Regeneration of the Cardiac Conduction System by Adipose Tissue-Derived Stem Cells

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**Background:** Adipose tissue is one of the sources of mesenchymal stem cells, which have the potential to differentiate into various types of cells, including myocytes. Whether brown adipose tissue (BAT)-derived cells might differentiate into the cardiac pacemaking-conducting cells, and have the potential to regenerate the cardiac conduction system (CCS), is investigated in this study.

**Methods and Results:** BAT was isolated from the interscapular area of mice and enzymatically digested before culture. Round or fusiform cells showed spontaneous beating at 4–7 days after culturing of BAT-derived cells. Reverse transcriptase-polymerase chain reaction analysis and immunocytochemical analysis revealed that BAT-derived cells expressed several cardiomyocytes, the CCS and pacemaker (PM) cell marker genes and proteins. Patch-clamp techniques revealed that spontaneous electrical activity and the shape of the action potential showed properties of cardiac PM cells. Next, a complete atrioventricular (AV) block was created in mice and green fluorescent protein-positive (GFP (+)) BAT-derived cells were injected intramyocardially around the AV node. At 1 week after transplantation, 50% of BAT-derived cells injected mice showed a sinus rhythm or a 2:1 AV block. Immunohistochemical analysis revealed that injected GFP (+) cells were engrafted and some GFP (+) cells co-expressed several cardiac PM cell marker proteins.

**Conclusions:** BAT-derived cells differentiate into the CCS and PM-like cells in vitro and in vivo, and may become a useful cell source for arrhythmia therapy. *(Circ J 2015; 79: 2703–2712)*

**Key Words:** Brown adipose tissue-derived cells; Cell transplantation; Conduction; Regeneration

Various types of stem cells have been reported to differentiate into cardiomyocytes, such as bone marrow-, adipose- and skeletal muscle-derived stem cells, endothelial progenitor cells, resident cardiac stem cells and pluripotent stem cells.1 However, clinical trials of stem cell therapy have shown a slight improvement of cardiac function, and if some improvement was observed, it is now thought to be attributable to paracrine effects but not transdifferentiation of transplanted stem cells into cardiomyocytes of transplanted cells.2 The major obstacles to current stem cell therapy are as follows. First, the efficiency of cardiomyocyte differentiation of cultured and transplanted stem cells is quite low. Second, phenotypes of cardiomyocytes differentiated from stem cells are not well characterized. Embryonic stem cell-derived cardiomyocytes have been reported to be highly heterogeneous, consisting of various cardiomyocytes such as atrial, ventricular and pacemaker (PM) cells.3 Spontaneous beating with myotube-like morphology and cardiomyocyte-like action potentials may not indicate ventricular cardiomyocytes.4,5

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Adipose tissue is classified into two types: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the primary site of energy storage, and BAT is an energy-expending tissue that regulates thermogenesis.6 Developmentally, BAT but not WAT is derived from a Myf5 (+) cellular lineage that also generates skeletal muscle,7 suggesting that bipotent muscle-brown fat precursors or more primitive stem cells may reside in BAT in adulthood. Thus, we hypothesized that these dormant...
stem cells might be able to differentiate into muscle lineage including cardiomyocytes, once cultured in an appropriate condition.

Here, we first report that BAT-derived cells differentiate into spontaneously beating cells, which have a typical character of the cardiac conduction system (CCS), but not working ventricular cardiomyocytes. When BAT-derived beating cells were injected into atrioventricular (AV) nodal regions of the mouse models of complete AV block, transplanted cells expressed conduction system-specific gap junction proteins such as connexin40 (Cx40) and connexin45 (Cx45), and the conduction disturbance was corrected. These results suggest that adipose tissue-derived cells are an effective cell candidate for the biological PM, which can create a stable physiologic rhythm from an optimal site in the individual heart without immune rejection.

