen or hoechst 33258. When the sections were labeled with HRP-conjugated secondary antibody, signals were visualized by 3, 3’-diaminobenzidine and H₂O₂, and observed with light microscope. Fluorescent signals were observed with fluorescence microscope. When quantitative analyses on immunopositive nuclei were performed, the number of positive nuclei on entire section was counted (n=4).

Statistical analyses: The graphed data were expressed as means ± SE. Unpaired t-test was used to evaluate statistical differences between the groups. P values less than 0.05 were considered as statistically significant.

RESULTS

Histological analyses of skeletal muscle after electroporation: To confirm that electroporation induces degenerative/regenerative response in skeletal muscle, histological analyses were performed on rat TA muscles after electroporation of pCXN2-EGFP. HE staining revealed that on day 1 after electroporation, the skeletal muscle fibres showed degenerative changes and an infiltration of inflammatory cells were observed (Fig. 1A, Day 1). On day 3, a numerous number of mononucleated cells, presumably including macrophages, vascular endothelial cells and activated satellite cells, was present in degenerative area (Fig. 1A, Day 3). On days 5 and 7, the number of mononucleated cells was decreased, and myotube formation, as revealed by the presence of multinucleated cells, was evident, indicating the progress of skeletal muscle fibre regeneration (Fig. 1A, Day 5 and 7). On day 10, immature skeletal muscle fibres with central nuclei were observed mainly (Fig. 1A, Day 10), and the regeneration appeared to be completed on day 14 as revealed by the presence of mature skeletal muscle fibres (Fig. 1A, Day 14). Immunohistochemical analyses of desmin revealed that the appearance of desmin-positive mononucleated cells was not seen on day 1 after electroporation, but became evident on day 3, suggesting the occurrence of activation and proliferation of satellite cells as well as the proliferation of vascular endothelial cells (Fig. 1B, Days 1 and 3). On day 5, desmin-positive mononucleated cells were scarcely seen, and instead, newly formed multinucleated myotubes showed intense staining for desmin (Fig. 1B, Day 5). The diameter of desmin-positive myotubes was increased on day 7, and the localization of desmin was shifted to peripheral region within the newly formed skeletal muscle fibres on days 10 and 14 (Fig. 1B, Days 10 and 14). These results confirmed that electroporation induces degenerative/regenerative response in skeletal muscle and the regeneration process appears to be completed on day 14 after electroporation.

Immunohistochemistry of MyoD and myogenin: The above results clearly indicated that electroporation of plasmid DNA into skeletal muscle induces its degeneration followed by regeneration, and suggested that the desmin-positive mononucleated cells that appeared on day 3 after electroporation includes activated satellite cells. To further confirm that the activation of satellite cells indeed occurred,
immunohistochemical analyses of early myogenic factors, MyoD and myogenin, were performed. As expected, both the numbers of MyoD- and myogenin-positive cells were peaked on day 3 after electroporation (Fig. 2A and B), indicating that activation and myogenic determination of satellite cells are taking place.

**Electroporation of DNA into skeletal muscle can transfected satellite cells:** Electroporation of plasmid DNA into skeletal muscle has been shown to transfect satellite cells with exogenously introduced gene [11]. In order to determine if electroporated plasmid DNA is indeed introduced into satellite cells, frozen sections of rat TA muscle on day 7 after electroporation of pCXN2-EGFP were immunostained with anti-desmin [1], and co-localization of EGFP and desmin was examined. As shown in Fig. 3, EGFP expression was co-localized with desmin in myotubes. Since introduction of exogenous plasmid DNA into satellite cells should occur only when the electric pulses were applied and myotube formation should be resulted from the fusion of activated satellite cells, this result indicates that electroporation of plasmid DNA to skeletal muscle can transflect satellite cells.

**Electroporation of PPARγ gene into skeletal muscle:** Our previous study indicated that transfection of determined myoblasts with PPARγ gene is sufficient to induce their transdifferentiation to adipocytes in vitro [23]. To examine if this could also be the case in *in vivo* situation, pCXN2-PPARγ is introduced into rat TA muscle by electroporation. Immunohistochemical analyses of PPARγ revealed that significant number of PPARγ-positive nuclei could be observed in mononucleated cells on day 3 after electroporation of pCXN2-PPARγ, and these PPARγ-positive mononucleated cells possibly includes activated satellite cells (Fig. 4A). Quantitative analyses indicated that the number of PPARγ-positive cells in PPARγ-introduced TA muscle peaked on day 3 and then decreased thereafter. On the other hand, PPARγ-positive cells were constantly seen in EGFP-introduced TA muscle, and their number did not change significantly throughout the degeneration/regeneration process (Fig. 4B). In order to examine if single introduction of
pCXN2-PPARγ could induce adipogenesis in skeletal muscle, frozen sections were prepared from rat TA muscle obtained on days 7 and 14 after electroporation, and subjected to HE staining. There was no histological difference between EGFP- and PPARγ-introduced TA muscles, and no sign of the presence of mature adipocyte was seen even when pCXN2-PPARγ was introduced (Fig. 5). These results indicated that introduction of exogenous PPARγ gene is insufficient to induce adipogenesis in skeletal muscle in vivo.

