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The Expression of *NPPA* Splice Variants During Mouse Cardiac Development

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Natriuretic peptide precursor-A (*NPPA*) is an early and specific marker for functional myocardium of the embryonic heart. *NPPA* gene encodes for a precursor of atrial natriuretic peptide (ANP). So far, three alternatively spliced variants have been reported for *NPPA* in human. In mouse, no alternatively spliced transcript of *NPPA* has been reported. In the current study, we investigated the expression of *NPPA* gene during cardiac differentiation of mouse adipose-tissue-derived stem cells (ADSCs) and embryonic stem (ES) cells. As revealed by reverse-transcription polymerase chain reaction analysis, 2-week-differentiated cells expressed some cardiac-specific makers, including *ANP*. Three additional intron-retained splice variants of *NPPA* were also detected during cardiac differentiation of the ADSCs and ES cells. In addition, we detected three intron-retained splice variants of *NPPA* in 8.5-day mouse embryonic heart. In the mature cardiomyocytes of 1-week-old mice, only the correctly spliced isoform of *NPPA* gene was expressed. Freshly isolated stromal vascular fraction also expressed one intron-retained isoform of *NPPA* gene. In conclusion, our findings have provided evidence for the expression of intron-retained splices of *NPPA* mRNA during the early stages of mouse cardiogenesis as well as in the mouse adipose tissue.

Introduction

Natriuretic peptide precursor-A (*NPPA*) is an early and specific marker for functional myocardium of the embryonic heart (Houweling *et al.*, 2005). *NPPA* gene encodes for a precursor of atrial natriuretic peptide (ANP) that has a regulatory effect on blood pressure (Newton-Cheh *et al.*, 2009). In cardiomyocytes, ANP is translated from the *NPPA* gene as prepro-ANP. This 151-amino-acid peptide is then cleaved by signal peptidase to produce signal peptide and pro-ANP (126 amino acids). Pro-ANP is stored in the dense core secretory granules, and at the time of secretion, it is cleaved by the enzyme corin to produce N-terminal (NT)-pro-ANP and bioactive ANP. All three components of prepro-ANP, including signal peptide, NT-pro-ANP, and bioactive ANP, are measurable in the circulation (Yandle, 1994; Martoglio *et al.*, 1997; Dultz *et al.*, 2008; Pemberton *et al.*, 2012).

The expression pattern of *NPPA* during development of cardiac chamber is highly dynamic (Zeller *et al.*, 1987; Houweling *et al.*, 2005). So far, three alternatively spliced variants have been reported for *NPPA* mRNA in human, including a correctly spliced transcript and variants with either retained intron 1 or retained introns 1 and 2. The correctly spliced variant has its highest expression level in heart, while it is moderately expressed in some other tissues, including prostate, pancreas, and small intestine (Annilo *et al.*, 2009). In mouse, no alternatively spliced isoform of *NPPA* gene has been reported previously (Annilo *et al.*, 2009).

Stem cells have been used as valuable models to investigate patterns of gene expression during cellular differentiation. In the current study, we studied the expression profile of *NPPA* gene during cardiac differentiation of mesenchymal and embryonic stem (ES) cells. For this purpose, ES cells were differentiated at the presence or absence of bone morphogenetic protein-4 (BMP4). Cardiac differentiation of the adipose-tissue-derived stem cells (ADSCs) was induced by 5-azacytidine or BMP4, which are known cardiogenic factors (Schultheiss *et al.*, 1997; Rangappa *et al.*, 2003b; Zhang *et al.*, 2005; Taha and Valojerdi, 2008), as well as by direct or indirect coculture with mouse cardiomyocytes.

As previously discussed (Rangappa *et al.*, 2003a), chemical and physical components of the microenvironment play a critical role in determining the ultimate fate of developing cells. In direct coculture of ADSCs with viable cardiomyocytes, both chemical and physical factors can influence the differentiation of ADSCs (Rangappa *et al.*, 2003a). However, when the ADSCs are cocultured with paraformaldehyde-fixed cardiomyocytes, just the physical contact between the ADSCs and cardiomyocytes can affect and direct the differentiation (Badorff *et al.*, 2003). In an indirect coculture method using culture inserts, there is no physical contact between different cell types, and they can influence each other only by soluble factors (Liu *et al.*, 2012). Using these models, we identified three alternatively spliced transcripts of *NPPA* for the first time. We further isolated the cardiac area of 8.5-day mouse embryos and showed the

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expression of intron-retained isoforms of *NPPA* mRNA at this early stage of embryonic heart development.

Materials and Methods

Isolation and culture of mouse ADSCs

All animal works conducted in this study were according to the regulations of animal care and use committee of the National Institute of Genetic Engineering and Biotechnology. Inguinal fat pads of 10–12-week-old NMRI mice were isolated and digested using 2 mg/mL collagenase A (Roche Applied Science), as described previously (Taha and Hedayati, 2010). Isolated cells were plated at 5×10^4 cells/mL in 6-cm tissue culture plates and cultured in a growth medium containing Dulbecco's modified Eagle's medium (DMEM) and 20% fetal bovine serum (FBS) (both from Gibco®, Life Technologies). The cells were daily observed and passaged after reaching 80–90% confluency. The culture media were changed every other day.

Cardiac differentiation of the ADSCs

Third-passaged ADSCs were cultured and propagated to a confluent state. Cardiac differentiation of the ADSCs was induced either by 5-azacytidine or BMP4 treatment or by coculture with mouse cardiomyocytes.

5-Azacytidine treatment

5-Azacytidine (Sigma) with a final concentration of 10 μM was added to the culture medium for 24 h. ADSCs were cultured in DMEM plus 10% FBS.

BMP4 treatment

The confluent ADSCs were cultured in a medium containing 15% Knockout™ Serum Replacement and 10 ng/mL bone morphogenetic protein 4 (BMP4; Sigma) during the first 5 days of differentiation. After this period, BMP4 was removed, and differentiation of the ADSCs was continued in DMEM containing 10% FBS without any additives.

Coculture of the ADSCs with mouse cardiomyocytes

Ventricular cardiomyocytes were isolated from the hearts of 4–7-day-old mice. The hearts were surgically removed, collected in tissue culture plates, and rinsed in cold PBS to remove blood and cell debris. Ventricles were dissected, minced, and dissociated by 0.2% collagenase II in PBS containing 1% BSA (Sigma). Cell suspension was transferred to gelatinized tissue culture plates and cultured for 1–1.5 h to remove cardiac fibroblasts. The same procedure was repeated.