Methods

Adult (8–12-week old) male wild-type mice (C57BL/6Jmqlc) were purchased from Japan SLC Inc. All protocols were approved by the institutional Animal Care and Use Committee of Chiba University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. BAT- and WAT-derived cells were isolated according to the method by Björntorp et al. Additional details are provided in the Supplementary data.

Results

Cells Derived From Adult Murine BAT Differentiate Into Spontaneously Beating Cells

When BAT-derived cells were cultured in 1% methylcellulose/Isoc сот’s Modified Dulbecco’s Medium containing hematopoietic cytokines, a colony of small round cells emerged on day 3 (arrowhead in Figure 1A). Five days after seeding, some of small round cells started beating (Figure 1A; Movie S1), and spontaneous beating was observed also in fusiform cells on day 7 (arrows in Figure 1A). On day 14, lots of round and fusiform cells showed regular beating (Figure 1A; Movie S1). The beating cells expressed cTnT, SA-actinin, α-MHC, and β-MHC showing fine sarcomere, which was characteristic to striated muscle cells (Figure 1B). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed that BAT-derived cells cultured for 7 days, including beating cells, expressed Nkx2.5, GATA6, Mef2c, ANF,
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α-MHC, β-MHC, MLC2a, MLC2v, but not GATA4 (Figure 1C). The expression levels of MLC2a and ANF were very low. Skeletal muscle specific transcription factor, MyoD, Myogenin, Myf5 was also expressed at day 7 (Figure 1D). Immunocytochemistry revealed that expression of atrial natriuretic factor was frequently observed in small round cells in the beating colonies, which were Sarcomeric α-actinin negative (−) (Figure 1D). Some of ANF (+) mono-nuclear fusiform cells co-expressed SA-actinin (Figure 1D Upper right), but not MyoD (Figure 1D Lower right). The expression of MyoD was restricted to large multi-nuclear myotube-like cells, where ANF was not detected (Figure 1D Lower right). These results suggest that BAT-derived cells are committed to the cardiac lineage and then a part of them is directed toward the skeletal muscle during in vitro culture.

BAT-Derived Cells Differentiate Into the Cells of the CCS

GATA6 has been reported to be expressed in the CCS. Expression of GATA6 but not GATA4, and existence of spontaneously beating small round cells suggest that BAT-derived cells differentiate into cardiac PM or CCS rather than working ventricular-type cardiomyocytes. To prove this possibility, we performed an electrophysiological study. BAT-derived beating both round and fusiform cells (Figure 2A Upper left) and fusiform cells (Figure 2A Lower left) showed a negative maximum diastolic potential of approximately −60 mV and prominent phase-4 depolarization. The shape of these action potentials was characteristic to cardiac premature PM cells. Two immunocytochemical images of typical round (white arrowhead) and fusiform cells. sarcomeric α (SA)-actinin in red. Nuclei are stained in blue. Scale bars, 10 μm. (B) Heart beat rate change of differentiated BAT-derived cells treated with isoproterenol and carbamylcholine. The Mann-Whitney U-test (isoproterenol, n=47) and the Kruskal-Wallis test, followed by the Steel-Dwass test (carbamylcholine, n=46) were used for statistical analysis (**P<0.01).

Figure 2. Beating brown adipose tissue (BAT)-derived cells have several features of cardiac pacemaker (PM) and conduction system cells. (A) Action potentials obtained from beating round (Top) and fusiform cells (Bottom). Typical immunocytochemical images from beating round and fusiform cells. sarcomeric α (SA)-actinin in red. Nuclei are stained in blue. Scale bars, 10 μm. (B) Heart beat rate change of differentiated BAT-derived cells treated with isoproterenol and carbamylcholine. The Mann-Whitney U-test (isoproterenol, n=47) and the Kruskal-Wallis test, followed by the Steel-Dwass test (carbamylcholine, n=46) were used for statistical analysis (**P<0.01).

