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Spontaneous Cardiomyocyte Differentiation From Adipose Tissue Stroma Cells

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Abstract—Cardiomyocyte regeneration is limited in adult life. Thus, the identification of a putative source of cardiomyocyte progenitors is of great interest to provide a usable model in vitro and new perspective in regenerative therapy. As adipose tissues were recently demonstrated to contain pluripotent stem cells, the emergence of cardiomyocyte phenotype from adipose-derived cells was investigated. We demonstrated that rare beating cells with cardiomyocyte features could be identified after culture of adipose stroma cells without addition of 5-azacytidine. The cardiomyocyte phenotype was first identified by morphological observation, confirmed with expression of specific cardiac markers, immunocytochemistry staining, and ultrastructural analysis, revealing the presence of ventricle- and atrial-like cells. Electrophysiological studies performed on early culture revealed a pacemaker activity of the cells. Finally, functional studies showed that adrenergic agonist stimulated the beating rate whereas cholinergic agonist decreased it. Taken together, this study demonstrated that functional cardiomyocyte-like cells could be directly obtained from adipose tissue. According to the large amount of this tissue in adult mammal, it could represent a useful source of cardiomyocyte progenitors. (*Circ Res*. 2004;94:223-229.)

Key Words: cardiomyocytes ■ adipose tissue ■ differentiation ■ stem cells ■ cell therapy

Cardiomyocyte differentiation mainly takes place during neonatal and perinatal life. In adult life, the regenerative potential of cardiac tissue is limited and is not sufficient to prevent from the degeneration occurring in pathological conditions such as myocardial infarction.[1] Cell transplantation seems to be an alternative to overcome this problem.[2] This led to numerous investigations to identify a putative source of transplanted cells and to better understand cardiomyocyte proliferation and differentiation in order to drive the fate of stem cells toward this process. Different sources, ie, embryonic, fetal, and adult cells have been investigated and tested.[3–8] Among them, mesenchymal stem cells (MSCs) isolated from bone marrow are accepted to give rise to connective tissue cell types. Their differentiation toward cardiomyocyte was obtained in vitro and in vivo. However, immortalized MSCs and treatment with the DNA demethylation agent 5-azacytidine was necessary to reveal their cardiogenic potential in vitro.[6]

Adipose tissues are mesodermic tissues, which develop during perinatal and postnatal life.[9] They are strongly involved in various metabolic disorders such as obesity. Tremendous changes in adipose mass are achieved through highly controlled processes such as angiogenesis, precursor recruitment, proliferation, differentiation, dedifferentiation, and apoptosis as well. More recently, differentiation of cells derived or purified from adipose tissue toward phenotypes different from adipocyte was described.[10–13] However, the characterization of cells with such potential remains to be done. The present statement is that adipose tissue consists of mature adipocytes and the stroma vascular fraction (SVF). SVF is a heterogeneous cell population. Among them the identified ones are vascular cells (endothelial, smooth muscle cell, circulating blood cells) and an undefined fibroblast-like cell population recently described as a multipotential stem cell population for various mesodermic lineage.[10–13]

This led us to test the in vitro cardiomyogenic potential of cells purified from adipose tissue. In the results reported in this study, adipose-derived cells (SVF cells) from primary culture spontaneously differentiate into cells with morphological, molecular, and functional properties of cardiomyocytes.

Materials and Methods

Animals
Six-week-old adult male C57Bl/6N mice (Harlan, France) were housed in conventional animal quarters (SPS barrier facility). Mice were killed by cervical dislocation under CO_2 anesthesia. All procedures were performed according to SELASA norms.

RT-PCR Primer Sequences and Product Size

Gene Product	Sense	Antisense	Product Size, bp
GATA4	5′-AAGACGCCAGCAGGTCCTGCTGGT-3′	5′-CGCGGTGATTATGTCCCCATGACT-3′	340
Nkx2.5	5′-CAGTGGAGCTGGACAAAGCC-3′	5′-TAGCGACGGTTCTGGAACCA-3′	217/217*
MLC-2a	5′-GATCATCTGCAAATCAGACCTG-3′	5′-CTGGGTCATGAGAAGCTGCTTG-3′	253/1049*
MLC-2v	5′-GCCAAGAAGCGGATAGAAGG-3′	5′-CTGTGGTTCAGGGCTCAGTC-3′	499
ANF	5′-TTGGCTTCCAGGCCATA-3′	5′-AAGAGGGCAGATCTATCGGA-3′	282/365*
β-Actin	5′-ATCGTGGGCCGCCCTAGGCACCA-3′	5′-TTGGCCTTAGGGTTCAGAGGGG-3′	243/330*
MyoD	5′-GGAAGAGTGCGGCTGTGT-3′	5′-CTGTTCTGTGTCGCTTAGGG-3′	150

*Size from cDNA amplification/size from genomic DNA amplification.