Monolayers of ventricular cardiomyocytes were prepared by culturing the cells in gelatinized tissue culture plates and cocultured with third-passaged ADSCs directly by cell–cell contact or indirectly using culture inserts. For the direct coculture, monolayers of the cardiomyocytes were fixed by incubation with 2% paraformaldehyde, and after three washes with PBS, third-passaged ADSCs were cultured on the top of these cells (Badorff *et al.*, 2003). As mentioned by Badorff *et al.* (2003), paraformaldehyde-fixed cells cannot fuse with other cells but provide an intact cell surface for coculture. After coculture, differentiated ADSCs were isolated by a brief trypsinization. Then, the cells were cultured in gelatinized tissue culture plates for 24 h. Immediately after attachment of the ADSCs, the cultures were washed to remove all nonviable unattached cells. In this way, only the differentiated cells are retained and paraformaldehyde-fixed cardiomyocytes are eliminated.

For the indirect coculture, about 8×10^4 cardiomyocytes were seeded on 0.4-μm porous Transwell-Clear inserts (Greiner Bio-One GmbH). Culture inserts were placed in six-well tissue culture plates containing third-passaged ADSCs (Liu *et al.*, 2012). Medium of both the coculture systems consisted of DMEM and 10% FBS (both from Gibco®, Life Technologies). Third-passaged ADSCs were cocultured with the same cells served as the control group.

Cardiac differentiation of ES cells

The mouse ES cell line Royan B1 (Royan Stem Cell Bank, Royan Institute, RSCB0001) was cultured on top of a feeder layer of mitomycin C-treated mouse embryonic fibroblast (MEF) at the presence of leukemia inhibitory factor (LIF; Chemicon, ESGRO), as previously described (Taha *et al.*, 2007). The ES cells were dissociated from the feeder layer, and differentiation was initiated with embryoid body (EB) formation using hanging-drop method (Taha *et al.*, 2007). In summary, 20 μL of hanging drops containing about 800 cells was cultured for 2 days and then EBs were maintained in suspension culture for 5 days. Seven-day-old EBs were plated in 0.1% gelatin-coated tissue culture plates and cultured for 3 weeks. Differentiation medium consisted of Knockout™ Dulbecco's modified Eagle's medium (Ko-DMEM, high-glucose, with sodium pyruvate; Gibco®, Life Technologies), 15% FBS (FBS, ES cell qualified; Gibco®, Life Technologies), 0.1 mM β-mercaptoethanol (Sigma), 1 mM L-glutamine, 1% nonessential amino acid stock, and 1% penicillin-streptomycin (all from Gibco®, Life Technologies). Some EBs were also treated with 10 ng/mL BMP4 during the suspension stage.

Reverse-transcription polymerase chain reaction

Total RNA was extracted from the cells using High Pure RNA Isolation Kit (Roche Applied Science) and treated with DNase I (Roche Applied Science) according to the manufacturer's instructions. For cDNA synthesis, 1 μg of total RNA was reverse transcribed to cDNA using oligo-dT primers and RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific, Inc.). cDNA samples were used in PCR using primers described in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/dna). Negative controls lacking either template or enzyme were included in the reverse-transcription polymerase chain reaction (RT-PCR) experiments. The PCR products were size fractionated by electrophoresis on 2% agarose gel.

Sequencing analysis

PCR products were extracted from the agarose gel using Accuprep Gel Purification Kit (Bioneer) and sequenced (Macrogen, Inc.). DNA sequencing results were visually analyzed by Sequence Scanner v1.0 (Applied Biosystems) aligned to the mouse genome using BLAST® alignment tool

(NCBI, NIH). The novel sequences detected in this study were submitted to GenBank database and were acquired accession numbers (KC526925, KC526926, and KC526927).

Immunocytochemistry

Differentiated cells were fixed by incubation with 4% paraformaldehyde, permeabilized by 0.5% Triton X-100 (Sigma), and blocked with 10% goat serum (Gibco®, Life Technologies). The cells were incubated with primary and secondary antibodies for 45 min each at 37°C. Antibodies used in this study included monoclonal antibodies for cardiac muscle troponin-I (CTnI; Chemicon) and anti-mouse FITC-conjugated IgG (Sigma), both with dilution of 1:100. Preparations were examined and photographed by an inverted fluorescence microscope connected to a high-resolution digital camera (Eclipse TE 2000U; Nikon).

Results

Isolation and culture of mouse ADSCs

After three passages, the ADSCs developed a homogenous fibroblast-like morphology (Supplementary Fig. S1A). We previously studied the molecular phenotype of the third-passaged ADSCs and showed that more than 98% of the cells express CD29 (beta-1 integrin), CD44 (hyaluronate receptor), and CD105 (endoglin) (Taha and Hedayati, 2010; Jafarzadeh *et al.*, 2014), which are known mesenchymal stem cell markers (Dominici *et al.*, 2006; Mitchell *et al.*, 2006; Schaffler and Buchler, 2007).

Cardiac differentiation of the ADSCs

Cardiac differentiation of the ADSCs was induced by 5-azacytidine, BMP4, or by direct or indirect coculture with mouse cardiomyocytes. During the first week of differentiation, some round-shaped cells appeared in all the experimental groups (Supplementary Fig. S1B). During the next weeks, these cells grew in size and proliferated. Moreover, some cells developed a tube-like morphology (Supplementary Fig. S1C–G).

The expression of cardiac transcription factors and cardiac-specific genes was detected in the heart tissue of 1-week-old mice as the positive control. GATA binding protein-4 gene (*GATA4*) and myocyte enhancer factor 2C (*MEF2C*) were weakly expressed, due to maturation of the cardiomyocytes (Fig. 1).

Third-passaged ADSCs expressed cardiac transcription factors and cardiac-specific genes, including α- and β-myosin heavy chain genes (α- and *β-MHC*), *ANP*, and myosin light chain-2V gene (*MLC-2V*) (Fig. 1). This may reflect potential of the ADSCs for cardiac differentiation. Two weeks after cardiac induction, the expression of *GATA4*, *MEF2C*, *α-MHC, β-MHC,* and *MLC-2V* was detected in all the experimental groups (Fig. 1).

As revealed by immunocytochemistry, ADSC-derived cardiomyocyte-like cells of the control, 5-azacytidine, BMP4, and coculture groups showed positive immunostaining for cardiac troponin-I, which is a cardiac-specific protein (Fig. 2A–E). Cardiomyocytes isolated from the hearts of 1-week-old mice were used as the positive control (Fig. 2F). Immunostaining of the third-passaged ADSCs for cardiac troponin-I protein was negative.