Next, we examined expressions of genes of the PM/CCS. After 7 days of culture, BAT-derived cells expressed Tbx3, Tbx5, HF-1b, Cx40, Cx45, MinK, and HCN 1 to 4 (Figure 3A). To evaluate expression levels quantitatively, we performed real-time PCR analysis of key channel and gap junction proteins. Relative expression levels of HCN2 and HCN4 were 24% and 3% of right atrium including sinoatrial node, respectively. Relative expression levels of Cx40 and Cx45 were 123% and 292% of RA+SAN (Figure 3B). Next, we examined the time-
As shown in Figures 1 and 4, 3 different types of morphology were observed in BAT-derived SA-actinin (+) myocytes: small mononuclear round, mononuclear fusiform, and large multinucleated tube-like myocytes. To examine whether the muscle phenotype (i.e. cardiac or skeletal muscle) differs depending on the morphology, we double-stained the BAT-derived cells with cardiac-specific cTnI and skeletal muscle-specific ACTN3 antibodies, and calculated the percentage of cTnI (+), ACTN3 (+), or double (+) cells for the total of immune-reactive cells. Specificity of the antibodies was confirmed immunohistochemically; there was no cross reactivity between cardiac and skeletal muscle tissue (Figure 5A). On day 5 of culture, most of the round and fusiform cells were cTnI (+), and approximately 80% of these cTnI (+) cells co-expressed ACTN3. Almost all of the multi-nucleated tube-like cells were only ACTN3 (+), suggesting that these cells
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We next examined the feasibility of the BAT-derived myogenic cells to correct conduction abnormality. Forty-five mice were subjected to an injection of glycerol solution into the AV node region. Complete AV block was produced in 21 out of 45 mice, as judged by the appearance of the typical dissociation between atrial P waves (Figure 7A, white arrowhead) and ventricular GRS waves (Figure 7A, black arrowhead). Four days after the operation, we examined electrocardiography to judge whether stable complete AV block was successfully produced. One mouse was excluded because of spontaneous reversion to normal sinus rhythm. Our preliminary results showed that spontaneous recovery from complete AV block did not occur for as long as 15 days when the block continued for the first 4 days. We injected differentiated GFP (+) BAT-derived cells into the AV nodal region. We also performed control injections of non-myogenic BAT-derived cells, which lost their myogenic and contracting activities after 2 passages. Twelve out of 20 mice survived after injection of the cells. Seven days

were skeletal myotubes (Figure 5C). Typical immunofluorescent images of BAT-derived cells are shown on the right side of each graph.

Next, we examined the frequency of cardiac PM/CCS in the colony of SA-actinin (+) BAT-derived cells. The frequency of HCN2 (+) cells in the SA-actinin (+) round, fusiform, and multi-nucleated tube-like cells was 62.1±11.5%, 50.9±21.6%, and 10.2±7.3%, respectively (round vs. multi-nucleated tube; **P<0.01, fusiform vs. multi-nucleated tube; **P<0.01; Figure 6A). Frequency of HCN4 (+) cells in the SA-actinin (+) round, fusiform, and multi-nucleated tube-like cells was 61.6±16.4%, 40.0±36.5%, and 7.14±12.3%, respectively (round vs. multi-nucleated tube; **P<0.01, fusiform vs. multi-nucleated tube; **P<0.01; Figure 6B). Frequency of Cx40 (+) cells in the SA-actinin (+) round, fusiform, and multi-nucleated tube-like cells was 53.4±15.4%, 29.5±23.8%, and 3.8±3.9%, respectively (round vs. multi-nucleated tube; *P<0.05; Figure 6C). Therefore, SA-actinin (+) round and fusiform cells contained a higher percentage of CCS and PM cells in comparison with multi-nucleated tube-like cells.

**Figure 4.** Immunocytochemical images of brown adipose tissue-derived cells double-stained with sarcomeric α (SA)-actinin (green) and one of the following proteins, hyperpolarization-activated cyclic nucleotide-gated (HCN) 2, HCN3, HCN4, MinK, connexin (Cx) 40 or Cx45 (red). Nuclei are stained in blue. Phase contrast images are presented in the right side column. White arrows indicate multinucleated tube-like cells. Scale bars, 10 μm.

