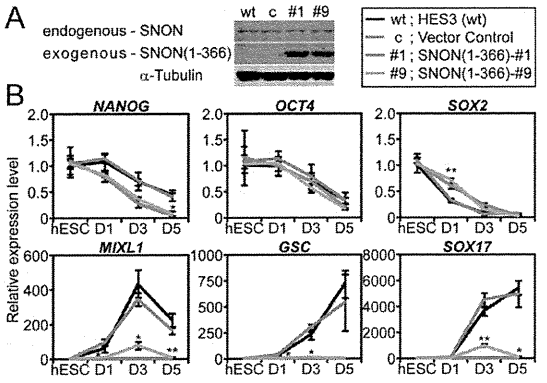


**Figure 3.** SNON overexpression inhibits DE formation. (*A*) Western blot analysis detects abundant C-terminally truncated, constitutively active SNON(1–366) in two stable, clonally selected lines (#1 and #9). (*B*) Expression analysis of the indicated genes by qPCR. hESCs were differentiated according to the schematic in Figure 1A. Data were normalized against *GUSB* and are shown relative to parental wild-type undifferentiated hESCs (= 1.0). The "vector control" HES3 line contains only the pCAG-IRES-Puro construct. *P*-values were calculated according to the Student's *t*-test. (*) $P < 0.05$; (**) $P < 0.01$.

and clones #1 and #9 (Fig. 3B; Supplemental Fig. 3C). HES3 and the vector control cell line predictably showed up-regulation of the PS markers (*BRACHYURY, WNT3, FGF8, MIXL1,* and *EOMES*) as well as mesendodermal (*LHX1* and *GSC*) and DE (*FOXA2* and *SOX17*) markers beginning on day 1 (Fig. 3B; Supplemental Fig. 3C). Clones #1 and #9 failed to activate the expression of PS, mesendodermal, and DE marker genes, all of which are known Activin/Nodal targets and bound by SMAD2/3 (Fig. 3B; Supplemental Fig. 3C; Schmierer and Hill 2007; Arnold and Robertson 2009). This result is entirely consistent with the ability of SNON(1–366) to strongly suppress the synthetic ARE-lux Activin/Nodal pathway reporter, which contains three repeats of a *Xenopus* Activin response element (ARE) that binds SMAD2/4 and the forkhead transcription factor FOXH1. Human GSC-Luc2 and MIXL1-Luc2 reporters containing their respective endogenous AREs are similarly repressed by SNON(1–366) (Supplemental Fig. 3D). When confronted with culture conditions tailored to produce cardiomyocytes (Hudson et al. 2012), SNON(1–366)-overexpressing clones also fail to up-regulate early mesodermal progenitor markers (Supplemental Fig. 3E). SNON(1–366) therefore serves as a potent inhibitor of both mesoderm and DE formation downstream from Activin/Nodal signals. Consequently, SNON(1–366)-expressing clones initiate extraembryonic lineage differentiation in response to Activin A and BMP4 treatment, as evidenced by the up-regulation of the trophoblast marker genes *CDX2, CGA, CYP19A, GCM1, KRT7, LGALS16,* and *VGLL1* and the visceral/parietal endoderm marker *SOX7* (Supplemental Fig. 3C). In addition, no induction of early neuroectodermal markers (*NEUROD1* and *SOX1*) was observed (Supplemental Fig. 3C).

### *SNON occupies SBEs in mesendodermal genes*

To formally test whether SNON binds and represses PS and mesendodermal genes in undifferentiated hESCs, we performed SMAD2, SMAD3, and SNON ChIP followed by qPCR for enrichment at select SBEs. Given the high percentage of amino acid identity between SMAD2 and SMAD3 and the potential for antibody cross-reactivity, we used monoclonal anti-SMAD2 and anti-SMAD3 antibodies that specifically distinguish between these two proteins (Supplemental Figs. 1K, 4B). Among "stem cell factor" genes (*NANOG, OCT4, SOX2, DPPA4,* and *LIN28*) that are highly expressed in hESCs, SNON occupancy at their respective SBEs was low to undetectable (Fig. 4A; Supplemental Fig. 4A). Only *SUZ12* showed statistically significant enrichment for SNON ($P < 0.05$). However, *SUZ12,* like the other stem cell factor genes analyzed, was insensitive to SNON(1–366) overexpression (Supplemental Fig. 3C). In contrast, SNON highly occupied PS (*FGF8, WNT3, EOMES,* and *MIXL1*) and mesendodermal (*GSC* and *LHX1*) genes (Fig. 4A; Supplemental Fig. 4A). SMAD2 bound all SBEs analyzed by ChIP (Fig. 4A; Supplemental Fig. 4A). Consistent with the ability of SNON to recruit HDACs, acetylated H3 and H4 levels at PS/mesendodermal genes were low in undifferentiated HES3 cells but increased with differentiation (Supplemental Fig. 4C; Xi et al. 2011). The converse was observed at stem cell factor gene promoters, with declining acetylation mirroring the decrease in pluripotency gene expression during differentiation (Supplemental Figs. 1A, 3C, 4C). Taken together, these data are wholly consistent with SNON in combination with SMAD2/SMAD4 acting as a key transcriptional repressor of differentiation-specific genes in pluripotential hESCs (Fig. 4B).

Our ChIP data also reveal a bias of SMAD3 toward stem cell factor gene promoters (Fig. 4A; Supplemental Fig. 4A). In a very recent study, Mullen et al. (2011) reported that OCT4 and SMAD3 are predominantly associated with transcriptionally active genes in hESCs and, importantly, co-occupy the genome by binding adjacent DNA sites. Our results show that no repression of pluripotency genes was observed in hESC clones overexpressing SNON(1–366), which retains its ability to bind SMAD3 but cannot be targeted by E3 ligases (Fig. 3B; Supplemental Fig. 3C). These genes include *NANOG,* whose regulation by TGFβ/Activin/SMAD2/3 in hESCs is extremely well characterized (Xu et al. 2008; Vallier et al. 2009). If SNON were a general repressor downstream from TGFβ-related signaling, modulating the overall activity of the TGFβ pathway in hESCs, ectopic expression of SNON(1–366) would be predicted to suppress *NANOG* and other crucial pluripotency genes and promote differentiation. This is not what we observed (Fig. 3B; Supplemental Fig. 3D). We therefore propose that OCT4 serves a central "protective" role in averting SNON repression of SMAD3-bound pluripotency genes (Fig. 4B). In contrast, selective recruitment of SNON represses PS/mesendoderm genes in hESCs until extrinsic cues trigger its degradation and allow differentiation to unfurl.

One outstanding question is thus the identity of the E3 ligases specifically responsible for the rapid clearance of SNON at the onset of differentiation. Genetic evidence in mice favors a role for Arkadia because *Arkadia*-null mutant embryos succumb to severe gastrulation defects due to impaired Nodal/Smad2/3 signaling (Episkopou et al. 2001; Mavrakis et al. 2007). One interpretation of this phenotype is that in the absence of Arkadia, SnoN associates with crucial Nodal/Smad2/3 target genes,

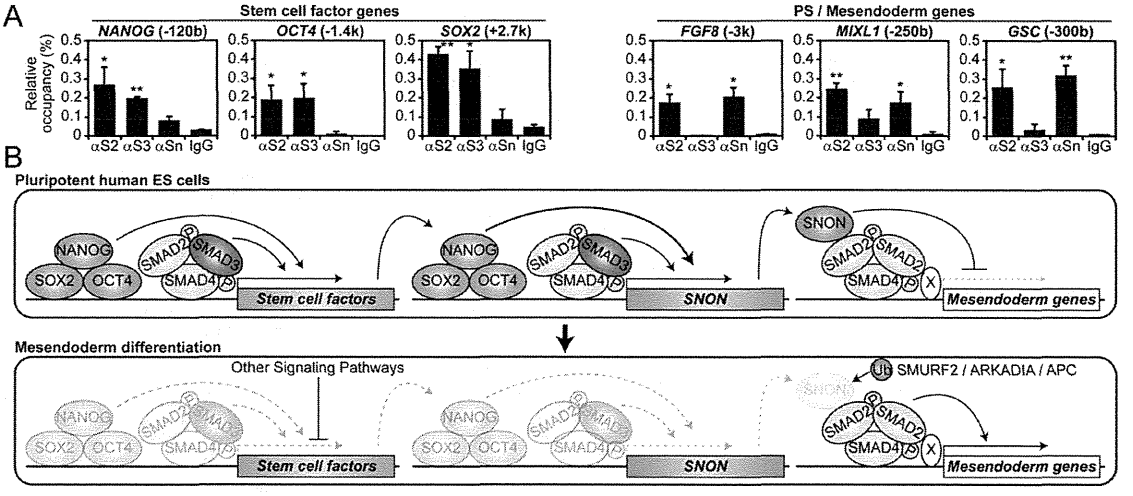


**Figure 4.** SNON highly and selectively occupies the promoters of PS and mesendodermal genes in hESCs. (*A*) ChIP-qPCR analysis of SNON-binding (αSn), SMAD2-binding (αS2), and SMAD3-binding (αS3) sites at the indicated gene regions in HES3 cultured in mTeSR1 medium. Relative occupancy values are shown as the apparent immunoprecipitation efficiency (percentage) (ratio = immunoprecipitated DNA/input DNA). (IgG) Normal rabbit IgG as negative control. *P*-values were calculated according to the Student's *t*-test. (*) $P < 0.05$; (**) $P < 0.01$. (*B*) Proposed model for SNON function in hESCs. In pluripotent hESCs, extrinsic signals (e.g., TGFβ/Activin/Nodal) regulate stem cell factor gene expression through activated SMAD2/3/4 complexes. OCT4/SOX2/NANOG positively regulate their own promoters and activate *SNON* transcription. SMAD2/3 also regulate *SNON*. SNON is selectively recruited to SMAD2-bound mesendodermal genes and suppresses their transcription. During early differentiation, stem cell factor gene expression and SMAD2/3/4 occupancy at the SNON promoter/enhancer decline. Consequently, *SNON* expression decreases. Existing SNON protein is rapidly degraded in a ligand-dependent manner by E3 ubiquitin ligases (e.g., SMURF2, ARKADIA, or APC). This leads to the derepression of early PS and DE target genes (collectively referred to as mesendoderm). (X) Tissue-specific transcriptional coactivators such as FOXH1.

preventing their activation. Consistent with this prediction, SnoN protein accumulates in *Arkadia*-deficient mouse ESC lines (Nagano et al. 2007). Thus, SNON degradation in hESCs may similarly require the stimulus-dependent activation of ARKADIA or the synergistic involvement of other ligand-dependent E3 ligases, such as SMURF2 (Bonni et al. 2001). It is important to note that there are >600 E3 ligases and substrate recognition subunits encoded by the human genome (Li et al. 2008), many of which are enriched in hESCs (Assou et al. 2009). This finding presages the heightened sensitivity that hESCs exhibit to proteasome inhibitors (Assou et al. 2009; Vilchez et al. 2012; data not shown) and further suggests that there are additional hitherto uncharacterized regulators that act alongside ARKADIA, APC, and SMURF2 to target SNON to the proteasome.

## Materials and methods

### *Cell culture*

HES3 cells were maintained as previously described (Suemori et al. 2006). For feeder-free culture, HES3 and S4TR5 cells (Zafarana et al. 2009) were maintained in mTeSR1 medium (Stem Cell Technologies) on 1:200 GFR-Matrigel-coated (Becton Dickinson) dishes. DE differentiation was carried out as previously described (Teo et al. 2012).

### *Tet-inducible shRNA knockdown*

Previously validated human *SNON* shRNA target sequences were ligated into pSUPERIOR-Zeocin (Supplemental Material; Sarker et al. 2005; Zhu et al. 2007). S4TR5 cells were transfected with the shRNA constructs using FuGene HD (Roche) and selected for 2 wk using 2.5 μg/mL Zeocin (Invitrogen).