Cell Culture

Cells from the stroma vascular fraction were isolated according to Björntorp et al[14] with slight modifications. Briefly, inguinal and interscapular adipose tissues were digested at 37°C in phosphate buffer PBS containing 2% BSA and 2 mg/mL collagenase for 45 minutes (collagenase A, Roche). After filtration through 25-μm filters and centrifugation, isolated SVF cells were suspended in PBS, counted with a hemocytometer, and plated at 7.10^3 cells/mL in 1.5 mL of MethoCult GF M3534 (StemCell Technologies). The cultured conditions referred as minimum medium contained basic 1% methylcellulose in Iscvove's MDM (MethoCult M3134) added with 1% bovine serum albumin, 15% fetal bovine serum, 2-mercaptoethanol (10^{-4} mol/L), L-glutamine (2 mmol/L), recombinant human insulin (10 μg/mL), and human transferrin (200 μg/mL). The complete methylcellulose medium mostly used in our experiments (MethoCult GF M3534) consisted of the same medium enriched with recombinant murine IL-3 (10 ng/mL), recombinant human IL-6 (10 ng/mL), and recombinant mouse SCF (50 ng/mL). SVF cells plated in methylcellulose were observed every 2 days under an inverted phase-contrast microscope and number as well as morphology of developing clones was followed.

RNA Extraction and RT-PCR Analysis

Total RNA from cells dissected in contracting areas from SVF culture and RNA from mouse heart were extracted using RNABle extraction solution (Eurobio). cDNA was synthesized from 2 μg total RNA using 100 U of reverse transcriptase (SuperScript II, GibcoBRL) and 0.5 μg oligodT (Amersham).[15] After a 10-minute denaturation at 70°C, buffer containing 2 mmol/L dNTP and 40 U RNAsin inhibitor was added in a 10-μL final volume. After 5 minutes at 42°C, RT was added and reverse transcription was performed for 50 minutes at 42°C and stopped by heating 15 minutes at 72°C. cDNA samples were subjected to PCR amplification with primers as described in the Table. For GATA-4, MLC-2a, MLC-2v, ANF, and Nkx2.5 amplifications, 2 μL of the RT reaction mixture was added to a PCR mix containing 1.5 mmol/L MgCl$_2$, 0.05 mmol/L dNTP, and 0.2 pmol/L of each primer and 1.5 U of Taq polymerase (Promega). For MyoD amplifications, 2 μL of the RT reaction mixture was added to a PCR mix containing 1 mmol/L MgCl$_2$, 0.05 mmol/L dNTP, and 0.2 pmol/L of each primer and 0.625 U of Taq polymerase (Promega).

For GATA-4 and MyoD, PCR were performed over 36 cycles, consisting of an initial denaturation at 94°C for 5 minutes, then 94°C for 30 seconds, 58°C (GATA-4) or 60°C (MyoD) for 30 seconds, 72°C for 30 seconds, and was terminated by a final extension at 72°C for 8 minutes. Nkx2.5 PCR consisted of denaturation at 94°C for 5 minutes followed by 30 cycles of 95°C for 30 seconds, 58°C for 1 minute 30 seconds, 72°C for 1 minute, and a final extension at 72°C for 7 minutes. For MLC-2v, MLC-2a, and ANF, each sample was initially denatured at 94°C for 4 minutes, followed by 5 cycles of denaturation at 94°C for 45 seconds, annealing at 57°C, 58°C, and 60°C, respectively, for 1 minute, extension at 72°C for 2 minutes, and 30 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 1 minute, extension at 72°C for 2 minutes, and 72°C for 10

minutes. β-Actin was used as the control. The PCR products were size fractionated by 2% agarose gel electrophoresis.

Immunocytochemistry

For confocal microscopy, adherent cells grown in chamber slides were washed out from methylcellulose with PBS then fixed with 3% paraformaldehyde or cooled (-20°C) in methanol-acetone (50/50) solution, permeabilized with 0.1% Triton X-100, and labeled with mouse anti-sarcomeric α-actinin antibody 1:500 (Sigma), rabbit anti-MEF2C antibody 1:50 (Cell Signaling), mouse anti–β-MHC antibody, anti-connexin43 1:50 (Zymed), anti-connexin40 (Chemicon), anti-MLC2v 1:50 (Biocytex, France), and alexa546 conjugated anti-mouse or anti-rabbit IgG (Molecular Probes) (dilution 1:300). In situ immunostained proteins were visualized in 0.2-μm optically z-sectioned cells. To improve resolution and signal-to-noise ratio, images were restored using the Huygens software (Huygens 2.2.1, Scientific Volume Imaging) and visualized using Imaris (Bitplane). Calculations were performed on DELL Precision 450 workstations.