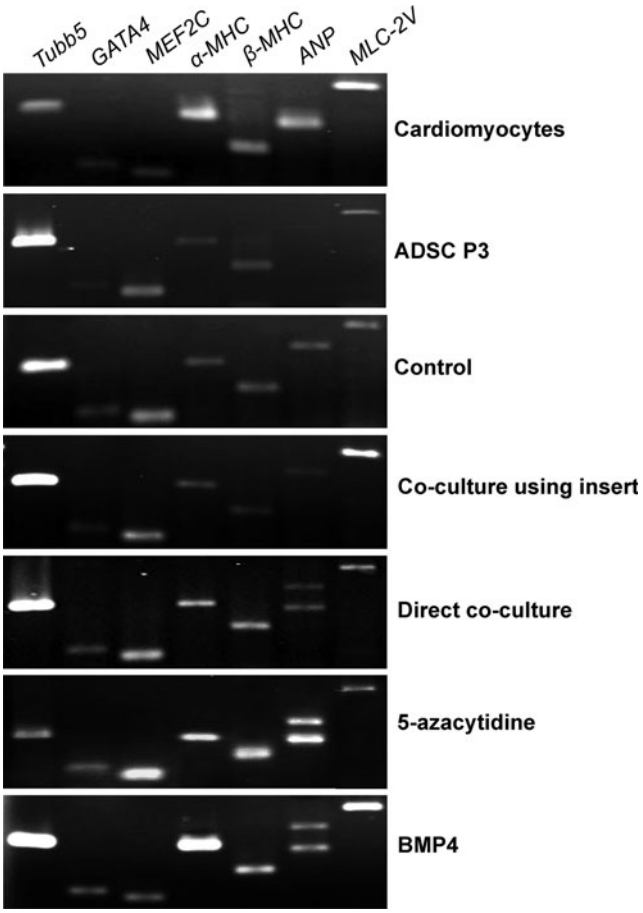

FIG. 1. The expression of some cardiac transcription factors and cardiac-specific genes in 2-week-differentiated, adipose-tissue-derived stem cells (ADSCs). The cardiomyocytes isolated from the hearts of 1-week-old mice were used as the positive control (*first upper row*). Third-passaged ADSCs (ADSC P3) were used as the initiating cells before induction (*second upper row*). The cells differentiated in 10% fetal bovine serum (FBS)–containing medium without any additional factors were used as the control group of differentiation (*third upper row*). Cardiac differentiation of the ADSCs was induced by direct or indirect coculture with mouse cardiomyocytes, as well as by 5-azacytidine and BMP4 treatment (the *last four rows*).

Functionality of the ADSC-derived cardiomyocytes

None of the experimental conditions we used were sufficient for differentiation of the ADSCs into functional beating cardiomyocytes.

The expression of alternatively spliced transcripts of NPPA *during cardiac differentiation of ADSCs*

PCR analysis using the *ANP* primers (F1 and R1 in Fig. 3A) in the control and indirect coculture groups amplified a product longer than the correctly spliced *ANP* mRNA (385 bp instead of 282 bp) (Fig. 1). In the direct coculture, 5-azacytidine, and BMP4 groups, two fragments with the different sizes of 282 and 385 bp were detected (Fig. 1). Sequencing of the 385-bp fragment and BLAST analysis revealed a *NPPA* splice variant with retained intron 1 (Fig. 3B).

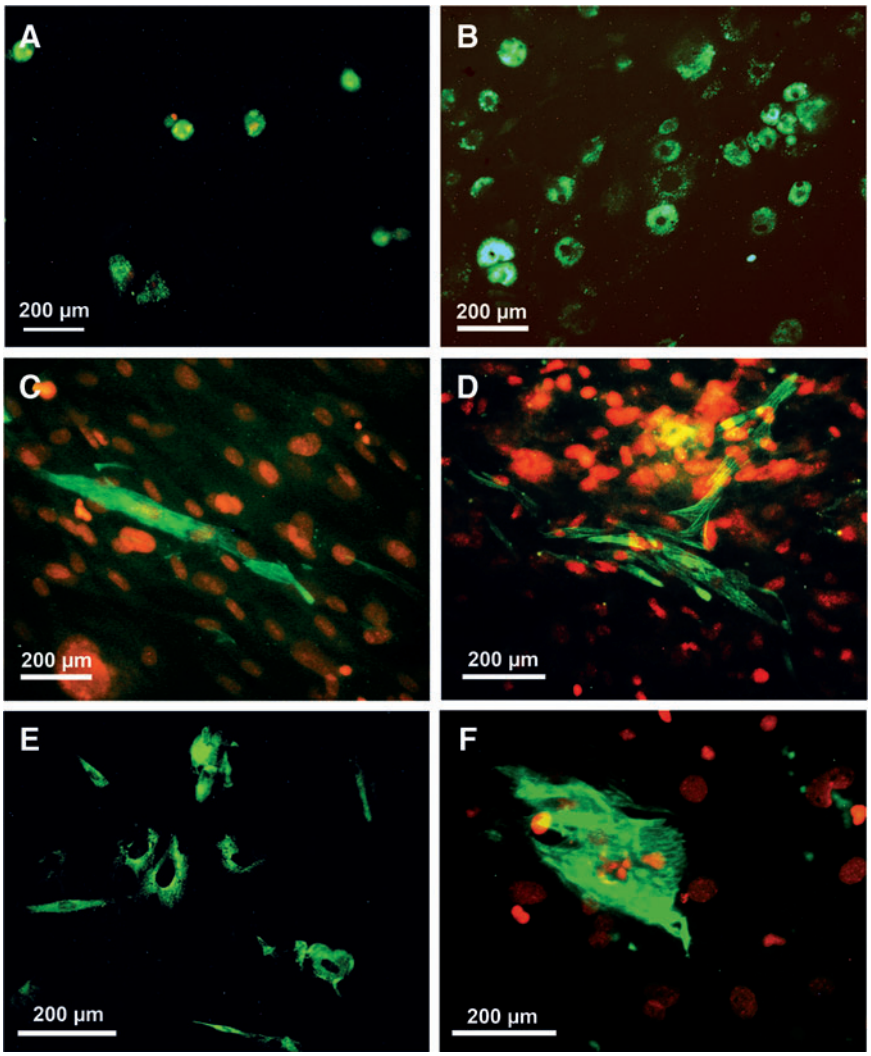

FIG. 2. Immunostaining of the ADSC-derived cardiomyocytes for cardiac troponin-I protein. **(A)** The cells that were differentiated in 10% FBS-containing medium without any additional factors were used as the control group of differentiation. **(B–E)** Three-week-differentiated ADSCs in the indirect coculture, direct coculture, 5-azacytidine, and BMP4 groups, respectively. 5-Azacytidine and BMP4 are known cardiogenic factors. In indirect coculture method, there is no physical contact between the different cell types, and only the soluble factors can influence the differentiation. When the ADSCs are cocultured with paraformaldehyde-fixed cardiomyocytes, just the physical contact between the ADSCs and cardiomyocytes can affect the differentiation. **(F)** The cardiomyocytes isolated from the hearts of 1-week-old mice were used as the positive control. *Green color*: Cardiac troponin-I immunostaining of the cardiomyocytes. *Red color*: Propidium iodide staining of the nuclei **(C–F)**. Color images available online at www.liebertpub.com/dna

Based on these findings, we asked whether *NPPA* splicing depends on the stage of cardiac development. To answer this question, we extended differentiation of the ADSCs in the control and indirect coculture systems for one additional week. At this time, RT-PCR analysis revealed the expression of both correctly and alternatively spliced isoforms of *NPPA* (Fig. 3C).