### *SNON overexpression*

Human *SNON*(1–366) cDNA was PCR-amplified and ligated into pCAG-IRES-Puro (Supplemental Material; Sumi et al. 2007). Feeder-free HES3 cells were transfected with pCAG-SNON(1–366)-IRES-Puro or empty vector using FuGene HD and selected for 2 wk using 1 μg/mL puromycin (Sigma).

### *qPCR analysis*

Total RNA was extracted using the RNeasy kit (Qiagen), and 0.5–2 μg was reverse-transcribed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR was performed using TaqMan Fast Advanced Master Mix or Power SYBR Green PCR Master Mix. Relative quantitation was performed using $2^{-\Delta\Delta CT}$ and normalized against *GUSB*. TaqMan gene expression assays and primers are listed in Supplemental Table 2.

### *Luciferase assay*

An ~5-kb segment of the human *SNON* promoter was PCR-amplified (Supplemental Table 1) and subcloned into pGL4.10[*luc2*] (Promega). pTK-*hRluc* was used for data normalization (Supplemental Material). Molar equivalents of each *luc2* reporter vector and 0.05 μg of pTK-*hRluc* were cotransfected into HES3 using FuGene HD. After 48 h, both firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega). Relative luciferase units were calculated by determining the ratio between firefly and *Renilla* luciferase activities and normalizing against the pGL4.10 vector control.

### *Immunofluorescence and immunocytochemical analysis*

For immunofluorescence, hESCs were fixed in 3.7% formaldehyde/PBS for 15 min, permeabilized with 0.2% Triton X-100/PBS for 5 min, and incubated with primary antibodies (Supplemental Table 3). After incubation with Alexa Fluor 488- or 594-conjugated secondary antibodies

(Invitrogen), nuclei were counterstained with DAPI (Sigma). For immunocytochemical staining of OCT4, hESCs were fixed, permeabilized, and incubated with anti-OCT4 antibody (Supplemental Table 3). After incubation with HRP-conjugated secondary antibody (Dako), antigens were visualized with DAB (Sigma).

### *Western blot analysis*

Cell lysates were separated by SDS-PAGE, transferred to Immobilon-FL PVDF membrane (Millipore), and probed with primary antibodies (Supplemental Table 3). After incubation with IRDye 800CW- or 680LT-conjugated secondary antibodies (LI-COR), proteins were detected using the Odyssey infrared imaging system (LI-COR).

### *ChIP and quantitative real-time PCR*

ChIP was performed as described previously (Boyer et al. 2005). Additional details are provided in the Supplemental Material.

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MINIREVIEW

# Tissue engineering and cell-based therapy toward integrated strategy with artificial organs

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**Abstract** Research in order that artificial organs can supplement or completely replace the functions of impaired or damaged tissues and internal organs has been underway for many years. The recent clinical development of implantable left ventricular assist devices has revolutionized the treatment of patients with heart failure. The emerging field of regenerative medicine, which uses human cells and tissues to regenerate internal organs, is now advancing from basic and clinical research to clinical application. In this review, we focus on the novel biomaterials, i.e., fusion protein, and approaches such as three-dimensional and whole-organ tissue engineering. We also compare induced pluripotent stem cells, directly reprogrammed cardiomyocytes, and somatic stem cells for cell source of future cell-based therapy. Integrated strategy of artificial organ and tissue engineering/regenerative medicine should give rise to a new era of medical treatment to organ failure.

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**Keywords** Biofabrication · Stem cell · Reprogramming · Direct conversion · Clinical trial

## Introduction

The human body is made up of approximately 60 trillion cells but can be traced back to one fertilized egg created by the union of an ovum and a sperm. The fertilized egg divides repeatedly, creating various cells that coordinate with each other to form all the different tissues and organs, ultimately leading to the formation of a complete individual. Whereas the human genome has been almost completely decoded and the genes involved in various mechanisms of the body are becoming known, many parts of this epic developmental process remain unclear. However, because these developmental mechanisms are closely related to the homeostatic maintenance and the regenerative mechanisms of organs and tissues, the field of regenerative medicine, which aims to use these mechanisms to treat diseases, is expanding rapidly.

## Tissue engineering

### Fusion protein

Biological tissue is composed not only of cells but also of a surrounding environment that is crucial in maintaining cell function in vivo and in homeostasis. Most importantly, the extracellular matrix is known to have dynamic and functional roles, such as providing a scaffold for cell adhesion (basement membrane and fibronectins) as well as maintaining and providing growth factors (heparan sulfate). Technological development with respect to manipulating

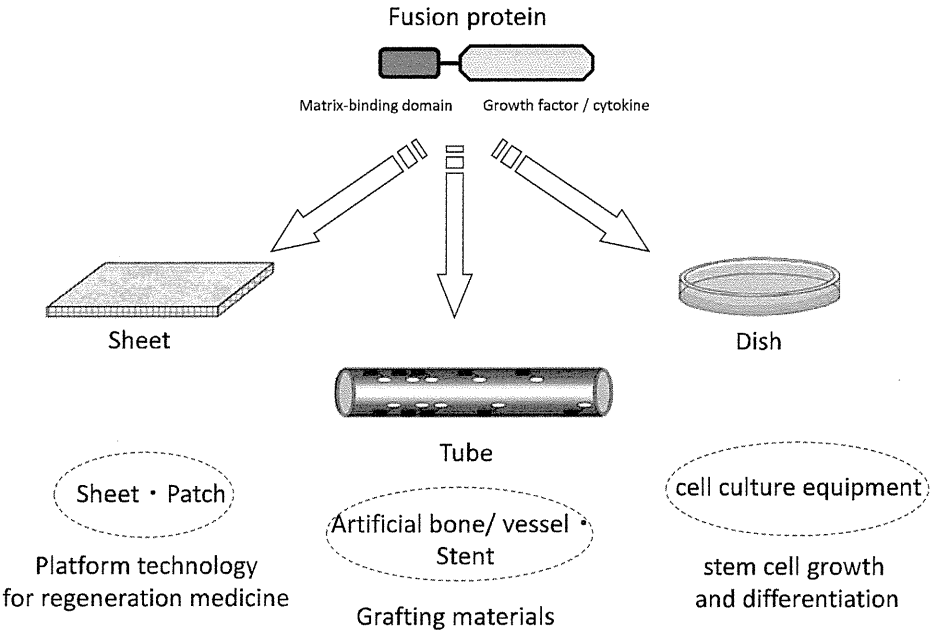


**Fig. 1** Tissue engineering for regenerative medicine

this extracellular matrix in order to control tissues and cells, and its subsequent application in regenerative medicine, is underway. For example, studies have revealed that a variety of growth factors play important roles in wound healing, and some of these growth factors are in clinical use. However, the short-term effects of these growth factors pose some limitations on their use. An example is provided by fibrin, which is released in the wounded area when tissue damage occurs. An increasing amount of research is being conducted on the use of fibrin as a material for tissue regeneration. If a protein produced by the fusion of a fibrin-binding domain (FBD) to epidermal growth factor (EGF) is added to an epidermal wound-model culture system, binding of the growth factor to the fibrin released from the wound leads to healing by stimulating growth in the surrounding cells [1]. This phenomenon presumably occurs not because of the independent function of the growth factor, but because the growth factor stabilizes after binding to fibrin, and the FBD–EGF complex causes continuous cell stimulation. This suggests that the process of altering the combination of extracellular matrix and growth factors can be of therapeutic value in a variety of conditions. Another example can be considered with respect to vascular grafts. The development of small-caliber vascular grafts, such as those used to treat coronary artery disease, has slowed down because these grafts tend to fail at an early stage owing to thrombotic occlusion. To prevent this, prompt graft endothelialization and prevention of blood clot adherence is necessary. The use of a protein produced by fusion of the collagen-binding domain (CBD)—which binds collagen (a component of the extracellular matrix)—to hepatocyte growth factor (HGF) has been considered in such cases, and it has been shown that this complex (CBD–HGF) effectively promotes growth of endothelial cells [2]. Furthermore, this type of fusion protein could be placed onto a biodegradable sheet of extracellular matrix and affixed to the wounded area, where it may stimulate vascular cell growth. This has the potential for a wide application in medicine (Fig. 1).

## Three-dimensional tissue engineering

A substantial amount of tissue engineering research has been performed on the three-piece that are cell, growth factors, and scaffolds. There are, however, various limitations to using scaffolds. First, cells tend to be distributed over the surface of the scaffold, thus making it difficult to form a solid tissue. Second, a 3D array and structure cannot be controlled when multiple cell types are used. Third, the concentration gradient of growth factors cannot be controlled. Fourth, there are certain limitations to the process of creating the vasa vasorum by tissue engineering techniques. In recent years, the concept of the scaffold has been put aside, and attempts to construct 3D tissue with cells and growth factors are now frequently reported. This method is generally called biofabrication [3], and the techniques of bioprinting [4] and organ printing [5] also fit into this category. In addition, although 3D structures using inkjet printer technology have already appeared as rapid prototyping, a 3D printer with an inkjet nozzle from which

droplets with a volume identical to that of cells are embossed, and which can be operated in a sterile environment, has been developed [6]. This could make the construction of 3D tissues possible [7]. Biorapid prototyping, a method in which many cellular spheres are used together with arbitrary structures to create 3D tissue, has also been reported [8]. This is expected to be an extremely promising methodology despite many issues, such as those related to cell solvents.

### Cell sheets

Of all the recently developed tissue engineering techniques, practical application of cell sheets has advanced the most. This technology is based on the properties of a temperature-responsive polymer, poly(*N*-isopropylacrylamide). Culture dishes coated with this material are hydrophobic at 37°C and hydrophilic <32°C. When cells are cultured to confluence, they can be recovered as a sheet without enzymatic digestion [9]. This technique has been made available from Japan for worldwide application in the development of regenerative medicine-related products [10]. It was reported that stratification, which was initially limited to a few layers, could evolve to include many layers with neovascularization. So far, cell sheets have been made that consist of myoblasts [11], mesenchymal stem cells [12], cardiac progenitor cells [13], and a mixture of fibroblasts and endothelial progenitors [14]. Osaka University is coordinating a clinical trial using autologous myoblast sheets in patients carrying a left ventricular assist device (LVAD) with the aim of providing a bridge to recovery. In France, a clinical trial using epithelial cell sheets for corneal regeneration is being conducted by a venture company.

### Whole-organ tissue engineering

The technology of perfusion decellularization of organs is a unique method of tissue production using scaffolds that has been reported in recent years. Intracellular structures can be completely eliminated by perfusing the heart using a Langendorff coronary perfusion apparatus for more than 12 h with the surfactant sodium dodecyl sulfate. It has also been reported that components of the extracellular matrix, including collagen type I/III, laminin, and fibronectin, can be preserved without disturbing their array structure; furthermore, the structure of valves and basal membrane of the epicardial vessels are not affected [15]. A heartbeat, albeit faint, has been achieved using this technique. The feasibility of whole-organ decellularization has been demonstrated in the pig heart [16] and rat liver [17]. Although the process of cellularization has its flaws, it is a creative initiative that holds promise for future developments.

## Regenerative medicine

### Induced pluripotent stem cells (iPSC)

The term regenerative medicine was introduced in 2000. Clinical applications have increased greatly since then, beginning with research on human embryonic stem cells (ESC) and confirmation of the plasticity of somatic stem cells. Amid frustration that human ESC could not be applicable not only to medicine, but also in biological research, the phenomenon of initialization via nuclear transplantation has been achieved in an elaborately planned experiment with four gene transfers. Now, these cells, called induced pluripotent stem cells (iPSC), certainly appear to be a major topic in regenerative medicine. Basic research into the clinical application of iPSC demonstrated the successful treatment of model mice for Parkinson's disease [18], sickle cell anemia [19], and hemophilia [20] with mouse iPSC. These reports indicate the same scheme could be applicable to human diseases. However, problems in iPSC application include the development of teratomas from undifferentiated cells, carcinomas due to gene transfer, and infection with xenogeneic materials used in cell cultures. These problems have attracted the interest of a large number of researchers, and many proposals for solutions to them have been reported (Fig. 2).