For phase-contrast microscopy, adherent cells grown in chamber slides were washed out from methylcellulose with PBS then fixed and permeabilized overnight at 4°C in 3.7% paraformaldehyde/PBS buffer. Endogenous peroxidase activity was removed by a 10-minute incubation in peroxidase blocking reagent (DAKO A/S, Glostrup, Denmark). After 1 hour of blocking in 1% milk, 0.25% Triton X-100/PBS buffer, cells were incubated for 1 hour with primary antibody monoclonal anti-smooth muscle actin 1:50 and monoclonal anti-MyoD 1:50 (DAKO). After washing in 1% milk PBS buffer, secondary antibody HRP conjugate (Jackson) was added for 45 minutes. Visualization was achieved through the peroxidase AEC system (DAKO). Negative controls were performed with purified mouse IgG and yielded no staining. anti–β-MHC antibody was kindly given by Dr D. Mornet, INSERM, Montpellier, France.

Transmission Electron Microscopy

Cells were washed in phosphate buffer and fixed with 3% glutaraldehyde. Samples were postfixed with 0.5% osmium, rinsed, dehydrated, and embedded in araldite (Durcupan, Fluka). Semithin sections (1.5 μm) were cut with a diamond knife and stained lightly with 1% toluidine blue. Semi-thin sections were re-embedded in an araldite block and detached from the glass slide by repeated freezing (liquid nitrogen) and thawing. The block with semithin section were cut in ultrathin (0.05 μm) sections with a diamond knife, stained with lead citrate, and examined under a Jeol 100CX electron microscope.

Electrophysiological Recordings

Cellular electrical activity was recorded at room temperature (20 ± 2°C) to reduce beating activity in current-clamp by using the whole cell configuration.[16] The bath solution had the following composition (in mmol/L): NaCl 140, KCl 5.4, MgCl$_2$ 1, CaCl$_2$ 1.8, BaCl$_2$ 1, MnCl$_2$ 2, HEPES 5.5, and glucose 5. Pipette solution contained (in mmol/L): KCl 40, NaCl 10, CaCl$_2$ 2, MgATP 2, GTP 0.3, EGTA 5, and HEPES 10. Action potentials (AP) data were acquired using an Axopatch-200 patch-clamp amplifier (Axon Instruments). Pipettes (3 to 4 MΩ) were prepared on a horizontal pipette puller (Sutter Instrument CO). Spontaneous electrical activity
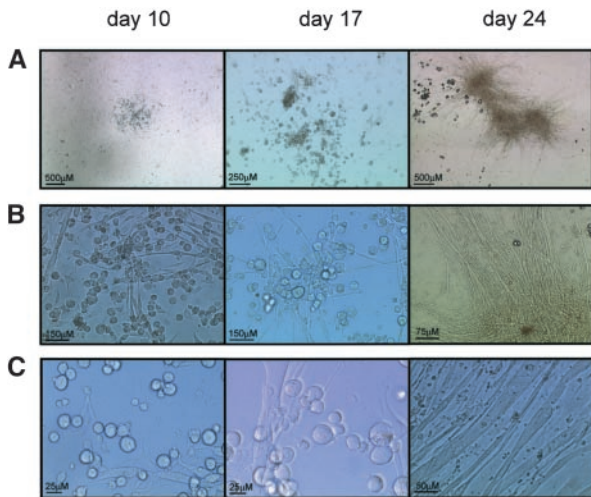
Figure 1. Phase-contrast microscopy of contracting clones at days 10, 17, and 24. Isolated SVF cells were plated into methylcellulose (7000 cells per milliliter) and observed every 2 days under an inverted phase-contrast microscope at ×2.5 (A), ×10 (B), and ×20 (C). Clones gradually increased in size with time due to cell proliferation. Early cells were rounded (day 10), then elongated cells appeared (day 17), and the mature clone was essentially composed of aligned and branched myofibrils (day 24).

appeared between −40 and −60 mV. AP amplitude, time-to-peak, half amplitude, AP duration at 50% repolarization, rise time 10% to 90%, and instantaneous frequency were determined using the program Clampfit 9 (Axon Instruments).