DISCUSSION

Our data showed that forced expression of PPARγ is insufficient to induce adipogenesis of satellite cells in vivo, and suggested that some factors other than expression of PPARγ are required to activate their adipogenic program in vivo. Forced expression of PPARγ has been shown to induce transdifferentiation of predetermined myoblasts into adipocytes in vitro [8, 23]. In addition, our preliminary experiments also confirmed that transfecting satellite cells with PPARγ expression vector could induce them to enter adipogenesis in vitro (data not shown). However, in all the cases, culturing cells in adipogenic differentiation medium (ADM) that consists of insulin, dexamethazone, isobutylmethylxanthine, and troglitazone is required for them to differentiate to adipocytes. It should be noted that, amongst the reagents included in ADM, troglitazone is a potent stimulator of PPARγ [17], and omitting troglitazone from ADM failed to induce adipogenesis in PPARγ-transfected myoblasts in vitro [23]. Thus, one of the factors required for the achievement of adipogenesis of PPARγ-transfected satellite cells may be an endogenous stimulator of PPARγ though its identification is yet to be undertaken.

Another possible explanation for the lack of adipogenesis in PPARγ-transfected satellite cells is the inappropriate niches. Several experimental models for the study of skeletal muscle regeneration have been established so far [2, 7, 13, 20]. However, not all models show similar regeneration process. For example, when BPVC [13] or cardiotoxin [20] is used to induce degeneration of skeletal muscle, the subsequent muscle fibre regeneration progresses relatively uniformly and synchronously, and is completed without accumulation of adipocytes [13, 20]. On the other hand, when the skeletal muscle degeneration is induced by glycerol injection [2] or freeze injury [7], the regeneration process does not take place uniformly and is occasionally accompanied by accumulation of adipocytes. Though the exact nature that leads to different outcomes in terms of the appearance of adipocytes during regeneration between these 2 experimental models is unknown, it is plausable that this is due to the difference in the niches surrounding satellite cells. In our previous study, we have found that injection of BPVC into skeletal muscle increases the number of cells that are capable of differentiating to adipocytes when cultured in ADM in vitro [25], while there appears no adipocyte during BPVC-induced regeneration. This is in agreement with the idea that appropriate niches will be required for achievement of adipogenesis in vivo even if the cells with adipogenicity are present.

When skeletal muscle fibres are injured, migration of macrophages into degenerated area occurs, and removal of degenerated fibres by phagocytosis of macrophages is indispensable for the subsequent regeneration of muscle fibres [6]. In the present study, PPARγ-positive cells were also seen in EGFP-introduced TA muscle and their number, though not statistically significant, showed tendency to increase on day 3 after electroporation. Since PPARγ is not solely exclusive marker for adipocytes, and macrophages are also one of the cells that express PPARγ [18], the PPARγ-positive cells in EGFP-introduced muscle would contain macrophages. Several inflammatory cytokines are produced by macrophages and some of them have been reported to inhibit adipogenesis [5, 15]. Thus, it is possible that the presence of migrated macrophages in electroporation-induced regenerating muscle would have inhibited adi-
Fig. 3. EGFP expression in newly formed myotubes in regenerating rat TA muscle after electroporation. Frozen section of rat TA muscles obtained on day 7 after electroporation of pCXN2-EGFP was subjected to immunohistochemistry of desmin, and examined for the expression of EGFP. White arrowheads indicate both desmin- and EGFP-positive myotubes. Bar, 200 µm.

Fig. 4. PPARγ expression in rat TA muscle after electroporation of pCXN2-PPARγ. Frozen sections of rat TA muscle obtained on day 1, 3, 7 and 14 after electroporation of pCXN2-EGFP or pCXN2-PPARγ were subjected to immunohistochemistry of PPARγ (A, representative photos from day 3), and the number of PPARγ-positive nuclei on entire section was counted (B). In A, black arrowheads indicate PPARγ-positive nuclei. Bar, 100 µm. *, p<0.05 vs. EGFP.
pogenesis of PPARγ-introduced satellite cells. As a supporting evidence for this possibility, it has been reported that the lack of migrating macrophages into degenerated skeletal muscle results in incomplete regeneration of muscle fibres and the appearance of adipocytes [6].

In summary, we have shown in the present study that forced expression of PPARγ is not sufficient for inducing adipogenesis of satellite cells in vivo, and suggested that appropriate niches are required for promotion of adipogenesis in skeletal muscle. Further study to clarify the exact factors that affect adipogenesis in skeletal muscle will be necessary for elucidating the mechanism of adipose tissue development within skeletal muscle.

ACKNOWLEDGEMENTS. This study was supported by Grant-in-Aid for Scientific Research (19380167) from the Japan Society for the Promotion of Science, and the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

REFERENCES