Teratoma formation in mice can reportedly be prevented by eliminating stage-specific embryonic antigen-1-positive cells [18]. If the target of interest is the heart, enrichment with mitochondria could prevent teratoma formation [21]. Of the four genes transferred during iPSC initialization, which are considered to be reprogramming genes, it was feared that the existence of c-Myc, in particular, which is an oncogene, would lead to cancer; carcinogenesis through c-Myc reactivation was actually observed in vivo. In addition, because the basic protocol uses a retrovirus as the vector for gene transfer, the possibility of carcinogenesis after its insertion into a genome is a problem. It was subsequently reported that just three factors (excluding c-Myc) induced iPSC, albeit at a low frequency [22]. However, recently, induction of iPSC with RNA [23] and proteins [24] of reprogramming factors has been reported in an attempt to circumvent carcinogenesis because of the methodology. Of the four factors, Sox2 and c-Myc could be replaced with transforming growth factor-α receptor antagonists [25], the nuclear acceptor Esrrb could be replaced with Klf4 [26], and Oct4 could be replaced with nuclear acceptor Nr5a2 [27].

Currently, xenogeneic materials are used in various processes in standard ESC/iPSC culture (Fig. 3). Feeder cells are used to maintain the undifferentiated state of both ESC and iPSC; usually, mouse embryonic fibroblasts (MEF) treated with mitomycin C to arrest their growth are

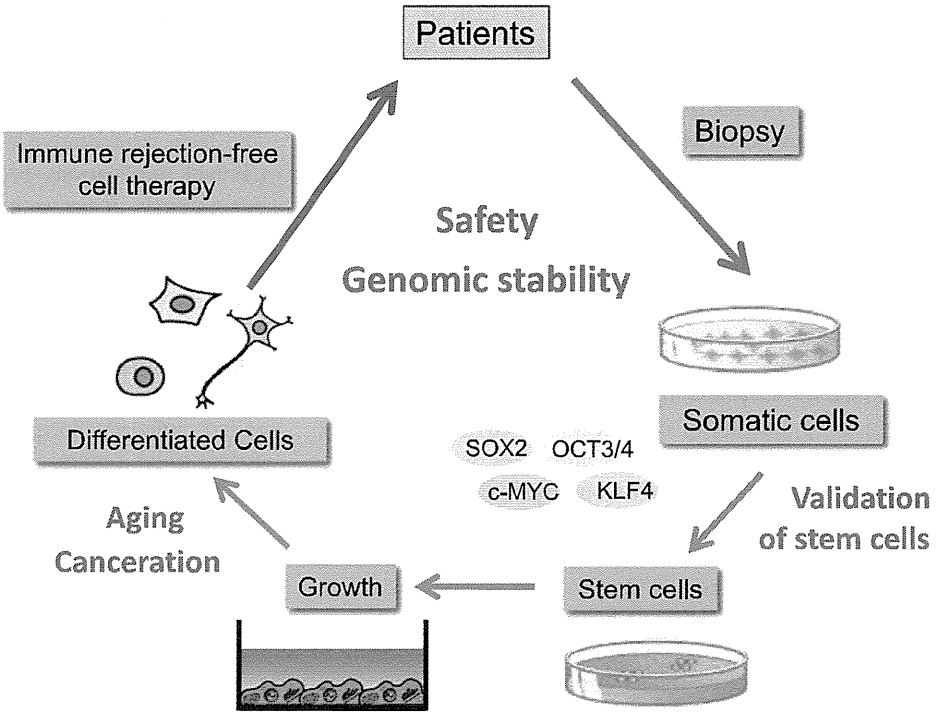


**Fig. 2** Order-made stem cell therapy

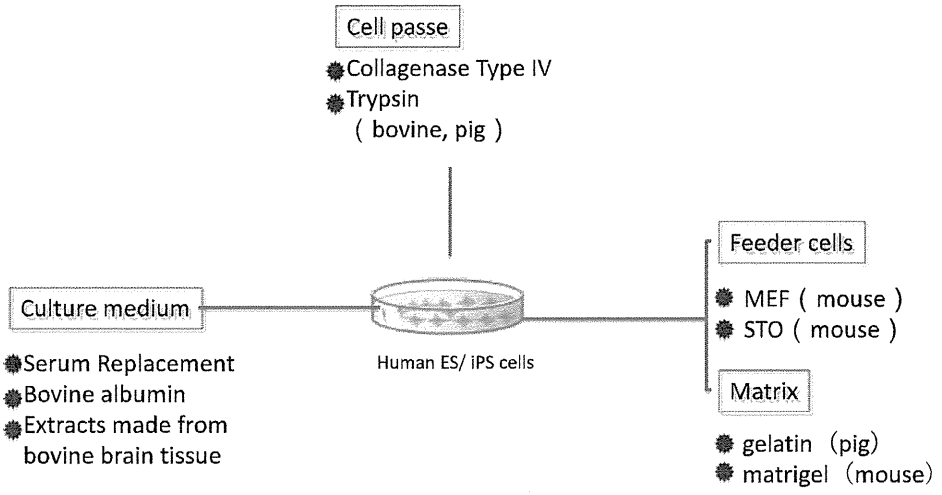


**Fig. 3** Xenogeneic factors and materials in human embryonic stem cell/induced pluripotent stem cell (ESC/iPSC) culture

used as feeder cells. It was feared that if these xenogeneic cells were used in clinical situations, contamination with xenogeneic cells may lead to infection. This did, in fact, occur: the presence of non-human-derived Neu5Gc was confirmed on the cell surface of human ESC cultured onto MEF. Many individuals possess antibodies for this antigen, and an immune reaction can be provoked in these individuals [28]. In order to avoid xenogeneic contamination, coating cell culture dishes with fully synthetic compounds and the chemical defined-culture medium has been reported from several institutes. A 3D porous natural polymer scaffold consisting of chitosan and alginate was able to sustain human ESC self-renewal [29]. Recombinant vitronectin also supported cultivation of three human ESC under feeder-free conditions [30]. Moreover, suspension culture of human ESC and iPSC in chemically defined media supplied a scalable number of cells [31]. However, the ability of xeno-free protocols to maintain the self-renewal ability and pluripotency of human ESC or iPSC remains questionable. On the other hand, autologous

fibroblasts could be used as feeder cells in the culture of human iPSC [32].

Almost the entire process of reprogramming in iPSC remains poorly understood. It is still unclear whether iPSC reprogramming is equal to nuclear transplantation, which showed that the somatic nucleus reacquired totipotency. The following factors imply that multiple processes exist in reprogramming: the expression of stem cell-related genes differs between iPSC clones [33], and "memories" of the parent cells remain. It has been reported that trichostatin A, a histone deacetylase inhibitor, is a factor that promotes this phenomenon [34]. The necessity of using it under strictly controlled temporal and quantitative requirements indicates the preciseness of its mechanism.

## Direct conversion to differentiated cells

The phenomenon termed direct conversion to differentiated cells is a novel occurrence recently reported to occur in several organs. Although many researchers have searched for the master gene, such as MyoD, that can induce formation of skeletal muscle cells from fibroblasts, no such gene has been discovered. However, because the use of four genes allows the differentiated cell to have pluripotency, transformation with one batch of gene transfer is been investigated. A first report described successful differentiation of pancreatic exocrine cells into insulin-secreting beta-like cells by the transfer of three genes (Ngn3/Pdx1/MafA) [35]. Another report documented successful induction of cells expressing myocardial cell structural proteins through the transfer of Gata4/Tbx5/Baf60c into mouse mesodermal cells [36]. According to later reports, functional neurons can be induced by transferring Asc11/Brn2/Myt11 into fibroblasts [37], and myocardial cells can be induced by transferring Gata4/Tbx5/Mef2c into fibroblasts [38]. Thus far, the possibility that these cells only caused specific gene expression that exists in the lower area of transgenes cannot be denied. Functional and quantitative assessments of induced cells produced by direct reprogramming are required to determine their application in the clinical setting.

## Somatic stem cells

It was long believed that cardiomyocytes were in a state of terminal differentiation in adults and that the heart cannot heal itself or restore its homeostatic functions. These properties led researchers in cardiac regeneration to increased interest in somatic stem cells, including bone-marrow-derived stem/progenitor cells, mesenchymal stem cells, and adipose-derived stem cells. Because cells derived from fetal-related tissue (including the amnion, umbilical cord, and placenta) contain a multipotent population that shows plasticity, many organizations, institutes, and companies run banking systems for these cells. Transplantation of amniocytes caused cardiac regeneration in myocardial infarction in rats [39]. On the other hand, other researchers have continually asserted the heart has regenerative properties. Recently, clear evidence that cardiomyocytes can be reborn in the adult heart was reported. This evidence was based on cardiomyocyte age estimation by measuring carbon-14, which was generated by nuclear weapons testing during the Cold War [40]. The impetus for the expansion of this field of study was the reporting of a method that was apparently based on embryonic bodies [41]: by forming a sphere with cardiac-tissue-derived cells, a group of nearly undifferentiated cells could be enriched. Subsequently, several groups reported that stem cells and precursor cells exist in the heart. Several profiles were reported for these cells, including c-kit (+) [42], sca-1 (+) [43], and side population cells [44]. Whether this means that we are observing the process of differentiation as it develops or that multiple stem cell systems exist is an issue that needs to be addressed. Matsubara et al. [45], who reported that murine sca-1 (+) cells could be cardiac stem cells (CSC), performed a detailed preclinical study in pigs to treat ischemic heart disease [46] and are directing the world's first clinical trial using CSC. This clinical trial targets patients with severe chronic ischemic heart failure whose left ventricular ejection fraction is <35%. The method involves intramuscular injection of CSC during coronary artery bypass grafting. The injected stem cells are isolated from cardiac tissue collected during a previous biopsy from the right ventricular septal region. During cell culture, recombinant basic fibroblast growth factor (bFGF) is used rather than xenogeneic materials, and blood serum is obtained from autologous blood. The cells are injected through the epicardium, and the injection sites are covered with a basic sustained-release gelatin sheet of bFGF. Patients are not randomized, and six cases are scheduled for an open-label phase I/IIa clinical trial, with a planned 1-year follow-up study. It is assumed that after this trial, cases will accumulate in multifacility clinical studies and that this method will develop into a highly advanced medical technology.