Pharmacological Studies

Elongated fibers with a regular contractile activity were selected and the basal beating rate was recorded before and after replacement of methylcellulose culture medium by DMEM-F12 medium containing 10% of newborn calf serum (Life Technologies). Chronotropic responses were then assessed in DMEM-F12, NCS 10% by extracellular recording of the beating rate in the presence of the appropriate drug. Dose-response experiments were performed with 0.25 to 5×10^{-6} mol/L isoproterenol, 5 to 20×10^{-6} mol/L propranolol, 0.125 to 2.5×10^{-6} mol/L carbamylcholine, and 0.5 to 5×10^{-6} mol/L atropin. Antagonist was added after the maximal dose of agonist. The values were represented as mean±SEM.

Videotape Recording

The cultured cells were observed through an inverted-type phase-contrast video microscope (Leica) equipped with a CCD camera (Zeiss).

Results

Morphological Analysis

Isolated SVF cells from adipose tissue were directly plated in semisolid methylcellulose medium (MethoCult GF M3534) without previous cell expansion or culture selection. From 6 days of culture, emergence of various cell morphologies were identified: clusters of preadipocytes/adipocytes, large spreading cells with a highly branched shape and fibroblast-like appearance (data not shown), and clones of rounded cells together with small tube cells (Figure 1, day 10). From the latter, some rounded cells independently started a contractile activity at days 11 to 14 after plating. Within a few days, myotube-like structures appeared, grew in size and proliferated, and were still surrounded by some of the rounded cells
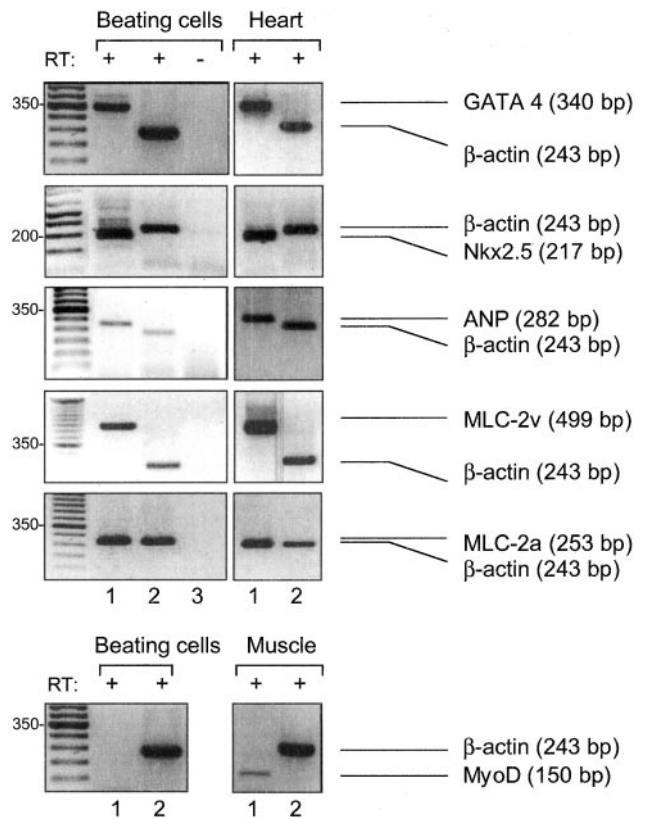

Figure 2. Expression of cardiac-specific genes and lack of skeletal mRNA in cells from contracting clones analyzed by RT-PCR. Cells from contracting clones were picked up and gene expression was compared with control cells from mouse heart (ventricle or atrium) and muscle. RT-PCR of indicated gene (lane 1) compared with β-actin control (lane 2) and to RT-negative control (lane 3) was performed. Expression of cardiac-specific mRNA, ie, GATA4, Nkx 2.5, ANP, MLC-2v, and MLC-2a was evidenced whereas MyoD mRNA was not detected. Results are representative of 3 to 5 separate experiments.

(Figure 1, day 17). After 20 to 30 days, the areas gave rise to a cohesive group of cells, with the presence of branching fibers and sharing tight connections. At this time, the entire area was beating at a single rate (Figure 1, day 24). The emergence of contracting clones was independent of the presence of 5-aza-2′-deoxycytidine and occurred whatever the tested adipose deposit was. However, the percentage of beating clones counted at day 20 varied according to the localization of the adipose tissue used to prepare SVF cells (from 0.02% to 0.07% of plated naïve SVF cells purified from interscapular and inguinal deposits, respectively). Another observation was that after starting beating, cells could be maintained for a long time (several months) in methylcellulose or in standard liquid medium.