We also performed additional PCR experiments with the second set of primers, F2 and R2 (Fig. 4A), which detects *NPPA* transcripts with retained introns 1 and 2 (Annilo *et al.*, 2009). PCR using this primer set resulted in the amplification of four products with different sizes. As shown in Figure 4B, the smallest band with the size of 601 bp represents the expression of correctly spliced variant, and the other bands show alternatively spliced variants of *NPPA* gene. Interestingly after 3 weeks of cardiac induction, differentiated cells expressed different splice variants of the *NPPA* gene. When the culture period was extended for another week, the variants of *NPPA* gene were disappeared and only a weakly expressed intron 1-retained variant was observable (Fig. 4C). Beating cardiomyocytes isolated from the hearts of 1-week-old mice only expressed the correctly spliced isoform of *NPPA* (Fig. 4C).

To develop more convincing evidence concerning the expression of *NPPA* splice variants during cardiac differentiation of mouse ADSCs, we assessed the expression of these splices after cultivation of the ADSCs in a noncardiogenic condition. The *NPPA* splice variants were not expressed in the ADSCs cultured in neurogenic differentiation media (Fig. 4C).

To ensure that amplification of the intron-retained splices of *NPPA* gene was not the consequence of contamination with genomic DNA, a third set of primers (F3 and R3) was designed to span exon–exon boundaries (Fig. 5A). These primers specifically attach to the nonintronic segments of cDNA. PCR using this set of primers resulted in the amplification of a 339-bp product (Fig. 5B). Additional PCR experiments were also performed with combinations of the fourth (Supplementary Table S1) and third sets of primers. As shown in Figure 6A, PCR using F3 and R4 primers led to amplification of three products. The first transcript with the size of 355 bp (**a** fragment) corresponds with the correctly spliced variant of *NPPA*. Sequencing and BLAST analysis of the two other fragments revealed that in the second product (**b** fragment; 497 bp), the first 10 bp and the terminal 132 bp of intron 2 were retained (Fig. 6B). The **c** fragment with a size of 745 bp contained full length of intron 2. Using F4 and R3 primers, we detected two separate products, one with the expected size of 358 bp for the correctly spliced

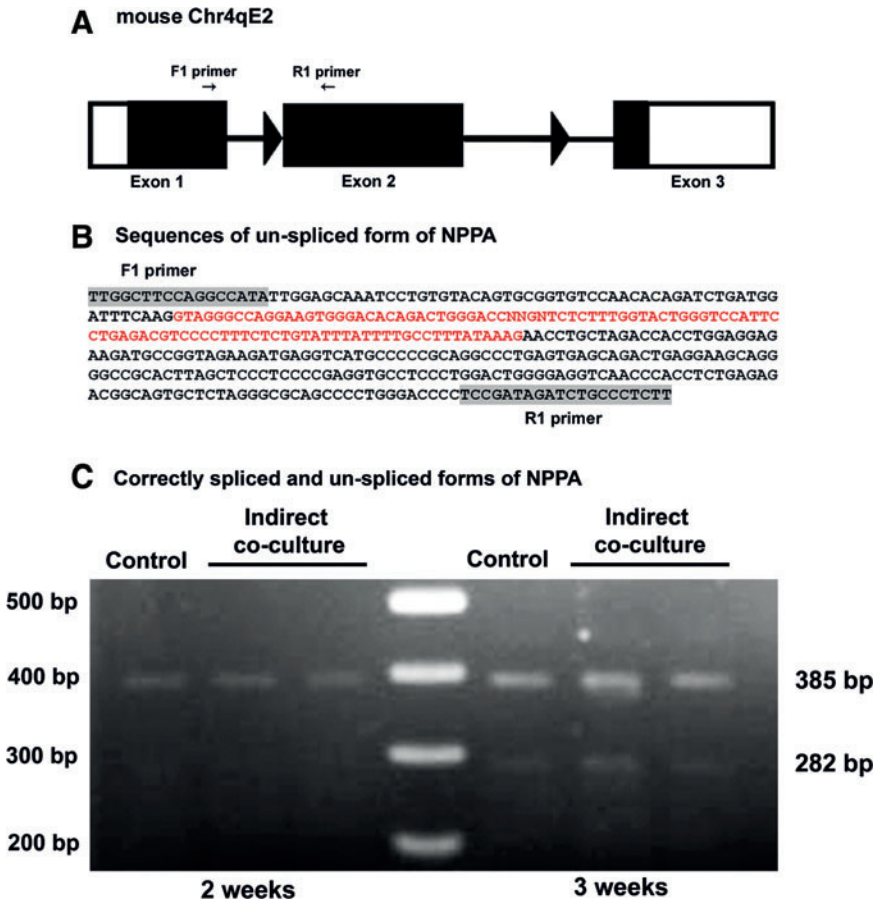

FIG. 3. **(A)** Genomic organization of natriuretic peptide precursor-A (*NPPA*) locus in mouse. *Arrows* marked as F1 and R1 indicate the location of primers and large *arrowheads* indicate the direction of transcription. **(B)** Sequencing of 385-bp PCR product of *ANP* primers revealed an isoform of *NPPA* with retained intron 1 (*red color*). **(C)** The expression of correctly spliced and intron-1-retained isoforms of *NPPA* in 2- and 3-week-differentiated ADSCs. Color images available online at www.liebertpub.com/dna

variant of *NPPA* gene (**d** fragment), and the other with the size of 461 bp (**e** fragment). DNA sequencing and BLAST analysis of the **e** fragment revealed a splice of *NPPA* gene with retained intron 1. Based on these sequencing data, we could identify the four fragments that were amplified by the PCR using F2 and R2 primers (Fig. 4B and Supplementary Fig. S2). The first band with the size of 601 bp represents the correctly spliced variant of *NPPA* gene. The other band with the size of 704 bp corresponds with the intron 1-retained splice of *NPPA* (GenBank accession number: KC526925). The longer *NPPA* transcript with the size of 846 bp contains intron 1 and a 142-bp fragment of intron 2 (GenBank accession number: KC526926). The longest band with the size of 1094 bp represents another *NPPA* variant with retained introns 1 and 2 (GenBank accession number: KC526927). In the case of these intron-retained splices of *NPPA,* the open-reading frame starts with the first methionine and encodes for a predicted protein with 88 amino acids (Fig. 6C). The N-terminal 40 amino acids of this protein are identical to the signal-peptide region and the first 16 amino acids of the prepro-ANP (Fig. 6C). However, due to retention of the intron 1, the coding sequence of prepro-hormone is terminated and the hypothetical peptide chain is continued with a 48-amino acid sequence (Fig. 6C). Therefore, the coding sequence of the alternatively spliced transcripts of *NPPA* does not translate to ANP hormone, but it may result in signal peptide and a different peptide with unknown biological activity.