Chemical pharmacology is faced with difficulty finding new classes of drugs despite increasing budgets. Cells and tissues, therefore, are likely to become important medical treatments. Moreover, integrated therapy of ventricular assist device (VAD) and regenerative medicine should have great potential to treat severe heart failure (Fig. 4). Although implantable VADs are being used with excellent prognosis, many issues remain; for example, right ventricular failure, infection, thrombosis, and device mechanical failure. A market report on VAD anticipates that the "bridge to recovery (BTR)" strategy will constitute more

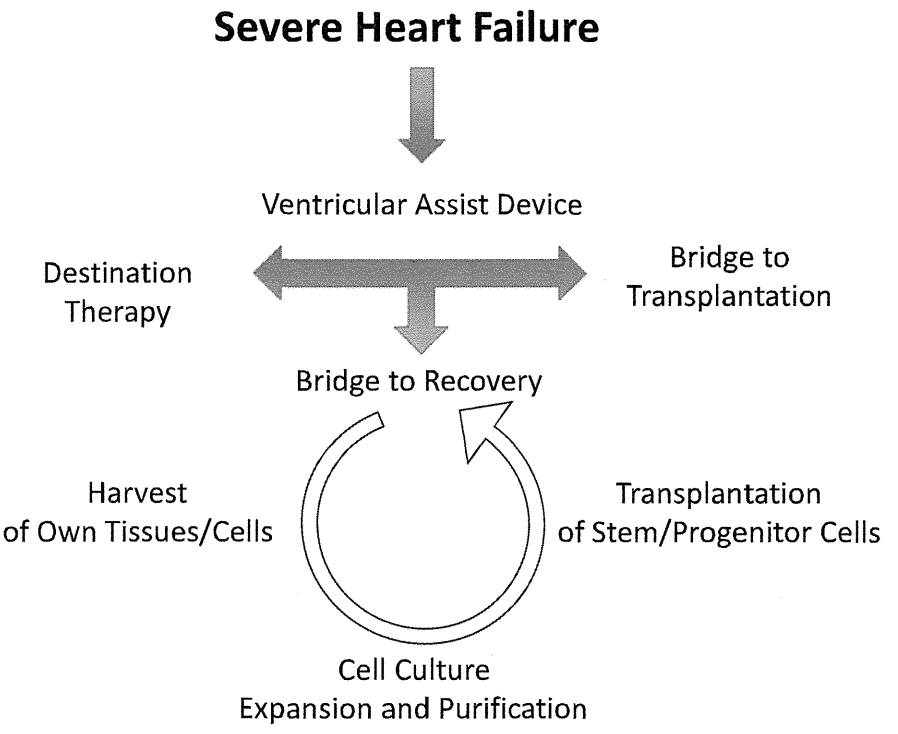


**Fig. 4** Integrated strategy to heart failure

than half of future VAD therapy. The emerging field of regenerative medicine will surely accelerate the trend to BTR therapy.

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# Treatment of Human Mesenchymal Stem Cells with Angiotensin Receptor Blocker Improved Efficiency of Cardiomyogenic Transdifferentiation and Improved Cardiac Function via Angiogenesis

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**Key Words.** Angiotensin • Bone marrow stromal cells • Transdifferentiation • Stem cell transplantation

ABSTRACT

**To improve the modest efficacy of mesenchymal stem cell (MSC) transplantation, the treatment of human MSCs with angiotensin receptor blockers (ARBs) was investigated. MSCs were cultured with or without the medium containing 3 μmol/l of ARBs before cardiomyogenic induction. After cardiomyogenic induction in vitro, cardiomyogenic transdifferentiation efficiency (CTE) was calculated by immunocytochemistry using anticardiac troponin-I antibody. In the nude rat chronic myocardial infarction model, we injected MSCs pretreated with candesartan (A-BM; $n = 18$) or injected MSCs without pretreatment of candesartan (BM; $n = 25$), each having survived for 2 weeks. The left ventricular function, as measured by echocardiogram, was compared with cardiomyogenic transdifferentiation in vivo, as determined by immunohistochemistry. Pretreatment with ARBs significantly increased the CTE in vitro ($10.1 \pm 0.8$ $n = 12$ vs. $4.6 \pm 0.3\%$ $n = 25$, $p < .05$). Transplantation of candesartan-pretreated MSCs significantly improved the change in left ventricular ejection fraction (BM; $-7.2 \pm 2.0$ vs. A-BM; $3.3 \pm 2.3\%$). Immunohistochemistry revealed significant improvement of cardiomyogenic transdifferentiation in A-BM in vivo (BM; $0 \pm 0$ vs. A-BM; $0.014 \pm 0.006\%$). Transplantation of ARB-pretreated MSCs significantly improved cardiac function and can be a promising cardiac stem cell source from which to expect cardiomyogenesis.** STEM CELLS *2011;29:1405–1414*

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

Regeneration therapies have attracted a great deal of medical attention. Various cellular resources such as embryonic stem cells [1], mesenchymal stem cells (MSCs) [2], mononuclear cells [3, 4], and endothelial progenitor cells (EPCs) [5] have been candidates for the regeneration therapies. The majority of cells derived from bone marrow (BM) consist of blood cells in various stages of differentiation; however, BM also contains, hematopoietic stem cells, EPCs, and MSCs. MSCs have characteristics of replication competence and multipotency [2, 6–8], as reported in numerous studies of MSCs.

Mesenchymal cells are classified as somatic stem cells and exist in BM stroma, dermis, skeletal muscle, uterine endometrial gland [9], umbilical cord blood [7, 10], placenta [11], amniotic membrane [6], etc. They are known to be capable of transdifferentiating into bone, cartilage, skeletal muscles, fats, ligaments, vascular endothelium, smooth muscle, and cardiomyocytes. Among the various mesenchymal cell sources, BM-derived MSCs (BM-MSCs) can be used in an autologous manner; therefore, there are no immunological problems in transplantations. However, in terms of cardiomyogenic transdifferentiation, the efficiency of human BM-MSCs is extremely low [8] in vitro, and efficiency of human BM-MSC transplantation is modest in in vivo [12, 13] and in clinical trials [14, 15]. The limited effect in clinical trials may be due to low angiogenic and paracrine effect of human BM-MSCs, low cardioprotective effect on host myocardium, and partially due to low cardiomyogenic transdifferentiation efficiency (CTE) [8]. We have previously shown that human mesenchymal cells derived from younger populations, that is,

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endometrial gland [9], umbilical cord blood [10], placenta [11], and amniotic membrane [6] have a high CTE and a beneficial effect on cardiac function. Therefore, we hypothesized that mesenchymal cells obtained from younger populations might have a better effect on regeneration therapies. As angiotensin receptor blocker (ARB) was known to have the potential to play a role in the anti-aging effect, we postulated that ARB might improve the efficacy of BM-MSCs on cardiac stem cell therapy.

Stimulation of angiotensin receptors is known to be related to adipogenic transdifferentiation of human BM-MSCs [16]. In the brain ischemic reperfusion model, BM-MSC transplantation significantly reduced the brain infarction area via improvement of brain blood flow and reduction of oxidative stress [17]. The effect of BM-MSC transplantation was abolished by knocking out the angiotensin-II (AT) receptor type-II (AT2R). On the other hand, this effect was restored by pretreatment with ARB for BM-MSCs in the culture. These facts suggest that ARB and stimulation of AT receptor may play a significant role in causing the angiogenesic effect of BM-MSC transplantation. Therefore, in this study, we investigated the effect of ARB on CTE of human BM-MSCs in vitro and in vivo, and efficacy of BM-MSC transplantation on cardiac function in the myocardial infarction (MI) model in vivo.

## Materials and Methods

### BM-Derived MSCs

Yub623 (RIKEN Cell bank, Cell No. HMS0017, Tokyo, Japan) cells were used as BM-MSCs in this study. Yub623 is a fibroblast-like shaped human MSC (hMSC) derived from neonatal human BM from a finger of patients with polydactyly. Cells were cultured in high-glucose supplemented Dulbecco's modified Eagle's medium containing 10% human serum.

### Cardiomyogenic Induction and Chemical Agents

The method of cardiomyogenic induction in vitro was described previously (Supporting Information Material and Method-1) [6, 8–11]. In short, enhanced green fluorescent protein (EGFP) labeled BM-MSCs were cocultured with murine cardiomyocytes. In this system, the incidence of cell fusion was approximately 0.3% and the evidence of cell fusion-independent cardiomyogenesis was extensively shown in the previous studies [6, 8–11, 18, 19]. BM-MSCs were preincubated with chemical agent-containing medium for 2 weeks before coculture and/or cultured with chemical agent-containing medium after coculture. In this study, we used 3 $\mu$mol/l of telmisartan (tel), candesartan (cnd), losartan (los), olmesartan (olm), and valsartan (val) as an AT receptor blocker (ARB), 3 $\mu$mol/l of PD123319 (pd) as a specific AT type-I blocker; enalaprilat (ena) and captopril (cap) as an angiotensin converting enzyme (ACE) inhibitor; 3 $\mu$mol/l of aliskiren (ali) as a direct rennin inhibitor; 1 $\mu$mol/l of AT; and 10 $\mu$mol/l of GW9662 (gw) as a peroxisome proliferators-activated receptor-$\gamma$ (PPAR-$\gamma$) blocker. Evaluation of efficiency of cardiomyogenic transdifferentiation was described previously [6, 10, 11]. In short, cocultivated BM-MSCs were enzymatically isolated, a smear sample was made, and then immunocytochemistry using mouse monoclonal antibody against anticardiac troponin-I (Trop-I, #4T21 Hytest, Euro, Finland) antibody was performed (described later). Isolated cells (spherical shape), in which Trop-I colocalized with EGFP at the cytoplasm were considered as Trop-I/EGFP double positive cells. The CTE was defined as the incidence of Trop-I/EGFP double positive cells in EGFP-positive BM-MSCs. The incidence of cell fusion was not affected by ARB treatment (0.30% to 0.39%) in this study.

### Immunocytochemistry and Immunohistochemistry

A laser confocal microscope (FV1000, Olympus, Tokyo, Japan) was used. As described previously [6, 8–11, 18, 19], samples were stained with Trop-I with mouse monoclonal antibody (sigma) and rabbit polyclonal anti-connexin 43 antibody (sigma) diluted 1:300 overnight at 4°C, then stained with TRITC-conjugated anti-mouse IgG antibody (Sigma) and Cy5-conjugated anti-rabbit IgG antibody (Chemicon) diluted 1:100, containing 4′-6-diamidino-2-phenylindole (Wako) at 1:300 for 30 minutes at 25–28°C.

### Enzyme-Linked Immunosorbent Assay

Angiogenic humoral factors (angiogenin, angiotensin-2, epidermal growth factor [EGF], basic fibroblast growth factor, heparin-binding EGF-like growth factor, hepatocyte growth factor, phosphatidylinositol-glycan biosynthesis class F protein, and vascular endothelial growth factor) in culture medium supernatant (cultured with 10% serum-containing medium for 7 days) were measured by enzyme-linked immunosorbent assay [19]. The assay was performed with Quantibody Human Angiogenesis Array I kit (RayBiotec, Inc. GA) and was conducted according to manufacturer recommended protocol.

### Gene Chip Analysis

Human genome-wide gene expression was examined with the Human Genome U133A Probe array (Affymetrix), which contains the oligonucleotide probe set for approximately 23,000 full-length genes and expressed sequence tags as described previously [11, 20].

### Transplantation of ARB-Pretreated BM-MSCs in MI Model In Vivo

MI was induced in the open chests of anesthetized female F344 nude rats (Clea Japan, Inc., 6 weeks of age) as described previously [6, 9, 19]. Two weeks after MI, 1–2 $\times$ $10^6$ of EGFP-labeled BM-MSCs were injected into the myocardium at the border zone of the MI. Two weeks after the first operation, rats with MI were randomized in a blind study of the following groups: the sham operated group (Sham), the (CNT), the CNT with plain BM-MSC transplanted group (BM), and the MI+candesartan-pretreated BM-MSC transplanted group (A-BM). After cellular transplantation, TCV-116 (stable form of candesartan; 0.5 mg/kg/day) was orally administered in some of the experiments (+A). Randomization occurred immediately before echocardiogram. Immediately before cell transplantation, two-dimensional and M-mode echocardiographic (8.5 MHz linear transducer; EnVisor C, Phillips Medical System, Andover, MA) images were obtained to assess left ventricular (LV) end-diastolic dimension and LV end-systolic dimension (LVESD) at the mid-papillary muscle level by a single blinded observer. Two weeks after the transplantation, a similar echocardiogram was performed again. LV percentage fractional shortening, thickness of anterior wall (AW), and thickness of posterior wall were calculated from five to six traces and averaged. LV pressure, brain natriuretic peptide (BNP), body weight, and heart weight (wet) were measured as described previously. Tissue samples were obtained by slicing along the short axis of the left ventricle, for every 1 mm of depth. After masson trichrom staining, the area of fibrosis was digitized from each slice, and then the percentage fibrosis volume in the LV myocardium was calculated as described previously [6, 19]. Immunohistochemical analysis was performed to observe CTE in vivo as described previously (Supporting Information Material and Method-2). Immunohistochemical analysis was performed using anti-rat CD34 antibody (1:200 R&D Systems; AF4117) to evaluate vascular density. Then, biotinylated goat immunoglobulins (Dako; E0466) were used as a second antibody, next, strept avidin biotin complex (ABC) complex/horseradish peroxidase (HRP) (Dako; K0377), and, finally, 3,3′-Diaminobenzidine substrate (Wako; K3183500) were used. The images were digitized and the percentage brown pixel area of the capillary vessels was counted in the peri-infarct normal zone (NZ) and the center of the MI