**Implantation of Differentiated BAT-Derived Cells Regenerates the CCS**

We next examined the feasibility of the BAT-derived myogenic cells to correct conduction abnormality. Forty-five mice were subjected to an injection of glycerol solution into the AV node region. Complete AV block was produced in 21 out of 45 mice, as judged by the appearance of the typical dissociation between atrial P waves (Figure 7A, white arrowhead) and ventricular GRS waves (Figure 7A, black arrowhead). Four days after the operation, we examined electrocardiography to judge whether stable complete AV block was successfully produced. One mouse was excluded because of spontaneous reversion to normal sinus rhythm. Our preliminary results showed that spontaneous recovery from complete AV block did not occur for as long as 15 days when the block continued for the first 4 days. We injected differentiated GFP (+) BAT-derived cells into the AV nodal region. We also performed control injections of non-myogenic BAT-derived cells, which lost their myogenic and contracting activities after 2 passages. Twelve out of 20 mice survived after injection of the cells. Seven days
It has been reported that paracrine factors play an important role in the beneficial effects of transplanted cells. As shown in Figure 1, BAT-derived cells cultured in Methocult contained skeletal and cardiac striated muscle cells. When myogenic activity was measured as the SA-actinin (+) area, BAT-derived cells showed the higher myogenic activity in comparison with WAT-derived cells cultured in Methocult (Figure 8A). Hepatocyte growth factor (HGF) is expressed in growing and regenerating skeletal muscle and stimulates myogenic precursor cell proliferation. In addition, HGF promotes angiogenesis and reduces cardiac fibrosis and cardiomyocyte death. As shown in Figure 8B, differentiated BAT-derived cells cultured in Methocult secreted a larger amount of HGF compared with WAT-derived mesenchymal cells.

It was later found that complete recovery of AV conduction was detected in 2 out of 8 mice after differentiated BAT-derived cell injection (Figure 7A Middle panel and B). Partial improvement of AV conduction was detected in 2 out of 8 mice (Figure 7A Lower panel and B). In contrast, there was no mouse that showed improvement of AV conduction after an injection of non-myogenic BAT-derived cells (Figure 7B). To verify the survival and functional integration of grafted cells, histological sections around the injection sites were examined by using immunohistochemical methods. Figure 7C shows the images obtained from the mouse that recovered AV conduction completely. The grafted myogenic cells were identified as GFP and SA-actinin double (+) cells in the muscular ventricular septum (Figure 7C). Some of the GFP (+) grafted cells expressed Cx45 and Cx40 (Figure 7C), suggesting that the phenotypes of CCS and PM cells were maintained.

Figure 5. Phenotype of brown adipose tissue (BAT)-derived myocytes differs depending on the morphology. (A) Tissue-specific staining of cardiac troponin I (cTnI) and α-actinin skeletal isoform 3 (ACTN3) antibodies. Paraformaldehyde-fixed frozen sections of heart and skeletal muscle were double-stained with cTnI (red) and ACTN3 (green) antibodies. Nuclei are stained in blue. Pictures merged with phase contrast images are shown. Scale bars, 20 μm. (B) and (C) Percentage of cTnI, ACTN3, or double (+) cells in the total positive round, fusiform, or multinucleated tube-like BAT-derived cells cultured for 5 (B) and 14 days (C). Mean of the data obtained from 2 (B) and 4 (C) experiments. BAT-derived cells double-stained with cTnI (red) and ACTN3 (green) antibodies are shown in the right side of each graph. Nuclei are stained in blue. Pictures merged with phase contrast images are shown. Red arrowheads, green arrowheads, and yellow arrowheads indicate cTnI (+), ACTN3 (+), and double (+) cells, respectively. Scale bars, 20 μm.
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Cardiomyocytes, and the addition of Noggin or Dkk1 was effective to increase cardiomyocytes. These authors defined the differentiated cells as cardiomyocytes based on the expression of cardiac specific-proteins, sarcomere structure, and action potentials, but did not examine the precise characteristics of cardiomyocytes.