The expression of NPPA *variants in the freshly isolated stromal vascular fraction*

In the current study, freshly isolated stromal vascular fraction (SVF) expressed an isoform of *NPPA* with retained introns 1 and 2 (Fig. 4D).

The expression of NPPA *splice variants during cardiac differentiation of ES cells*

The expression of *NPPA* splice variants was studied in the ES-cell-derived EBs of the control and BMP4 treatment groups. Two weeks after plating, both groups showed positive immunostaining for cardiac troponin-I (Fig. 7A, B). Nevertheless, spontaneously contracting cardiomyocytes of the control group formed thicker and more branching bundles. When the EBs were assessed for the expression of *NPPA* splices, EBs of the control group showed the expression of correctly spliced variant of *NPPA* mRNA, while the BMP4-treated EBs expressed correctly spliced and intron-1-retained isoforms of *NPPA* mRNA (Fig. 7C).

The expression of NPPA *variants in the mouse embryonic heart*

To assess the expression of *NPPA* splice variants during heart development, the cardiac area of 8.5-day mouse embryos was isolated surgically. Total RNA was isolated and RT-PCR analysis was performed with F2 and R2 primers. This revealed

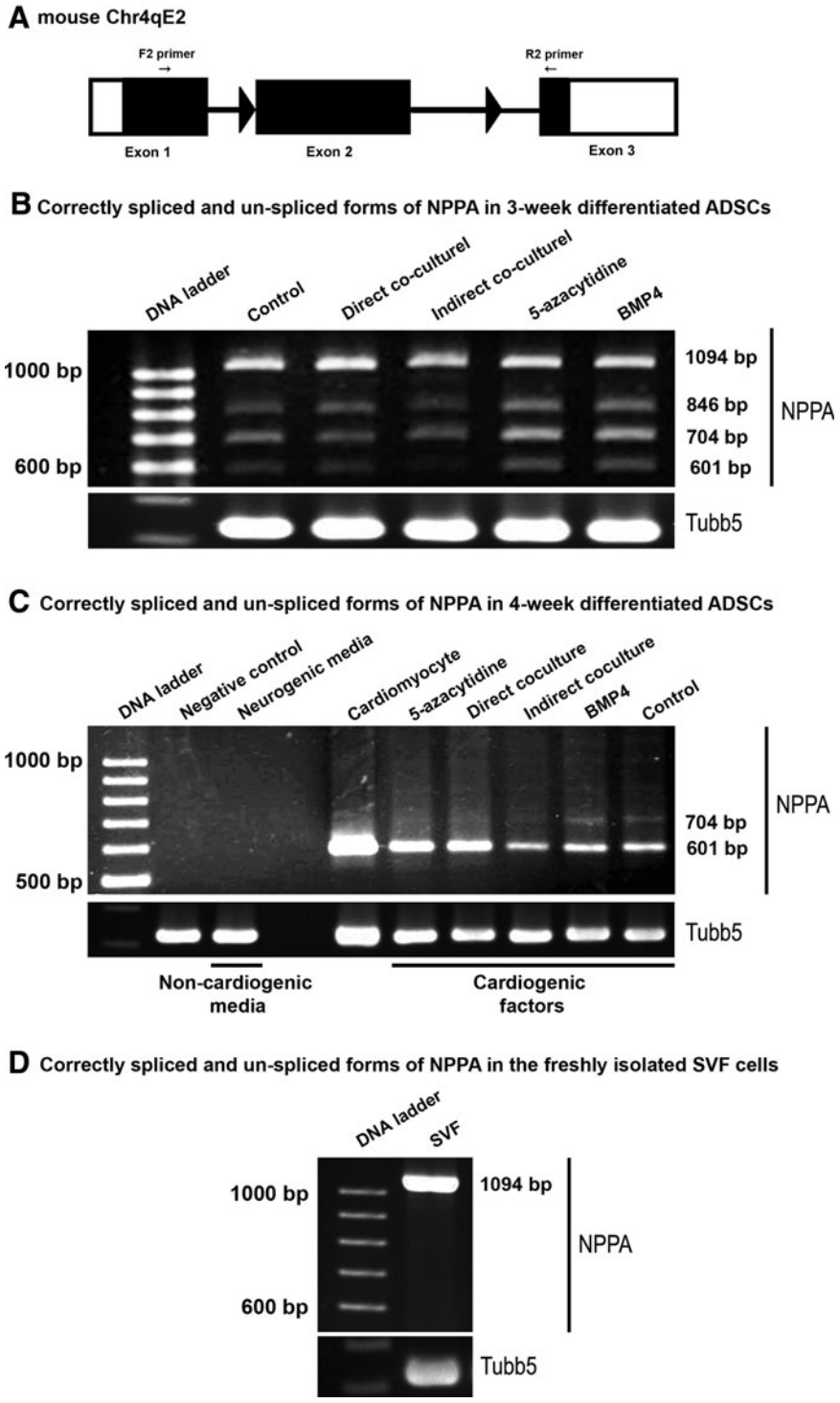

FIG. 4. **(A)** Genomic organization of *NPPA* locus in mouse. *Arrows* marked as F2 and R2 indicate location of the second set of primers. **(B)** The expression of alternatively spliced variants of *NPPA* in the differentiated ADSCs, after 3 weeks and **(C)** after 4 weeks of cardiac induction. PCR was performed using the second set of primers. Neurogenic medium was used as the noncardiogenic condition and mouse cardiomyocytes were used as the control. **(D)** The expression of one intron-retained isoform of *NPPA* in the freshly isolated SVF cells.

the expression of the same four *NPPA* mRNA splices as observed in 3-week-differentiated ADSCs (Fig. 7D).