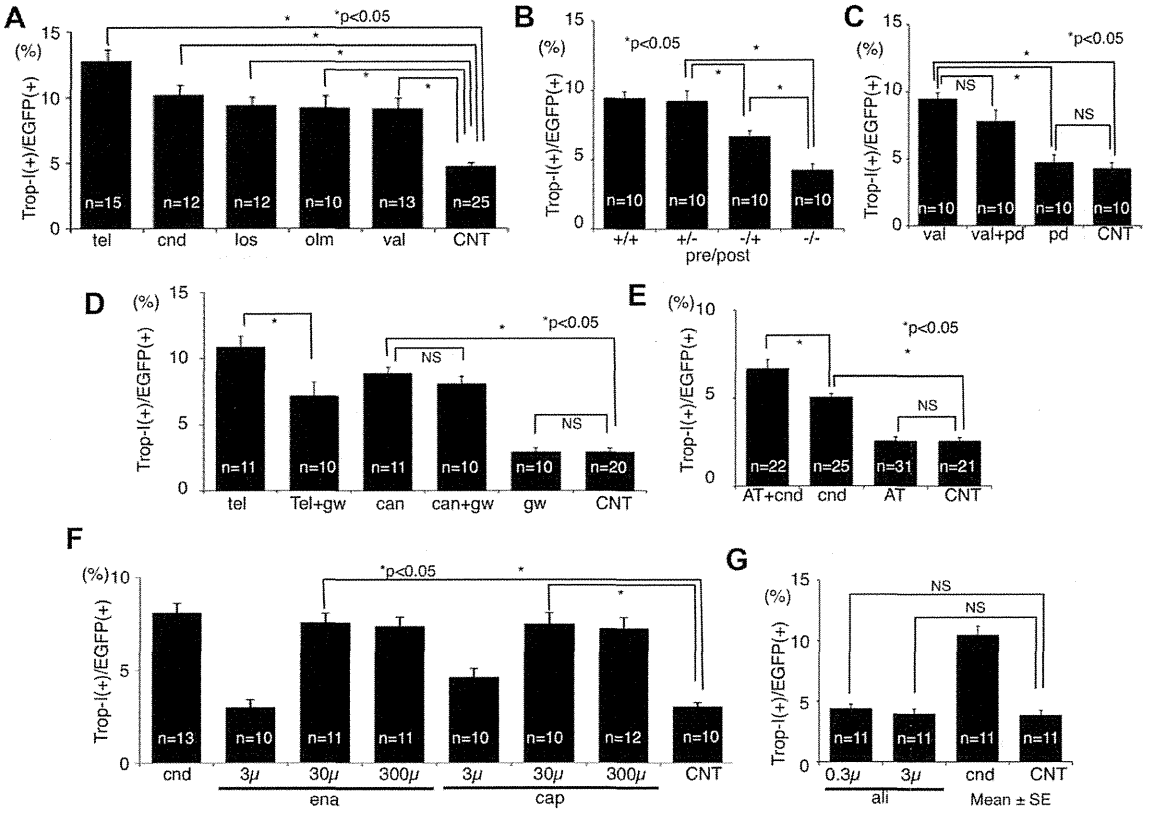


**Figure 1.** Improvement of cardiomyogenic transdifferentiation efficiency (CTE) of bone marrow-derived mesenchymal stem cells (BM-MSC) by blockade of renin-angiotensin system in vitro. The calculated rate of cardiac troponin-I positive cells in enhanced green fluorescent protein-positive cells are averaged and shown as CTE. **(A):** The effect of pretreatment with telmisartan (tel), candesartan (cnd), losartan (los), olmesartan (olm), and valsartan (val) on CTE of human BM-MSCs are shown. CNT denoted CTE of control MSCs. These ARBs increase CTE significantly. **(B):** Condition of pretreatment of val (before slash) and val treatment after induction (after slash) are shown in the bottom. Pretreatment of val significantly increased CTE and was essential for val-induced CTE increase. Val treatment after induction moderately increased CTE. **(C):** The effect of combination of val as a specific angiotensin-II (AT) receptor type-I (AT1R) blocker and PD123319 (pd) as a specific AT2R blocker to CTE is shown. The pd did not affect CTE. **(D):** The effect of GW9662 (gw) as a specific peroxisome proliferators-activated receptor-γ (PPAR-γ) blocker on tel-induced CTE increase and cnd-induced CTE increase are shown. The blockade of PPAR-γ partially blocked the tel-induced CTE increase and did not affect cnd-induced CTE increase. **(E):** The effect of additional application AT in the presence or in the absence of cnd is shown. AT alone did not affect CTE; however, AT significantly increased CTE in the presence of cnd. **(F):** Dose-response effect of pretreatment with enalaprilat (ena) and captoril (cap) as angiotensin converting enzyme inhibitors (ACEI). ACEI significantly improves CTE in a dose-dependent manner. **(G):** The effect of aliskiren (ali) as a renin inhibitor on CTE is shown. Ali did not affect CTE. *p<0.05. Abbreviations: ali, aliskiren; AT, angiotensin-II; cap, captoril; cnd, candesartan; CNT, control; EGFP, enhanced green fluorescent protein; ena, enalaprilat; gw, GW9662; los, losartan; olm, olmesartan; pd, PD123319; Tel, telmisartan; Trop-I, troponin-I; val, valsartan.

zone (MI) using a light microscope at 10× magnification. The areas in five high-power fields were calculated and averaged.

### Statistical Analysis

All data are shown as mean value ± SE. The difference between mean values was determined with one-way analysis of variance (ANOVA) test or one-way repeated measures ANOVA test and Bonferroni post hoc test. Statistical significance was set at $p < .05$.

## Results

### Pretreatment with ARB Increased Efficiency of Cardiomyogenic Transdifferentiation Via AT2R

Administration of 3 μmol/l of popular ARBs (tel, can, los, olm, and val) did not cause any significant change in morphology of BM-MSCs (Supporting Information Fig. 1A, 1B), while improved CTE in vitro was observed (Fig. 1A and Supporting Information Fig. 1C–1P). In our pilot study, we tested dose-response effect of ARBs and confirmed that this effect was saturated at the concentration of 3 μmol/l (CTE at control, 0.03, 0.3, 3, and 30 μmol/l of cnd were 3.0 ± 0.3, 3.5 ± 0.2, 4.8 ± 0.3, 8.9 ± 0.4, and 8.1 ± 0.5%, respectively). Therefore, in this study, we selected 3 μmol/l as a default concentration of ARBs. To clarify the target of the ARBs, val was administrated only before the coculture or only after the coculture (Fig. 1B). Administration of val after the start of coculture (±) caused modest improvement of CTE; on the other hand, administration of val before the start of coculture (±) significantly increased CTE, suggesting that val modified the character of the BM-MSCs so as to be able to cause higher CTE. To determine whether the effect of the ARBs was mediated by AT receptor type-I (AT1R) or AT2R, we used val as AT1R specific blocker and pd as AT2R specific blocker (Fig. 1C). Administration of pd did not affect CTE, while val increased CTE significantly. Furthermore, CTE with both val and pd administered did not show an additional increase

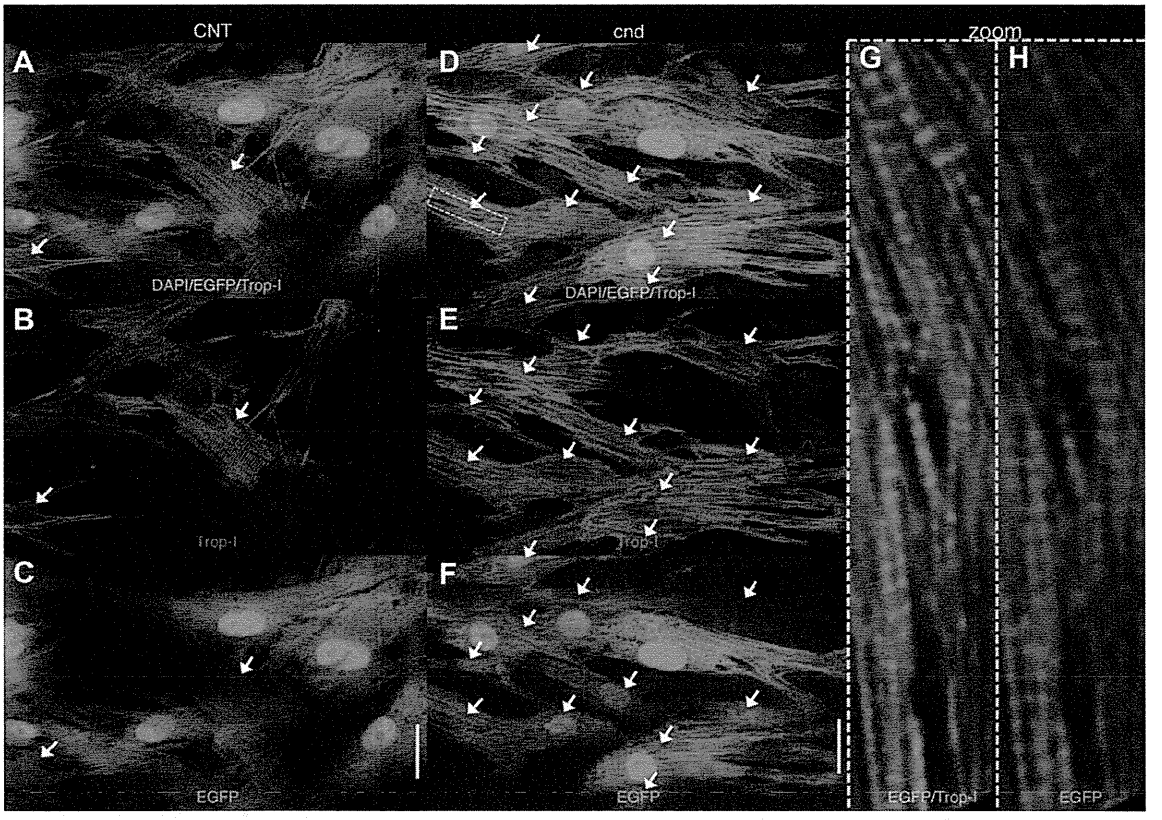


**Figure 2.** Confocal laser microscopic images of the immunocytochemical analysis of transdifferentiated cardiomyocytes. Confocal microscopic images of immunocytochemistry after cardiomyogenic induction using anti-cardiac troponin-I (red: Trop-I) revealed significant augmentation of enhanced green fluorescent protein (EGFP) (green)/Trop-I double positive cardiomyocytes (white arrow) by candesartan (cnd) (**D–F**) pretreatment, while EGFP/Trop-I double positive cells were rare in CNT (**A–C**). Area within the dotted yellow box is expanded and shown in (**G, H**). Clear striation staining pattern of Trop-I was observed in every EGFP-positive cell. The striating pattern of EGFP and Trop-I appeared in alternation, suggesting that the Trop-I was expressed in the EGFP-positive cells. Scale bar = 20 $\mu$m. Abbreviations: cnd, candesartan; CNT, control; DAPI, 4′-6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; Trop-I, troponin-I.