Evidence for Cell Development Into Various Cardiac-Like Phenotypes

In order to define the phenotype of contracting cells at molecular level, clones were picked up from methylcellulose culture by micropipette. Expression of several cardiac-specific genes was assessed using RNAs from atrium and ventricle as positive control. Figure 2 showed that beating
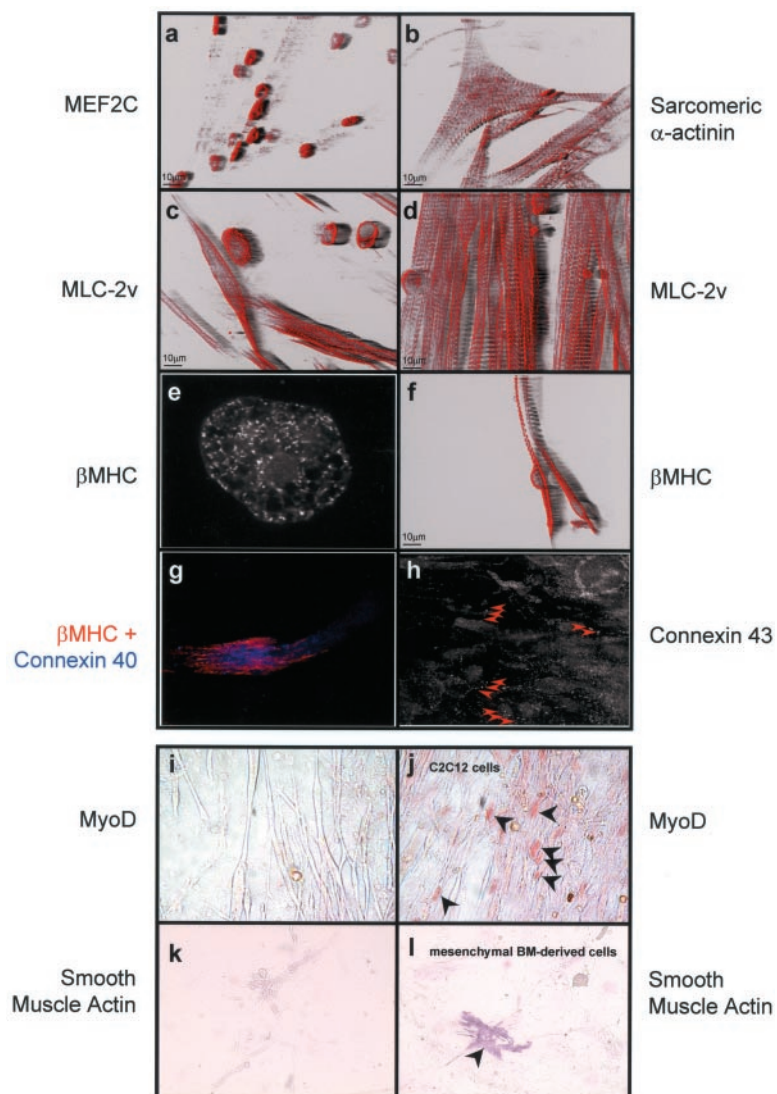
Figure 3. Immunostaining of contracting clone cells for muscle-specific proteins. Cells in contracting clones were specifically stained with anti-myosin enhancing factor 2C (MEF2C) (a), anti-sarcomeric α-actinin (b), anti-myosin light chain-2v (MLC-2v) (c and d), anti–β-myosin heavy chain (βMHC) (e, f, and g, red), anti-connexin40 (g, blue), and anti-connexin43 (h) antibodies. Tridimensional representation of contractile proteins emphasized cell ultrastructure (a through d and f). Red arrows point out specific location of proteins (h). No specific staining was obtained with the anti-MyoD (i) and anti-smooth muscle actin (k) antibodies compared with positive controls labeling nuclei of differentiated C2C12 cells (j, black arrows) and mesenchymal bone marrow–derived myofibroblasts (l, black arrows). Results are representative of 3 to 6 separate experiments.

clones expressed several cardiac-specific mRNA such as transcription factors, GATA-4 and Nkx2.5, the ventricular and atrial myosin light chain MLC-2v and MLC-2a, respectively, and a secretion product, the atrial natriuretic peptide (ANP). Muscle phenotype can be excluded because of the lack of MyoD mRNA detection (Figure 2). These data strongly argued for the cardiomyocyte nature of the contracting cells.