The myocardium contains the specialized CCS and PM cells, as well as the atrial and ventricular working cardiomyocytes. GATA6, MinK, Cx40, Cx45 and HF-1b have been reported to be markers of the CCS using the lacZ reporter mouse. Four HCN isoforms, HCN1-4, have been found in the heart and encode If-like currents. HCN4 transcripts are approximately 10-fold enriched in the adult SAN of a mouse compared with the atrium. In contrast, HCN2 transcripts are expressed equally in atrial and SAN cells at a low to moderate level. Jumabay et al have reported that WAT-derived cells differentiated into spontaneously beating cardiomyocytes, and the addition of Noggin or Dkk1 was effective to increase cardiomyocytes.

Discussion

In this report, we have demonstrated that BAT-derived cells differentiate into spontaneously beating cells, which possess characteristics of cardiac PM. An electrophysiological study showed that BAT-derived beating cells had a less negative maximum diastolic potential and prominent phase-4 depolarization, indicating the PM activity, reminiscent to the action potential of sinoatrial node (SAN). Differentiated BAT-derived cells expressed several PM activity-relating channels and gap-junctional hemichannel connexins, along with the appearance of beating cells. Transplantation of BAT-derived cells successfully regenerated the CCS.

It has been reported that adipose tissue contains stem cells that have the ability to differentiate into many lineages such as fat, bone, cartilage, and skeletal, smooth, and cardiac muscle. Recently, 3 groups have reported the cardiomyogenic capacity of adipose tissue-derived cells. Planat-Bénard et al have reported that stromal vascular fraction of murine adipose tissue differentiates into cardiomyocyte-like cells, when cultured in Methocult M3534. Yamada et al induced the similar myogenic cells from CD29-positive cells in BAT. Jumabay et al have reported that WAT-derived cells differentiated into spontaneously beating cardiomyocytes, and the addition of Noggin or Dkk1 was effective to increase cardiomyocytes.
Therefore, unlike SAN, HCN2 may account for a substantial component of the PM channel in BAT-derived cells. The physiological role of HCN2 has not been fully elucidated; however, a pivotal role of HCN2 in the normal rhythmicity of SAN myocytes has been reported from a study of HCN2-deficient mice. In HL-1 atrial myocytes, HCN2 functionally contributed to the spontaneous contractile activity. In addition, forced HCN2 expression induced ectopic PM activity in the atria, left bundle branch or ventricle of a canine model. Our immunocytochemical analysis revealed that HCN2 and HCN4 were mainly localized to the round and fusiform SA-actinin (+) cells in BAT-derived cells (Figures 4, 6). These results suggest that their spontaneous beating activity may be caused by HCN2 and to a lesser degree by HCN4. The mRNA levels of Cx40 and Cx45 were comparable or more than RA+SAN (Figure 3C). The dense fluorescent signals of Cx40 (Figures 4, 6) and Cx45 (Figure 4) were localized to the round and fusiform SA-actinin (+) cells; however, other types of SA-actinin (−) BAT-derived cells, such as mesenchymal cells or fibroblasts, might express Cx40 or Cx45 to some degree but not in a fully assembled form as previously reported. A low percentage of multi-nucleated tube-like cells expressed Cx40 or Cx45 to some degree but not in a fully assembled form as previously reported.24,25
Taken together, these results suggest that a part of differentiated SA-actinin (+) BAT-derived cells are CCS and PM cells.