(rather, tended to show a statistically nonsignificant decrease). These data suggest that blockade of AT1R plays a pivotal role in ARB-dependent CTE increase. We have previously reported that PPAR-$\gamma$ activator has an ability to increase CTE of BM-MSCs [19], and some of the ARBs, that is, tel, have a potential to activate the PPAR-$\gamma$. To clarify that the mechanism of ARB-induced CTE increase was mediated via PPAR-$\gamma$ activation effect, we used gw as a specific blocker for PPAR-$\gamma$ (Fig. 1D). The gw partially blocked tel-induced CTE increase; on the other hand, it did not block cnd-induced CTE increase. These data suggest that the effect of cnd on CTE was independent from PPAR-$\gamma$ activation. In our previous study, the effect of pio was completely blocked by gw [19]; therefore, the gw-insensitive tel-induced CTE increase was caused by a PPAR-$\gamma$-independent mechanism. On the other hand, administration of AT did not affect CTE in the absence of ARB, while administration of AT significantly increased CTE in the presence of ARB (Fig. 1E). These data suggest both blockade of AT1R and stimulation of AT2R increase CTE. The increase in CTE was also observed by administration of ACE inhibitors ena or cap (Fig. 1F), suggesting the source of AT in this system is autocrine of angiotensin-I from BM-MSCs and local ACE activity. Furthermore, the effect was not blocked by the specific renin blocker, ali (Fig. 1G); therefore, angiotensinogen does not play a role as an AT source in this system, but a local angiotensin-generating system may play a role in this phenomenon.

## The Effect of ARB-Treated BM-MSC Transplantation on Cardiac Function In Vivo

The BM-MSCs were transplanted into the hearts of nude rats with chronic MI, in vivo, and the effect on cardiac function was examined. Representative M-mode echocardiographic images at 2 weeks after transplantation are shown (Fig. 2A). In the CNT group, akinesis and thinning of AW are observed. There were no marked changes in the BM group, while in A-BM group, the motion of AW markedly improved. The same trend was also observed in the ARB orally administered group (+A group). The changes in echocardiographic parameters between the immediately before the transplantation group (post MI 2 weeks) and the 2 weeks after transplantation group (post MI 4 weeks) are compared (Fig. 3). Changes in LV ejection fraction ($\Delta$LVEF) were decreased as a function of time, even 2 weeks after the MI, which may be due to LV remodeling. The transplantation of plain BM-MSCs (BM) did not have an effect on $\Delta$LVEF; on the other hand, candesartan-pretreated BM-MSCs (A-BM) significantly improved $\Delta$LVEF. The degree of improvement was marked when candesartan was orally administered (A-BM-A). Change in end-diastolic diameter of LV ($\Delta$LVEDD) did not differ among the

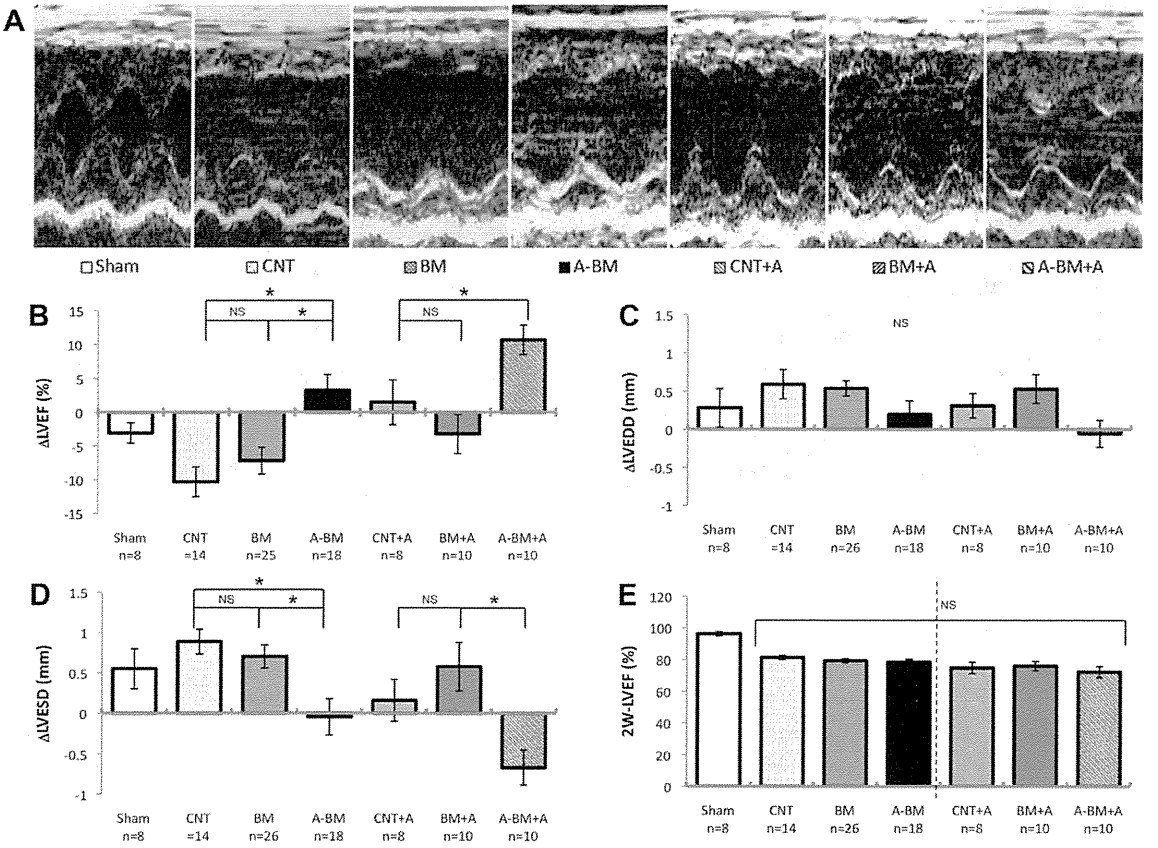


**Figure 3.** Effect of candesartan-pretreated bone marrow-derived mesenchymal stem cell (BM-MSC) transplantation and/or oral administration of candesartan on echocardiographic parameters in vivo. **(A):** Representative trace of M-mode echocardiogram from Sham-operated nude rats, control myocardial infarction (MI) (CNT), MI with BM-MSCs transplantation (BM), candesartan-pretreated BM (A-BM), and oral administration of candesartan after the transplantation (CNT+A, BM+A, A-BM+A) is shown. Changes in left ventricular ejection fraction (LVEF) from 2 to 4 weeks (**B**; ΔLVEF), LV end-diastolic dimension (**C**; ΔLVEDD), and LV end-systolic dimension (**D**; ΔLVESD) are averaged and shown. **(E):** Calculated LVEF from each group at 2 weeks after first operation are shown. There was no statistical significance; however, the degree of percentage EF tends to be worse in the oral administration series (right columns separated by dotted bar). Candesartan-pretreated BM significantly improved LVESD, consequently improved LVEF. *p<0.05. Abbreviations: BM, bone marrow; CNT, control; LVEDD, left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic dimension.

groups; on the other hand, change in LVESD (ΔLVESD) was significantly improved in A-BM group (vs. BM group) and A-BM+A group (vs. BM+A group), suggesting transplantation of candesartan-pretreated BM-MSCs significantly improved systolic function. Other echocardiographic parameter did not differ among the groups. There was no difference in the changes in body weight, serum BNP concentration, heart weight, LV systolic pressure, or LV end-diastolic pressure among the groups (Fig. 4). LV dP/dt was significantly improved by candesartan-pretreatment (A-BM vs. BM) with BM-MSCs; however, there was no additional effect of candesartan-pretreatment in the group of candesartan oral administration group (N.S. CNT-A vs. A-BM+A).

In this study, the beneficial effect was observed even in the ARB-pretreated BM-MSC transplantation group. The effect of ARB is known to cause an irreversible biological change in the cell, the "so-called" memory effect; therefore, such memory effect might affect cardiac function in vivo. To check this possibility, we cultured three groups of BM-MSCs: cells with candesartan for 2 weeks (ARB), cells without candesartan (CNT), and cells with candesartan for 1 week followed by 1 week without candesartan (1 week-ARB: washout for 1 week). The GeneChip analysis was performed among them, then the hierarchical clustering was used using the average distance method [20]. The gene expression pattern of 1 week-ARB was similar to CNT; therefore, the effect of ARB on BM-MSCs was reversible from the aspect of gene-chip analysis.

## Incidence of Myocardial Transdifferentiation of ARB-Pretreated BM-MSCs In Vivo

To evaluate myocardial transdifferentiation of BM-MSCs in vivo, immunohistochemical analysis was performed. Antibodies against cardiac troponin-I (Trop-I) and connexin 43 were used. Confocal laser microscopic images could not detect EGFP-positive cardiomyocytes having clear striation staining pattern of Trop-I in the BM group. Sometimes enucleated EGFP-positive fragments of the cell at the center of the MI zone were observed, but taking the number of the injected EGFP-positive cells into account, the incidence seemed to be rare, as was reported previously [6, 19]. On the other hand, EGFP-positive and Trop-I double positive cells with clear striation staining pattern were observed at the marginal zone of the MI area in the candesartan-pretreated BM-MSC transplanted group (A-BM, Fig. 5F–5I). The oral

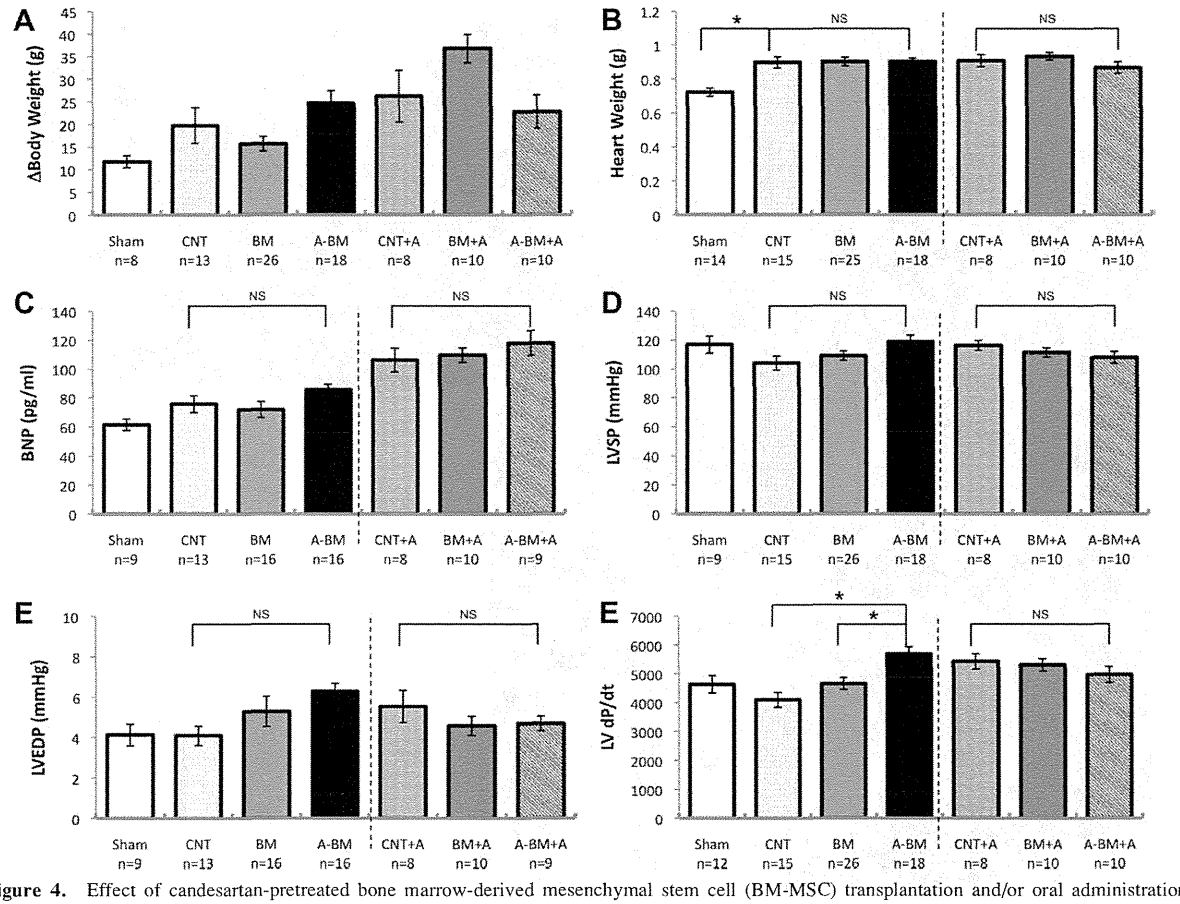


**Figure 4.** Effect of candesartan-pretreated bone marrow-derived mesenchymal stem cell (BM-MSC) transplantation and/or oral administration of candesartan on body weight, serum BNP concentration, and hemodynamic parameters. There was no difference in **(A)** changes in body weight, **(B)** heart weight, **(C)** BNP concentration, **(D)** left ventricular (LV) end-systolic pressure, or **(E)** end-diastolic pressure. **(D):** Effect of BM-MSCs on LV positive dP/dt is significantly improved by pretreatment with pioglitazone. **(F):** The LV dP/dt was significantly improved by transplantation of candesartan-pretreated BM-MSC (A-BM). *$p<0.05$. Abbreviations: BM, bone marrow; BNP, brain natriuretic peptide; CNT, control MI; LV, left ventricle; LVEDP, left ventricular end- pressure; LVSP, left ventricular systolic pressure.

administration of candesartan increased the incidence of survival of the EGFP/Trop-I double positive cells in vivo (A-BM+A, Fig. 5A–5E, 5J).