To further characterize cells present in contracting areas, immunocytochemistry experiments were performed. A specific positive staining was obtained with antibodies against the myosin-enhancing factor 2C (MEF2C, Figure 3a), the sarcomeric α-actinin (Figure 3b), the contractile protein MLC-2v (Figures 3c and 3d), the β-myosin heavy chain (βMHC, Figures 3e and 3f), and connexins (Figures 3g and 3h). In contrast, antibodies raised against the skeletal muscle protein MyoD (Figure 3i) or the smooth muscle actin (Figure 3k) did not stain the contracting cells compare to positive controls, ie, differentiated C2C12 cells (Figure 3j) and mesenchymal bone marrow–derived myofibroblasts (Figure 3l), respectively. Consistent with RT-PCR studies, these results

supported that cells identified in contracting clones were cardiomyocyte and not skeletal or smooth muscle cells.

In respect to cell shape and protein detected, the pattern of myofibrillar assembly was then analyzed by ultrastructural analysis (Figure 4). Electron microscopy revealed mononuclear cells and multinuclear cells (Figures 4A and 4E) with myofibrillar bundles oriented in a regular manner (Figures 4B and 4C). Nuclei of these multinuclear cells presented very similar morphology: nuclei were in the center of the myotubes with multiple nucleoli and laxe chromatin (Figures 4B and 4E). Cytoplasm had long mitochondria but presented a few organelles. Nuclei with a wrinkled surface and invaginations were also detected. Cells contain myofibrils with the definitive ultrastructure of an organized sarcomere with typical cross-striation and developed Z bands (1.2 to 1.3 μm) as shown in Figure 4D. The degree of myofibrillar organization varied within different areas and in certain areas, cell bifurcations further suggested these multinucleated cells to be cardiomyocytes.

In conclusion, such clonogenic culture conditions promoted SVF-derived cells to develop into ventricle-like cells
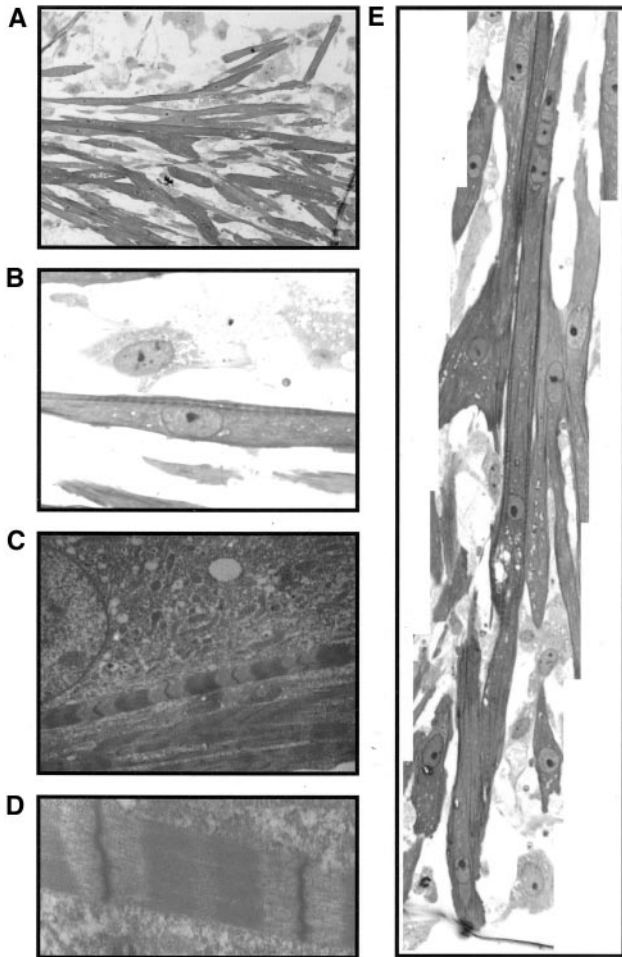
Figure 4. Ultrastructural analysis of contractile clone cells. Isolated SVF cells were plated in methylcellulose for 20 days, and transmission electron micrographs of SVF-derived beating colonies were performed and represented: semi-thin cut (A) low-power transmission electron micrographs (B and C), high-power micrograph with organized sarcomere between Z-lines (D), and low-power transmission electron micrographs that reconstitute an entire fiber (E).
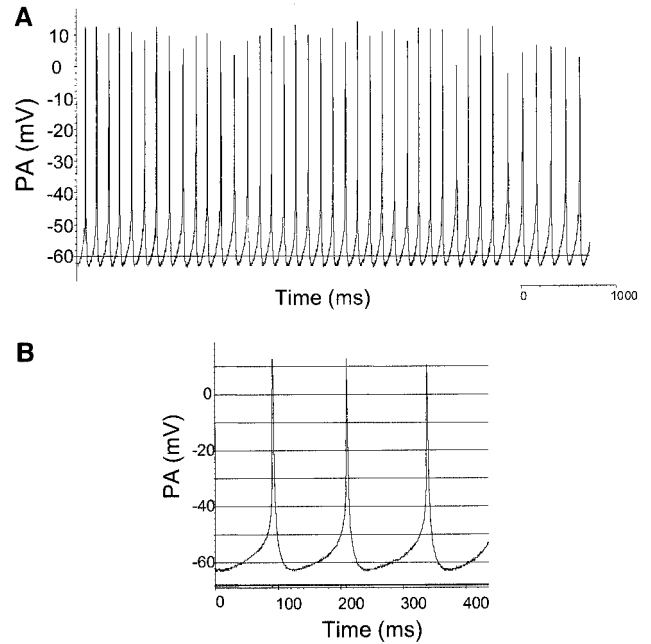