Discussion

Cardiac differentiation of the ADSCs

Cardiac differentiation of the ADSCs was induced by 5-azacytidine or BMP4, which are known cardiogenic factors (Schultheiss *et al.*, 1997; Rangappa *et al.*, 2003b; Zhang *et al.*, 2005; Taha and Valojerdi, 2008), or by direct or indirect coculture with mouse cardiomyocytes. The role of 5-azacytidine in cardiac differentiation of MSCs has been controversial. Several investigators have reported that 5-azacytidine alone is not sufficient for differentiation of mesenchymal stem cells (Martin-Rendon *et al.*, 2008; Lee *et al.*, 2009; Wan Safwani *et al.*, 2012). In our study, both 5-azacytidine and BMP4 treatment induced the expression of cardiac-specific genes and proteins, but they were not sufficient for differentiation of the ADSCs into functional contracting cells. Some additional growth and differentiation

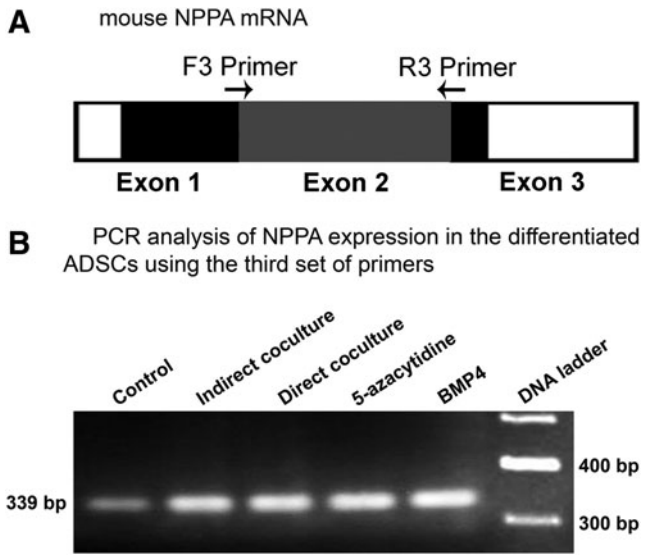

FIG. 5. **(A)** Location of the third set of *NPPA* primers (*arrows*) spanning the exon–exon boundaries. **(B)** The expression of a 339-bp fragment that was amplified using the third set of *NPPA* primers (F3 and R3).

factors may be required to induce functional maturation of the ADSC-derived cardiomyocytes. Moreover, direct and indirect coculture systems induced the expression of some cardiac markers, although neither the chemical niche created by viable cardiomyocytes nor the physical contact with nonviable cells was sufficient for generation of mature cardiac cells. As reported previously (Choi *et al.*, 2010; Metzele *et al.*, 2011), direct contact with the viable cardiomyocytes may be necessary for generation of functional beating cells.

The expression of alternatively spliced variants of NPPA *gene during cardiac differentiation of the ADSCs*

Our study has demonstrated the expression of four alternatively spliced transcripts of *NPPA* after differentiation of ADSCs into immature cardiomyocyte-like cells. Variants with retained intron 1 and retained introns 1 and 2 are similar to those previously reported for *NPPA* in human (Annilo *et al.*, 2009), while the 846-bp transcript that includes intron 1 and a 142-bp fragment of intron 2 is reported for the first time. Since in our culture conditions, the ADSCs could not generate functional cardiomyocytes, it may be suggested that the *NPPA* splice variants are expressed in the early stages of cardiac differentiation.

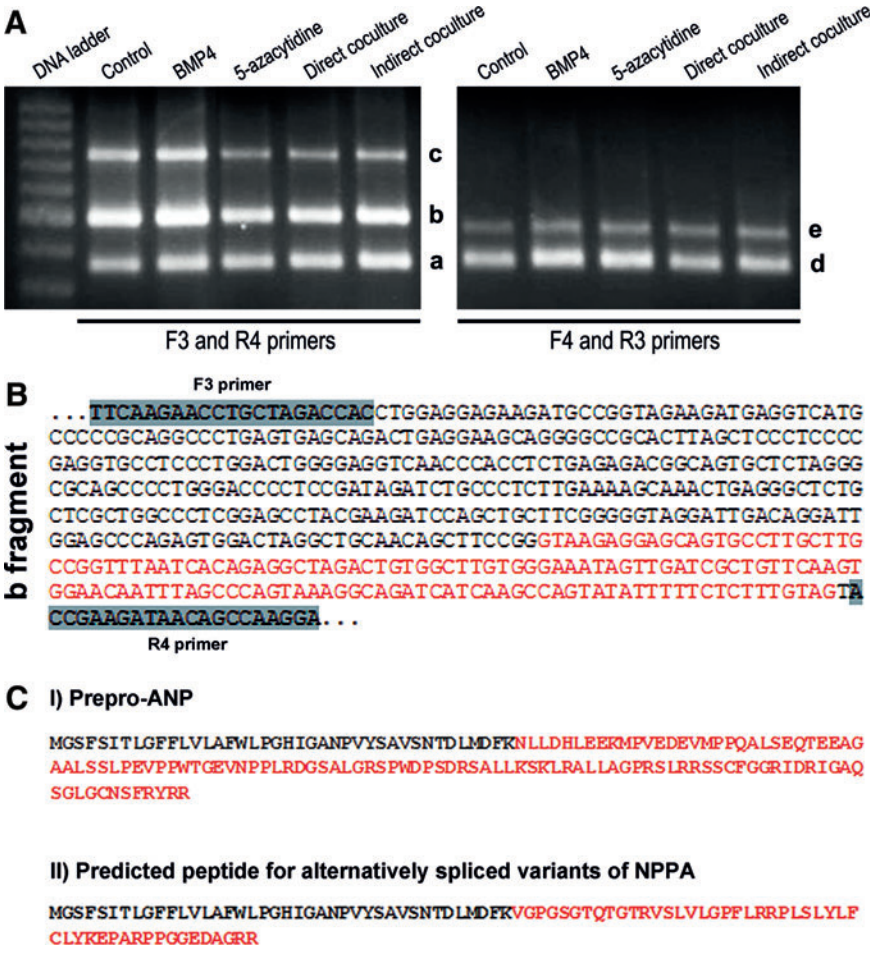

FIG. 6. **(A)** The expression of alternatively spliced variants of *NPPA* in 3-week-differentiated ADSCs. PCR was performed using the second and third set of primers, as indicated in **(A)**. **a** and **d** fragments represent the expression of correctly spliced variants and **b**, **c**, and **e** fragments indicate the expression of alternatively spliced variants of *NPPA*. **(B)** Sequencing of the **b** fragment revealed retention of the first 10 bp and terminal 142 bp of intron 2. Retained part of intron 2 has been marked by *red color* and the position of F3 and R4 primers has been marked by blue-shaded rectangles **(C)**. The amino acid sequence of prepro-ANP **(I)**. Predicted peptide sequence for the alternatively spliced variants of *NPPA* mRNA **(II)**. The N-terminal 40 amino acids of this protein are identical to the signal peptide region and the first 16 amino acids of the prepro-ANP, but due to retention of the intron 1, the coding sequence of prepro-hormone is terminated and the peptide chain is continued with a 48-amino-acid sequence. The different regions of the amino acid sequences in panels I and II have been marked by *red color*. Color images available online at www.liebertpub.com/dna