### Genesis of Angiogenic Humoral Factors Derived from BM-MSCs by ARB

Angiogenic humoral factors were detected in the supernatant of the culture medium of BM-MSCs, suggesting that they are secreted from BM-MSCs, as reported previously [19]. However, the administration of 3 $\mu$mol/l of candesartan did not significantly affect the concentration of these angiogenic factors (Fig. 6). On the other hand, the angiogenic effect of candesartan-pretreated BM-MSCs was observed in vivo (Fig. 7A, 7B). In the peri-MI NZ, a CD34 positive area was not different among CNT, BM, and A-BM groups (without oral administration of candesartan). On the other hand, in the MI area, a CD34 positive area was significantly higher in A-BM group (vs. BM group). Oral administration of candesartan, significantly increased the CD34 area (CNT+A vs. CNT) in the peri-MI normal area and significantly increased it in the MI area. Masson trichrome staining and calculated MI volume at 2 weeks after transplantation (Fig. 7C, 7D) showed significant reduction of MI volume by pretreatment with candesartan of engrafted BM-MSCs (BM vs. A-BM) and the effect of pretreatment was not significantly augmented by the oral administration of candesartan.

## Discussion

### The Effect of Pretreatment with ARB in Human Neonatal BM-MSCs

The ARB did not affect the morphology of BM-MSCs and did not increase secretion of angiogenic humoral factors from BM-MSCs. The pretreatment with ARB significantly increased the CTE in vitro and in vivo. As pretreatment with ARB was essential for the effect on CTE, we concluded that the effect of ARB is not mediated by murine cultured myocardium, but directly affects BM-MSCs themselves, modifying the character of BM-MSCs. As the effect was not mediated by PD123319 as a selective AT2R blocker, the effect of ARB was mediated by the blockade of AT1R. In our previous article [19], activation of PPAR-$\gamma$ significantly increased the CTE in BM-MSCs and the effect was completely blocked by GW9662, as a specific blocker of PPAR-$\gamma$ receptor. The effect of telmisartan, which is known to have the strongest PPAR-$\gamma$ activation activity among the ARBs, on CTE was partially blocked by GW9662, suggesting that the effect of ARBs is not mediated by PPAR-$\gamma$ receptor activation activity. The

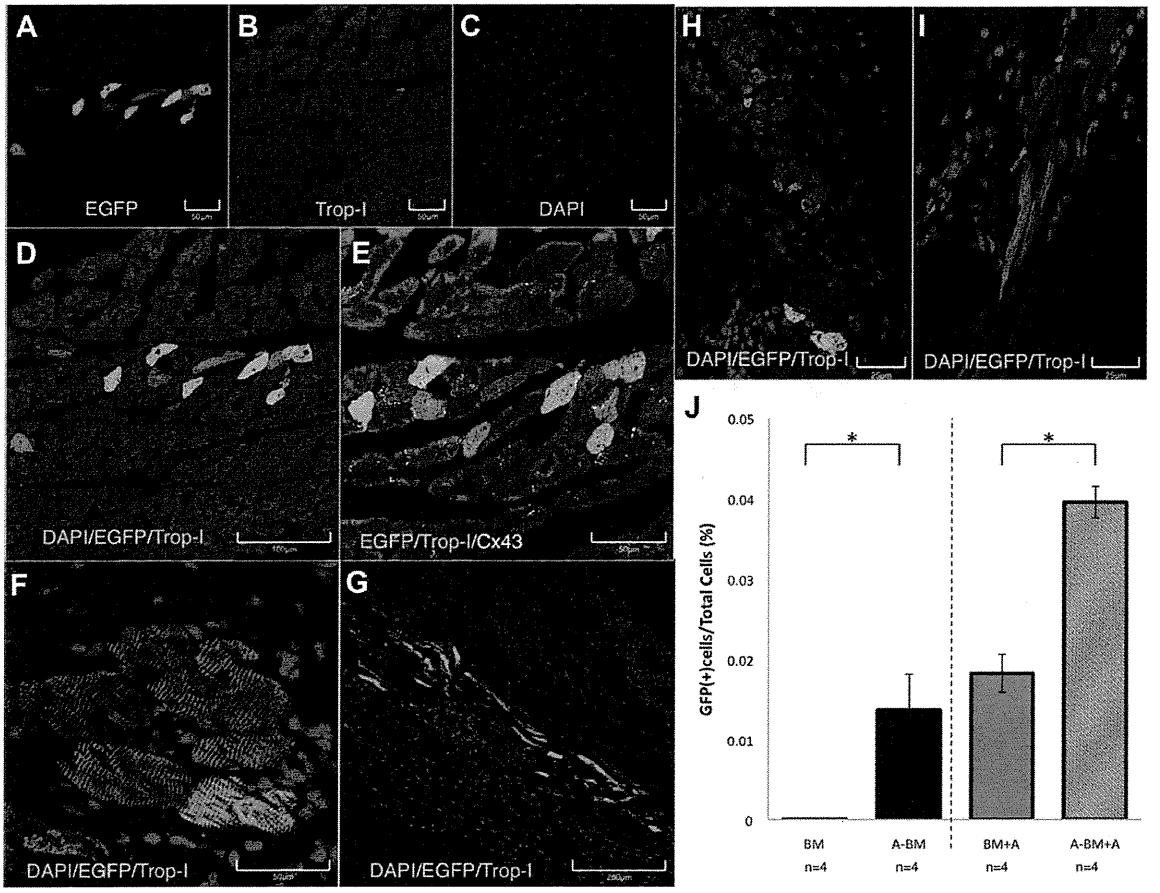


**Figure 5.** Both pretreatment and oral administration of candesartan significantly improved the incidence of survival of bone marrow-derived mesenchymal stem cell (BM-MSC)-derived cardiomyocytes in vivo. Confocal laser microscopic image of immunohistochemistry using anti-cardiac troponin-I antibody (red; Trop-I) is shown. **(A–C):** Lower magnification view for enhanced green fluorescent protein (EGFP) (green; **A**), Trop-I (**B**), and 4′-6-diamidino-2-phenylindole (Blue; **E**) is shown. After transplantation of candesartan-pretreated BM-MSCs in the presence of oral administration of candesartan (A-BM+A), EGFP-positive cells can be observed at the margin of the myocardial infarction (MI), but there were many EGFP/Trop-I double positive cardiomyocytes survived at the peri-MI zone (**A**). **(D):** Higher magnification view of merged image is shown. **(E):** The Trop-I positive cells are surrounded by dot-like staining of connexin 43 (white; Cx43). **(F):** Higher magnification view clearly shows striation staining pattern of Trop-I in the EGFP-positive cells. **(G):** At the center of MI zone (A-BM group), many EGFP-positive cells were enucleated and were negative for Trop-I. **(H, I):** However, there were some EGFP, Trop-I double positive rod-shaped cells at the center of MI zone. **(J):** The percentage of EGFP/Trop-I double positive cells in the injected EGFP-positive cells was averaged and is shown. By pretreatement with candesartan, the rate was significantly improved (A-BM vs. BM), and oral administration of candesartan additionally improved the incidence of EGFP/Trop-I double positive cells in vivo. Scale bars = 50 $\mu$m **(A–C, E, F)**, = 100 $\mu$m **(D)**. = 200 $\mu$m **(G)**, and = 25 $\mu$m **(H, I)**, respectively. *$p<0.05$. Abbreviations: BM, bone marrow; DAPI, 4′-6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; Trop-I, troponin-I.

molecular mechanism of the effect of ARBs on CTE is still unclear. Further experiments should be done.

In the absence of valsartan as an AT1R selective blocker, administration of AT did not affect CTE; however, in the presence of valsartan, AT significantly increased CTE, suggesting that the relative stimulation of AT2R increased CTE. Furthermore, AT in culture medium seems to be generated by ACE activity in BM-MSCs, as the administration of ACE inhibitor to the BM-MSCs in culture significantly increased CTE in vitro. Furthermore, aliskiren did not affect the CTE; therefore, rennin and angiotensinogen did not play a role, but the angiotensin-I in the culture medium or autocrine from BM-MSCs must be a major source for AT.

## Mechanism of Improving Systolic Function with ARB

Although EGFP-positive cardiomyocytes were observed in the candesartan-treated BM-MSC transplanted group, the number of them seems to be low for causing improvement in systolic function in vivo, as was seen in this study.

Concordant with the previous in vivo study [8] and clinical study [14], in the absence of BM-MSC transplantation, oral administration of candesartan suppressed the post-MI LV remodeling and progressive worsening of LVEF (CNT vs. CNT+A) at 2 weeks after MI. Furthermore, in this study, even in the absence of oral administration, the beneficial effect was observed in the candesartan-pretreated BM-MSC transplantation group. In this study, the effect of default BM-MSC transplantation was modest and there was no statistical significance from the control MI group. These data suggest that the ARBs modify the biology of BM-MSC, which play an important role in suppressing post-MI LV remodeling. This trend was observed in hemodynamic parameters and histological data. Pretreatment with candesartan significantly improved the efficacy of BM-MSC transplantation in augmentation of LV dP/dt and reduction in MI volume. Such