Figure 5. Electrophysiological analysis of early contractile clone cells. Generation of spontaneous action potential after application of a potential of −60 mV. Firing rate was 66 bpm (A). Details of 3 individual action potentials (B).

(presence of MLC-2v and connexin43), atrial-like cells (presence of MCL-2a, ANP, and connexin40) and pacemaker like-cells inducing the spontaneous contractions.

Evidence for Pacemaker Activity

To provide additional information regarding to the spontaneous beating activity, we performed electrophysiological studies very early in the time course of SVF cells culture (1 to 2 weeks after plating). At this time,, individualized cells could independently be recorded in methylcellulose culture. Cellular electrical activity was recorded on cells in current-clamp by using the whole cell configuration. Under current clamp, adipose-derived cardiomyocytes displayed spontaneous as well as triggered action potentials. Spontaneous electrical activity (instantaneous frequency=7±1Hz, n=4) appeared between −40 and −60 mV (Figures 5A and 5B). The calculated characteristics of this pace maker activity were as follows: peak amplitude=68±5 mV, time-to-peak=62±0.3 ms, half amplitude=34±3mV, time-to-half-amplitude

rise=60±1ms, and rise time 10% to 90%=31±9 ms (n=4). The shape of this action potential was characteristic to cardiac pacemaker cells.

Pharmacological Studies

To test the functionality of the putative cardiomyocytes, we examined the chronotropic response of mature contracting fibers to several agents known to control the heart rate. Pharmacological studies were performed on 30-day-old beating cells where the ventricle-like morphology predominated. As expected for cardiomyocyte-like cells, the β-agonist isoproterenol induced a dose-dependent increase of the spontaneous contraction rate measured as bpm (Figure 6A). Propranolol, a nonselective β-adrenergic antagonist, reversed the isoproterenol-induced acceleration (Figure 6A). By contrast, increasing concentrations of carbamylcholine, a nonselective acetylcholine agonist, significantly decreased to finally stop the spontaneous contractions (Figure 6B). Stopped cells started again to contract when atropin, a muscarinic antagonist, was added (Figure 6B). These chronotropic responses definitively demonstrated that these cells responded like functional cardiomyocyte-like cells. When analyzing the behavior of the different clones, a great heterogeneity appeared in the sensitivity to the different drugs. For instance, some cells that did not contract in control experiment started to beat under β-adrenergic stimulation (data not shown). However, when cells were responsive, similar pattern toward the drugs was always observed.

