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Adenovirus-mediated hypoxia-inducible factor 1α double-mutant promotes differentiation of bone marrow stem cells to cardiomyocytes

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Abstract The hypoxia-inducible factor 1α (HIF-1α) regulates transcriptional genes involved in cell proliferation, survival, and differentiation. Under normoxia, HIF-1α has a short half-life ($t_{1/2} \approx 5$ min) and low transcriptional activity. An HIF-1α mutant, produced by substitution of alanine (Ala) for proline (Pro) at position 564 and asparagine (Asp) at position 803, can prevent HIF-1α hydroxylation and results in a highly active form of HIF-1α (HIF-1α-Ala564-Ala803). We hypothesized that adenovirus (Ad)-mediated transfer of the active form of HIF-1α (pAd-HIF-1α-Ala564-Ala803) could effectively occur in bone marrow stem cells (MSCs) and promote MSC differentiation under normoxia. PCR-based site-specific mutagenesis was used to construct the Ad vector expressing HIF-1α-Ala564-Ala803. RT-PCR and immunostaining were used to study whether pAd-HIF-1α-Ala564-Ala803 affected MSC differentiation to cardiomyocyte (CMC). pAd-HIF-1α-Ala564-Ala803 exhibited higher transcriptional activity and stable HIF-1α protein expression. Under normoxia, an MSC–CMC co-culture treated with pAd-HIF1a-Ala564-Ala803 augmented TGF-β_1, Smad4, NKx2.5, and GATA4 expression. Higher expression of cTnT and α-actinin was observed by immunostaining in MSCs, compared with the control and contrast groups. Adenovirus-mediated hypoxia-inducible factor 1α double-mutant, pAd-HIF-1α-Ala564-Ala803, can stably express HIF-1α and promote its downstream genes and MSC differentiation to CMC in the MSC–CMC co-culture system under normoxia.

Keywords Bone marrow stem cells ·
Hypoxia-inducible factor 1α · Adenovirus · Differentiation

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Introduction

Myocardial infarction is a leading cause of morbidity and mortality in civilized countries. Bone marrow stem cells (MSCs) are considered promising cells for myocardial infarction therapy. Previous reports have identified MSCs as exciting candidates for cell-based cardiac therapies, because MSCs can differentiate into cardiomyocytes (CMCs) or CMC-like cells in ischemic hearts [1]. However, the differentiation ratio of MSCs to CMCs is a challenge. Co-culture and hypoxia have been suggested as two effective methods to promote the differentiation of MSCs. The co-culture of MSCs and CMCs can induce MSC differentiation to the cardiac lineage within a few days [2, 3]. Moreover, neonatal CMCs can commit stem cells towards the myocardial lineage to a greater extent than adult CMCs [4]. The presence of cardiac cells provides stem cells with a myocardial-like environment.

Hypoxia, which is a result of myocardial ischemia, is a powerful regulator of gene expression and is involved in cardiac cell growth, proliferation, and differentiation. MSCs transplanted into the infarcted myocardium can be induced to acquire the phenotype of CMCs, which suggests that MSC transdifferentiation might be related to the hypoxic microenvironment [5]. Previous reports have suggested that hypoxia can promote the differentiation of

MSCs into CMCs in a myocardial medium or in a CMC and MSC co-culture system [5, 6]. However, the signal transduction involved in the differentiation of MSCs under hypoxic conditions is unclear. Moreover, few studies have determined the roles of genes that promote the differentiation of MSCs under hypoxic conditions. Hypoxic-inducible factor 1α (HIF-1α) is the central point of the oxygen-sensitive signal pathway, which may upregulate more than 100 downstream target genes. We hypothesize that HIF-1α plays a crucial role in the differentiation of MSCs under hypoxic conditions by regulating its downstream target genes (e.g., TGF-β_1, Smad4, NKx2.5, and GATA4).