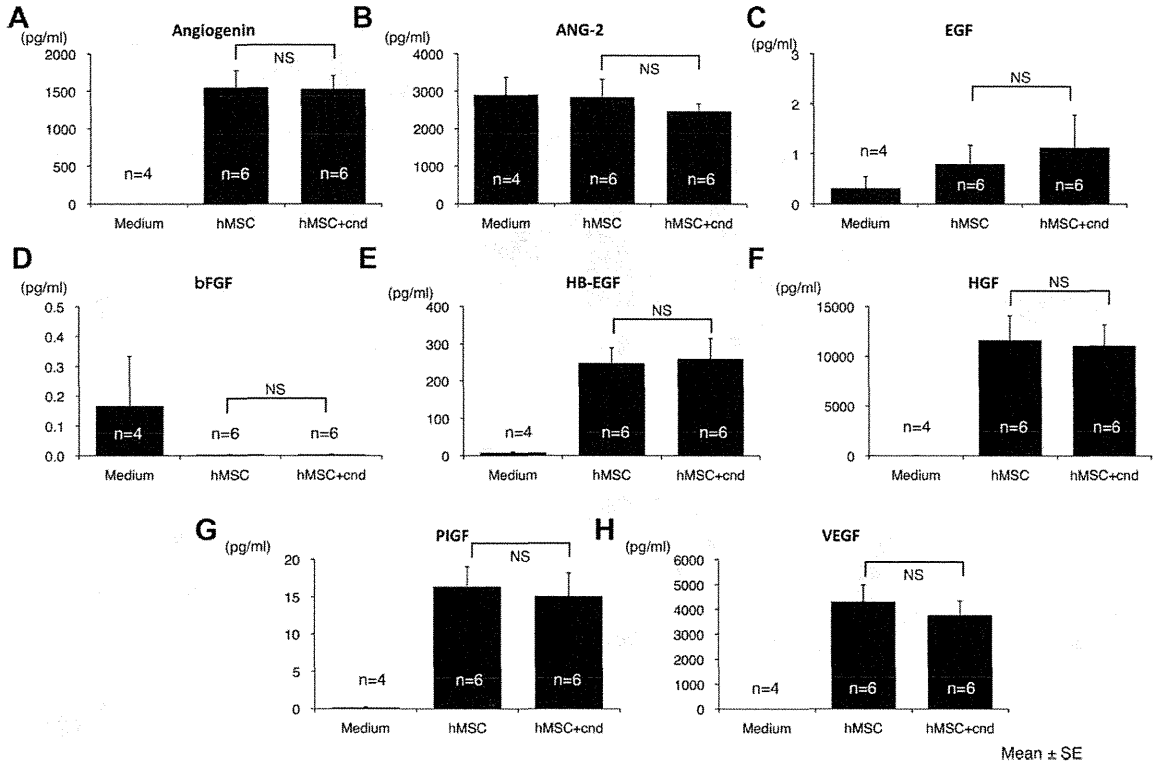


**Figure 6.** Secretion of angiogenic humoral factors from bone marrow-derived mesenchymal stem cells (BM-MSCs) into the culture medium supernatant and the effect of candesartan in vitro. Concentration of angiogenic humoral factors in **(A)** angiogenin, **(B)** angiotensin-2 (ANG-2), **(C)** epidermal growth factor (EGF), **(D)** basic fibroblast growth factor, **(E)** heparin-binding EGF-like growth factor, **(F)** hepatocyte growth factor, **(G)** phosphatidylinositol-glycan biosynthesis class F protein, and **(H)** vascular endothelial growth factor in culture medium was measured by enzyme-linked immunosorbent assay and averaged. Candesartan (cnd) treatment did not cause any significant change in angiogenic humoral factors secretion from BM-MSCs into the culture medium. Abbreviations: ANG-2, angiotensin-2; bFGF, basic fibroblast growth factor; cnd, candesartan; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; HGF, hepatocyte growth factor; hMSC, human mesenchymal stem cell; PIGF, phosphatidylinositol-glycan biosynthesis class F protein; VEGF, vascular endothelial growth factor.

cardioprotective effect of ARB-pretreated BM-MSCs may be due to augmentation of angiogenic effect and/or anti-apoptotic paracrine effect of BM-MSCs by pretreatment with ARB. The beneficial effect of ARB-pretreated BM-MSCs was also reported in the ischemia-reperfusion brain injury model [17], in which it was pointed out that both the stimulation of AT2R and blockade of AT1R have a significant effect on reducing brain damage in vivo and this data well correlated with our CTE data in vitro. In this study, the effect can be observed even by BM-MSC transplantation at 2 weeks after MI; therefore, the BM-MSC-induced angiogenesis might have suppressed ongoing post-MI LV remodeling. In this study, there was discrepancy between the angiogenic effect of ARB-pretreatment in BM-MSCs in vitro and in vivo. We speculated that additional angiogenic effect of BM-MSC transplantation by ARB-pretreatment might require graft-host interaction, that is, immunological reaction or inflammation in the host myocardium.

## Cell Fusion-Independent Cardiomyogenic Transdifferentiation

Extensive evidence of cell fusion-independent cardiomyogenic transdifferentiation of human MSCs was presented in our previous study [6, 9–11, 19]. In this study, the incidence of cell fusion was approximately 1% and it was not affected by ARB pretreatment; therefore, the increase in EGFP-positive cardiomyocytes by ARB treatment was due to an increase in efficiency of cardiomyogenic transdifferentiation in vitro. Furthermore, there were no EGFP/Trop-I double positive rod shaped cardiomyocytes in the default BM-MSC transplanted group; on the other hand, the appearance of significant numbers of EGFP/Trop-I double positive cardiomyocytes was observed in ARB-pretreated BM-MSC transplanted group. This suggests an improvement of CTE of BM-MSCs in vivo by ARB pretreatment. Taking into account our previous study and our present in vitro experiment, we concluded that our observed EGFP/Trop-I double positive cells in vivo are caused by cardiomyogenic transdifferentiation.

## Clinical Application

The efficacy of human BM-MSC transplantation had been modest [14, 15], and a new method for BM-MSC transplantation that will gain dramatic improvement in efficacy is expected. Genetic modification, that is, over-expression of the *AKT*-gene was reported to improve efficacy of BM-MSC transplantation in vivo [21]; however, use of such genetically modified cells raises a safety concern, that is, tumorgenecity. In comparison with the genetic modification, modification of BM-MSCs by ARBs, which are commonly used for heart failure patients, is a method that is ready to use for clinical patients.

In addition to the beneficial efficacy for cardiac function, this experimental model may also give us a clue to improving CTE in vivo, which is very essential for cardiac regenerative therapy. The precise mechanism for cardiomyogenic transdifferentiation of human BM-MSCs has been unclear. As the

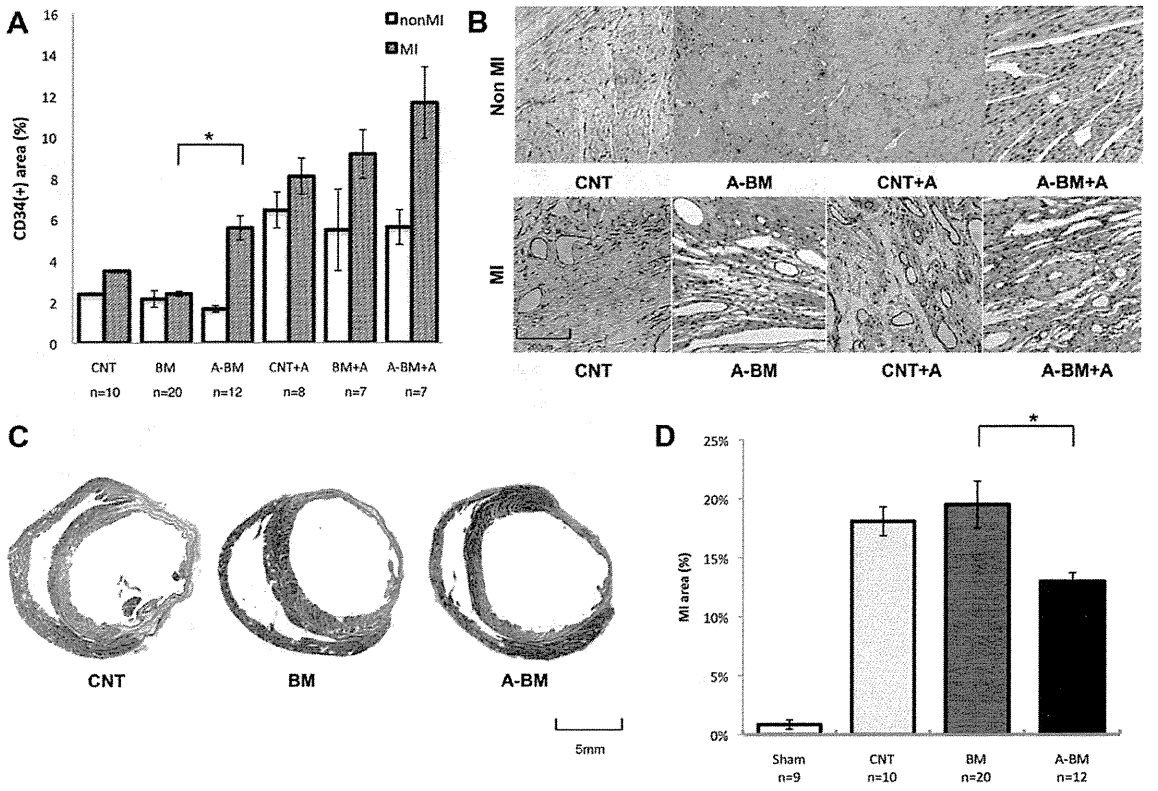


**Figure 7.** Effect of bone marrow-derived mesenchymal stem cell (BM-MSC) transplantation and/or treatment with candesartan on vessel density and infarction size in the heart in vivo. **(A):** The percentage of CD34 positive area in control myocardial infarction (MI) (CNT), MI with bone candesartan-pretreated BM-MSCs transplantation (BM), candesartan-pretreated BM (A-BM), and additional oral administration of candesartan after the transplantation (CNT+A, BM+A, A-BM+A) are calculated and averaged. **(B):** Representative microscopic image of immunohistochemistry using anti-CD34 antibody to detect vessels at center of MI zone and peri-MI normal zone (non-MI) are shown. Scale bar = 20 $\mu$m. Pretreatment with candesartan significantly increased vessel density at MI zone; on the other hand, oral administration of candesartan significantly increased vessel density at non-MI zone. **(C):** Representative masson-trichrom staining of the heart at the tendinous cord level of CNT, BM, and A-BM are shown. The digitized data were measured and calculated in **(D)**. By the candesartan-pretreatment, BM-MSC transplantation significantly decreased in percentage fibrosis volume. Scale bar = 5 mm. *p<0.05. Abbreviations: BM, bone marrow; CNT, control; MI, myocardial infarction.

incidence of cardiomyogenic transdifferentiation of human BM-MSCs is extremely rare, it has been impossible to statistically analyze the effect on CTE of various drugs or interventions in vivo. Therefore, there has been no systematic strategy for improvement of CTE of BM-MSCs until our previous article [6, 9–11, 19]. Our in vivo model of ARB-treated BM-MSCs is able to statistically analyze the effects of drugs on CTE, which is important for further improvement of CTE. In vitro, the pioglitazone's effect on CTE was independent from the effect of ARB; therefore, the additional administration of pioglitazone, as a PPAR-$\gamma$ activator may be expected to improve CTE further. Further experiments should be done.

## Study Limitation

In our previous study, we have used BM-MSCs obtained from a 41-year-old and a 90-year-old men. The CTE results were 1% and 0.3% in vitro [19], respectively. In this study, the CTE of default BM-MSCs from neonates was approximately 3%–5%. This data implies BM-MSCs obtained from younger generations that may have higher cardiomyogenic transdifferentiation ability. As ARB is known to have a potential for an anti-aging effect, the effect of ARB on BM-MSCs might increase the CTE by ARB's anti-aging effect on BM-MSCs. Further experiments should be done on this issue.

In vivo MI model was performed by two series (Sham, CNT, BM, A-BM series and CNT-A, BM-A, A-BM-A series) at different periods. As it was difficult to control the size of the MI at the coronary ligation, the size of the MI of later series are slightly larger (N.S.) than the former series. Therefore, we did not perform statistical analysis on some parameters between the series (separated by dotted line in the figures). The serum BNP level and the size of percentage MI volume are slightly larger in the later series. In this study, intra-individual difference values were compared with the values of the two series.

## Conclusion

Pretreatment with angiotensin receptor blockers (ARBs) in culture activate human marrow-derived mesenchymal stem cells by angiotensin-II receptor type 1 blockade. ARBs-pretreated human marrow-derived msenchymal stem cells was significantly improved cardiomyogenic transdifferentiation efficiency in vitro and in vivo, and transplantation of the ARBs-pretreated cells significantly improved cardiac function and can be a promising cardiac stem cell source from which to expect cardiomyogenesis.

## Acknowledgments

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## Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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