Discussion

This study is the first one to describe the spontaneous emergence of functional cardiomyocyte-like cells from adult tissues in primary culture. All molecular and functional
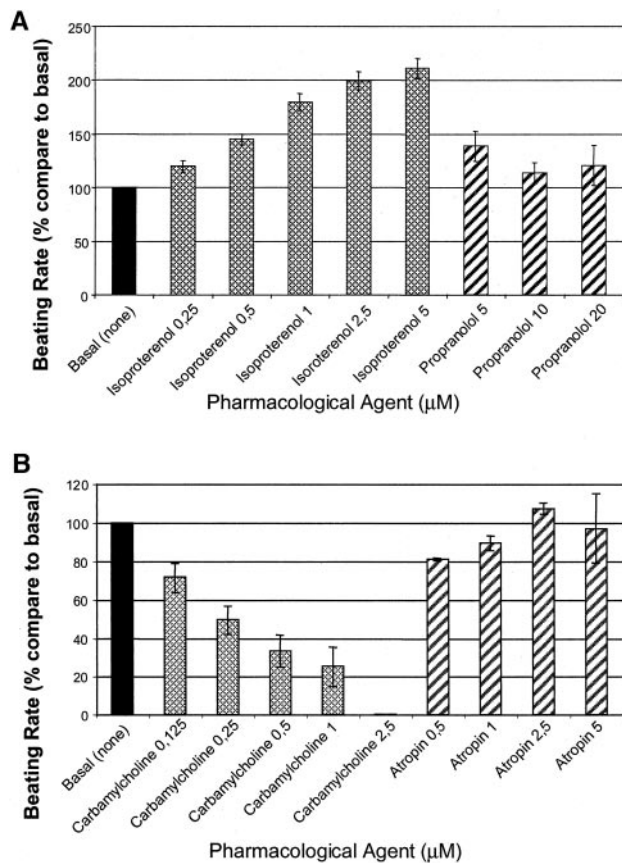
Figure 6. Chronotropic response of contracting cells to adrenergic and cholinergic stimulation. Isolated SVF cells were plated into methylcellulose for 30 days, then methylcellulose was gently removed and replaced by liquid medium DMEM-F12 10% NCS. Basal beating rate was recorded before and after methylcellulose replacement. Contracting rate was measured under treatment with the β-adrenergic agonist isoproterenol and antagonist propranolol (A) and with the cholinergic agonist carbamylcholine and muscarinic antagonist atropin (B). Results represent the mean of 3 to 9 separate experiments.

features are consistent with this conclusion and definitively exclude skeletal or smooth muscle phenotype. SVF-derived cells failed to express the skeletal marker MyoD and the smooth muscle actin. In contrast, these adipose tissue-derived cells expressed the cardiac-specific transcription factors Nkx2.5, GATA4, and MEF2C, the structural cardiac protein βMHC, as well as late-stage cardiac specification proteins MLC-2v, MLC-2a, and ANP.

Our results identified various cell shapes all along the clone development that could correspond to the shift cardiomyocyte undergoes from early to terminally differentiated stages. Early cells in contracting areas were roundish myocytes and changed into elongated fibers with sarcomeric-organized myofibrils. The morphological heterogeneity observed could also be correlated with the contractile proteins rearrangement occurring during cell maturation. For instance, myosin MLC-2v and βMHC organization evolved from circular or spots in rounded early cells (Figures 3c and 3e) to complete sarcomeric structures and oriented fibers in elongated differentiated cells (Figures 3d through 3f). In very late cultures, mature contractile cells were linked by tight connections evidenced with the presence of

connexins (Figure 3). Furthermore, at early stage of differentiation, they displayed electrophysiological properties (ie, pacemaker-like activity), characteristic of intermediate stage cardiomyocytes as previously defined in embryonic stem cell-derived cardiomyocytes.[17]

In contrast to all previous reports on medullar mesenchymal stem cells in vitro, this cardiomyocyte differentiation was achieved with the lack of 5-azacytidine as described for cardiomyocyte differentiation of embryonic stem (ES) cells.[6,17] Compared with cell culture usually performed in liquid medium, we chose the semisolid methylcellulose medium known to support hematopoietic clonogenic expansion that allowed 3-dimensional structure and microconcentration of cells.[18] Although the nature of the medium promoted contractile clone development, it cannot by itself explain our results. The same culture conditions failed to reveal cardiomyocyte potential of murine bone marrow stroma cells (data not shown). In addition, beating cells were observed in liquid medium even though the occurrence of such event was rare. To argue about the role of hematopoietic cytokines present in the methylcellulose medium, ie, IL3, IL6, and SCF, preliminary data suggested they were not absolutely required but seemed to enhance cardiomyocyte-like cells differentiation. Preliminary experiments indicated that contracting clone differentiation was achieved in minimum as well as in complete methylcellulose medium. The major difference came from the size and the emergence that was delayed in the very first steps when cultured in minimum medium. However, no more significant differences were observed between minimum and complete methylcellulose culture conditions at day 20 after plating. Nevertheless, cytokine effects and their interplay need further investigation to be finely characterized.

Previous reports described adipose tissue as a possible source of pluripotent stem cells and the differentiation of SVF cells toward the osteogenic, adipogenic, myogenic, neurogenic, chondrogenic, and hematopoietic lineages.[13,19] Our data are consistent with these findings. The cardiomyocyte phenotype was spontaneously obtained from cells derived from adipose tissues raising the hypothesis that putative cardiogenic progenitors reside in the tissue ready to express their potential in some specific conditions that need to be determined. Thus, a better understanding of cardiomyocyte differentiation from adipose-derived cells is required to foresee the use of adipose stroma–derived cardiomyocytes in cell based therapy of heart failure.

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