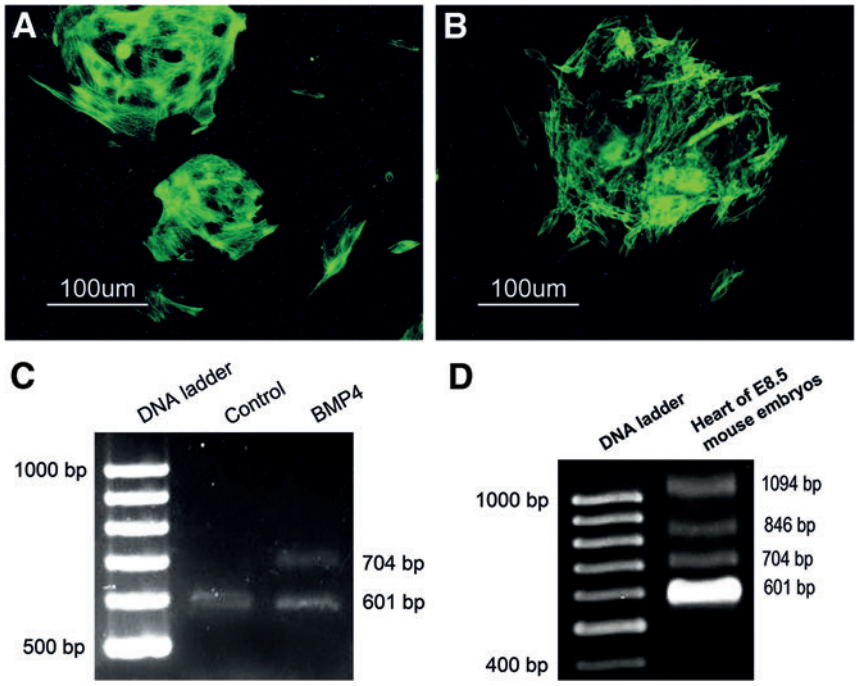

FIG. 7. Cardiac differentiation of mouse embryonic stem (ES) cells. **(A)** Immunostaining of the ES-cell-derived cardiomyocytes of the control and **(B)** BMP4-treated embryoid bodies (EBs) for cardiac troponin-I protein, after 2 weeks of differentiation. **(C)** The expression of two splice variants of *NPPA* mRNA in differentiated EBs of the control and BMP4 treatment groups. **(D)** The expression of *NPPA* splice variants in 8.5-day (E8.5) mouse embryonic heart. Color images available online at www.liebertpub.com/dna

The expression of alternatively spliced variants of NPPA *gene during cardiac differentiation of the ES cells*

We previously studied the role of BMP4 in cardiac differentiation of mouse ES cells and showed that BMP4 treatment decreases population of beating EBs, down-regulates the expression of cardiac-specific genes, and retards the ultrastructural and functional maturation of the ES-cell-derived cardiomyocytes in serum-containing media (Taha *et al.*, 2007). In the current study, we studied the expression of alternatively spliced transcripts of *NPPA* in the EBs of the control and BMP4 treatment groups. As revealed by RT-PCR analysis, contracting EBs of the control group showed the expression of correctly spliced variant of *NPPA* mRNA, while the BMP4-treated EBs expressed both the correctly spliced and intron-1-retained transcripts of *NPPA*. These findings provided more evidence regarding the stage-dependent expression of *NPPA* mRNA splices during cardiac development.

The expression of NPPA *variants in the freshly isolated SVF*

We showed that freshly isolated SVF cells express an isoform of *NPPA* with retained introns 1 and 2. So far, only the expression of one correctly spliced variant of *NPPA* gene has been reported in mouse tissues. Our findings confirm the expression of one intron-retained splice of *NPPA* gene in the adult mouse adipose tissue for the first time.

The expression of NPPA *variants in the mouse embryonic heart*

The cardiac area of 8.5-day mouse embryos was isolated and examined for the expression of alternatively spliced *NPPA* transcripts. PCR analysis revealed the expression of the same four *NPPA* mRNA splices as observed in 3-week-differentiated ADSCs. Based on previous studies, full maturation of mammalian cardiomyocytes only develops postnatally (Viragh and Challice, 1969). This might be a reason for the expression of four *NPPA* mRNA variants in the embryonic heart and the expression of the correctly spliced variant in the cardiomyocytes of 1-week-old mice.

Based on previous reports, *NPPA* expression is tightly regulated during embryonic development of heart (Zeller *et al.*, 1987; Houweling *et al.*, 2005). Annilo *et al.* (2009) found a strong expression of correctly spliced *NPPA* mRNA in human heart and a moderate expression in a number of human tissues, such as prostate, pancreas, and small intestine. In addition, they observed the expression of *NPPA* isoforms with retained intron 1 or retained introns 1 and 2 in several human tissues. However, according to these investigators, the expression profile of *NPPA* in mouse and human was different. In mouse, *NPPA* was strongly expressed in some tissues, such as brain, lung, and liver, where human *NPPA* was expressed weakly. They did not detect any alternatively spliced or intron-retained transcript of *NPPA* in mouse tissues, and this is in contrast to our result concerning the expression of intron-retained transcripts of *NPPA* during embryonic development and in the adult mouse adipose tissue.

Conclusions

In the present study, cardiac differentiation of the ADSCs was induced by 5-azacytidine, BMP4, or coculture with the mouse cardiomyocytes. Differentiated ADSCs expressed some cardiac markers but did not generate functional beating cardiomyocytes. After induction by different cardiogenic factors, ADSCs expressed four alternatively spliced variants of *NPPA* gene. When cardiac differentiation was continued for a longer duration, the expression of different splices of *NPPA* gene was diminished, while the expression of correctly spliced *NPPA* was enhanced. The 8.5-day

mouse embryonic heart expressed both the correctly spliced and three alternatively spliced variants of *NPPA*, while mature cardiomyocytes of 1-week-old mice and ES-cell-derived beating cardiomyocytes expressed the correctly spliced transcript of *NPPA*. The expression of alternatively spliced variants of *NPPA* during cardiac development in mouse has not been reported previously. We also showed for the first time that freshly isolated SVF cells express a splice variant of *NPPA* gene with retained introns 1 and 2. These findings indicate that alternatively spliced variants of *NPPA* gene are expressed during the early stages of mouse cardiogenesis as well as in the mouse adipose tissue. However, further studies would be required to clarify the expression of these variants in the other mouse tissues and the functional significance of these transcripts.