Our immunocytochemical analysis revealed that BAT-derived myogenic colonies contained cardiac and skeletal muscle cells. Existence of MyoD (+) multi-nucleated skeletal myotubes and mononuclear round or fusiform myoblast-like cells that express ACTN3 but not cTnI suggest active skeletal myofiber generation. This is in agreement with previous reports; BAT and skeletal muscle cells arise from common precursors, and brown adipose precursor cells express myogenic genes such as myoD and several isoforms of skeletal muscle myosin. Spontaneously beating cTnI (+) cardiomycocytes emerged earlier than skeletal muscle did (Figure 5B). These cardiomycocytes are characterized by their mononuclear round or fusiform shape and expression of several PM and CCS-related proteins and atrial natriuretic factor (Figures 1, 4–6). Interestingly, a part of cTnI (+) cells co-expressed ACTN3 (Figure 5). Takebayashi-Suzuki et al has reported that Purkinje cells of E16 embryonic chicken heart express low levels of skeletal muscle-specific MyoD by in situ hybridization and RT-PCR. It has been reported that the transient expression of the fetal skeletal myosin and slow skeletal troponin I isoform was observed in bovine SAN and embryonic rat AV node. In the ventricle, the persistent expression of atrial natriuretic factor was exclusively observed in the conduction system after birth. These reports and our findings suggest that cTnI and ACTN3 double (+) cells may be in the early stage of development into specialized cardiac muscle cells. Or otherwise, myogenic lineage of BAT-derived stem cells may further diverge into skeletal or cardiac cells via cTnI and ACTN3 double (+) cells.

Complete AV block is a serious clinical problem in infants and children as well as in adults, and although electronic PMs are often used as a palliative therapy, there are several shortcomings, such as malfunction due to some electrical or magnetic devices, and repeated replacement of the power pack and electrode. Therefore, there is an urgent need to recreate the lasting AV conduction pathway. Currently, only a few reports have been made with respect to the cell transplantation therapy targeting the AV node. Bunch et al transplanted fibroblasts to the normal AV node in combination with transforming growth factor-β1 in order to modify the rapid AV conduction during atrial fibrillation. Choi et al implanted skeletal myoblasts with the engineered constructs into the AV nodal groove and demonstrated AV conduction through implanted constructs in Langendorff-perfused rat hearts. The novelty of our report is as follows: first, we used a complete AV block model of mice by ablating the AV node, which mimics pathological status. Previously, several kinds of species such as rat, rabbit, and pig were used to develop a model of complete AV block; however, no report has been made about the cell therapy aimed for the repair of AV conduction by using such heart block models. Second, we transplanted BAT-derived myogenic cells, which certainly possess the characteristics of PMs and the CCS. Third, our results clearly showed that the CCS was regenerated at least in part from differentiating BAT-derived myogenic cells.

HGF was abundantly secreted from differentiated BAT-derived cells compared with non-myogenic BAT- and WAT-derived mesenchymal cells. WAT-derived cells cultured in Methocult, which showed little myogenic potential, secreted a low amount of HGF. These results suggest that BAT-derived mesenchymal cells in the myogenic milieu (ie, Methocult) and/or developing myocytes release HGF. Therefore, secreted HGF may promote survival of transplanted cells through its anti-fibrosis and anti-apoptotic effects. Although engrafted GFP and Cx double (+) cells did not show a continuous alignment in the AV node area (Figure 7C), it has been reported that functional connexin-based coupling is possible between cardiac and non-cardiac cells such as fibroblasts and mesenchymal cells in the absence of immunohistochemically detectable spatially clustered gap junctions. Therefore, the interstitial cells that circumvent from apoptosis and scar formation by paracrine factors such as HGF may support AV conductivity recovery.

BAT-derived cells have several advantages such as an intrinsic differentiating ability to PM cells without modification and the feasibility for autologous transplantation. Until recently, it was thought that BAT is rare tissue in adult human. However, positron emission tomography scans have identified active brown fat in many regions of adults including cervical, supra-
clavicular, and paravertebral regions. Stromal vascular fraction of WAT, which also possesses the ability to differentiate into beating cells, may become the substitute for BAT-derived cells. Therefore, adipose tissue-derived cells that have the potential to reconstitute the CCS, may be a good candidate as the cell source for biological PMs.

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