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No competing financial interests exist.

References

Annilo, T., Kepp, K., and Laan, M. (2009). Natural antisense transcript of natriuretic peptide precursor A (NPPA): structural organization and modulation of NPPA expression. BMC Mol Biol **10,** 81.

Badorff, C., Brandes, R.P., Popp, R., Rupp, S., Urbich, C., Aicher, A., Fleming, I., Busse, R., Zeiher, A.M., and Dimmeler, S. (2003). Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation **107,** 1024–1032.

Choi, Y.S., Matsuda, K., Dusting, G.J., Morrison, W.A., and Dilley, R.J. (2010). Engineering cardiac tissue *in vivo* from human adipose-derived stem cells. Biomaterials **31,** 2236–2242.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy **8,** 315–317.

Dultz, E., Hildenbeutel, M., Martoglio, B., Hochman, J., Dobberstein, B., and Kapp, K. (2008). The signal peptide of the mouse mammary tumor virus Rem protein is released from the endoplasmic reticulum membrane and accumulates in nucleoli. J Biol Chem **283,** 9966–9976.

Houweling, A.C., van Borren, M.M., Moorman, A.F., and Christoffels, V.M. (2005). Expression and regulation of the atrial natriuretic factor encoding gene Nppa during development and disease. Cardiovasc Res **67,** 583–593.

Jafarzadeh, N., Javeri, A., Khaleghi, M., and Taha, M.F. (2014). Oxytocin improves proliferation and neural differentiation of adipose tissue-derived stem cells. Neurosci Lett **564,** 105–110.

Lee, W.C., Sepulveda, J.L., Rubin, J.P., and Marra, K.G. (2009). Cardiomyogenic differentiation potential of human adipose precursor cells. Int J Cardiol **133,** 399–401.

Liu, J.L., Jiang, L., Lin, Q.X., Deng, C.Y., Mai, L.P., Zhu, J.N., Li, X.H., Yu, X.Y., Lin, S.G., and Shan, Z.X. (2012). MicroRNA 16 enhances differentiation of human bone marrow mesenchymal stem cells in a cardiac niche toward myogenic phenotypes *in vitro*. Life Sci **90,** 1020–1026.

Martin-Rendon, E., Sweeney, D., Lu, F., Girdlestone, J., Navarrete, C., and Watt, S.M. (2008). 5-Azacytidine-treated human mesenchymal stem/progenitor cells derived from umbilical cord, cord blood and bone marrow do not generate cardiomyocytes *in vitro* at high frequencies. Vox Sang **95,** 137–148.

Martoglio, B., Graf, R., and Dobberstein, B. (1997). Signal peptide fragments of preprolactin and HIV-1 p-gp160 interact with calmodulin. EMBO J **16,** 6636–6645.

Metzele, R., Alt, C., Bai, X., Yan, Y., Zhang, Z., Pan, Z., Coleman, M., Vykoukal, J., Song, Y.H., and Alt, E. (2011). Human adipose tissue-derived stem cells exhibit proliferation potential and spontaneous rhythmic contraction after fusion with neonatal rat cardiomyocytes. FASEB J **25,** 830–839.

Mitchell, J.B., McIntosh, K., Zvonic, S., Garrett, S., Floyd, Z.E., Kloster, A., Di Halvorsen, Y., Storms, R.W., Goh, B., Kilroy, G., *et al.* (2006). Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells **24,** 376–385.

Newton-Cheh, C., Larson, M.G., Vasan, R.S., Levy, D., Bloch, K.D., Surti, A., Guiducci, C., Kathiresan, S., Benjamin, E.J., Struck, J., *et al.* (2009). Association of common variants in NPPA and NPPB with circulating natriuretic peptides and blood pressure. Nat Genet **41,** 348–353.

Pemberton, C.J., Siriwardena, M., Kleffmann, T., Ruygrok, P., Palmer, S.C., Yandle, T.G., and Richards, A.M. (2012). First identification of circulating prepro-A-type natriuretic peptide (preproANP) signal peptide fragments in humans: initial assessment as cardiovascular biomarkers. Clin Chem **58,** 757–767.

Rangappa, S., Entwistle, J.W., Wechsler, A.S., and Kresh, J.Y. (2003a). Cardiomyocyte-mediated contact programs human mesenchymal stem cells to express cardiogenic phenotype. J Thorac Cardiovasc Surg **126,** 124–132.

Rangappa, S., Fen, C., Lee, E.H., Bongso, A., and Sim, E.K. (2003b). Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. Ann Thorac Surg **75,** 775–779.

Schaffler, A., and Buchler, C. (2007). Concise review: adipose tissue-derived stromal cells—basic and clinical implications for novel cell-based therapies. Stem Cells **25,** 818–827.

Schultheiss, T.M., Burch, J.B., and Lassar, A.B. (1997). A role for bone morphogenetic proteins in the induction of cardiac myogenesis. Genes Dev **11,** 451–462.

Taha, M.F., and Hedayati, V. (2010). Isolation, identification and multipotential differentiation of mouse adipose tissue-derived stem cells. Tissue Cell **42,** 211–216.

Taha, M.F., and Valojerdi, M.R. (2008). Effect of bone morphogenetic protein-4 on cardiac differentiation from mouse embryonic stem cells in serum-free and low-serum media. Int J Cardiol **127,** 78–87.

Taha, M.F., Valojerdi, M.R., and Mowla, S.J. (2007). Effect of bone morphogenetic protein-4 (BMP-4) on cardiomyocyte differentiation from mouse embryonic stem cell. Int J Cardiol **120,** 92–101.

Viragh, S., and Challice, C.E. (1969). Variations in filamentous and fibrillar organization, and associated sarcolemmal structures, in cells of the normal mammalian heart. J Ultrastruct Res **28,** 321–334.

Wan Safwani, W.K., Makpol, S., Sathapan, S., and Chua, K.H. (2012). 5-Azacytidine is insufficient for cardiogenesis in

human adipose-derived stem cells. J Negat Results Biomed **11,** 3.

Yandle, T.G. (1994). Biochemistry of natriuretic peptides. J Intern Med **235,** 561–576.

Zeller, R., Bloch, K.D., Williams, B.S., Arceci, R.J., and Seidman, C.E. (1987). Localized expression of the atrial natriuretic factor gene during cardiac embryogenesis. Genes Dev **1,** 693–698.

Zhang, F.B., Li, L., Fang, B., Zhu, D.L., Yang, H.T., and Gao, P.J. (2005). Passage-restricted differentiation potential of mesenchymal stem cells into cardiomyocyte-like cells. Biochem Biophys Res Commun **336,** 784–792.

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