HIF-1α contains an oxygen-dependent degradation domain (ODDD) and two transactivation domains, N-TAD and C-TAD. The hydroxylation of proline (Pro) residues 402 and 564 in the ODDD and asparagine (Asp) residue 803 of the N-TAD regulates protein stability and the transactivation function in an O_2-dependent manner [7–11]. Hypoxia inhibits hydroxylation of Pro residues 402 and 564 to stabilize the HIF-1α protein. Stabilized HIF-1α can then enter the cell nucleus, where interaction with CBP/p300 in the nucleus leads to activation of its transcriptional activities, which are also controlled by hydroxylation of Asp residue 803. Therefore, HIF-1α has a short half-life ($t_{1/2} \approx 5$ min) and a low transcriptional activity. However, the recombinant Pro residues 402 and/or 564 of ODDD and the Asp residue 803 of TAD, namely, the substitution of alanine (Ala) for Pro at position 564 and Asp at position 803 of HIF-1α (HIF-1α-Ala564-Ala803), can prevent the hydroxylation and rapid degradation of HIF-1α under normoxic conditions. The recombinant HIF-1α may provide a basis for studying biological functions under normoxia.

To investigate our hypothesis and increase our understanding of the role of HIF-1α in hypoxia-induced transitions of MSCs to CMC-like cells, we studied whether a recombinant adenovirus (Ad) expressing a HIF-1α double-mutant could promote MSC transdifferentiation to CMCs in an MSC–CMC co-culture system.

Materials and methods

Construction of recombinant adenovirus vector

The parental plasmid for point mutagenesis and expression of HIF-1α in mammalian cells, termed pShuttle2-HIF-1α, was provided by Professor PS Wu (Department of Cardiology, Nanfang Hospital, First Military Medical University, Guangzhou 510515, China). In order to construct an active form of HIF-1α (p-HIF-1α-Ala564-Ala803), Pro 564 and Asp 803 mutations of the parental plasmid were made with Quick ChangeII Site-Directed Mutagenesis Kits, according to the manufacturer's instructions (Stratagene, USA), and the mutations were confirmed by PCR. HIF-1α-Ala564-Ala803 fragments were excised from pShuttle2-HIF-1α-Ala564-Ala803 by PI-SceI and I-CeuI double digestion. The fragments were extracted with a QIAquick Gel Extraction Kit (Qiagen, Germany), and they were then ligated into Adeno-X System 1 Viral DNA (Clontech, USA) to generate the recombinant plasmids of pAd-HIF-1α-Ala564-Ala803, according to the manufacturer's procedure. The recombinant plasmids were transfected into DH5α, extracted and confirmed by PCR with an HIF-1α-specific primer, an Ad-specific primer, and XhoI enzymatic digestion. The mutation plasmid sequence was confirmed by Shanghai Biologic Technology (China). Ad with HIF-1α-Ala564-Ala803 was amplified in mammalian HEK293 cells. The Ad titer, determined by the median tissue culture infectious dose (TCID50), was estimated at 2×10^9 PFU/mL. To select the optimal multiplicity of infection (MOI) for this Ad-mediated gene transfer, MSCs were transfected with pAd-HIF-1α-Ala564-Ala803 at MOI 50, 100, 150, 200, 250, and 300 for 24 h to measure cytopathic effects. The optimal MOI value of 100 was thus determined.

Isolation and culture of MSCs

MSCs were harvested from the femur and tibia of adult Sprague–Dawley rats with a weight of 150–200 g, which were obtained from the Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China). Isolation and culture of MSCs were performed as described previously [12]. Briefly, MSCs were flushed and centrifuged in a 1.073 g/mL Percoll (Pharmacia, USA) density gradient, and the enriched cells were collected from the interphase, re-suspended in the culture medium and cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) at 37°C in a humidified atmosphere containing 5% CO_2. Seventy-two hours after seeding, the MSCs were adhered to the bottom of the culture plates, and the hematopoietic cells remained suspended in the medium. The medium was changed every three days. The subconfluent cells in the seed cultures were removed from the flasks by treatment with 0.25% trypsin (Sigma, USA) 10–14 days after the initial plating. They were labeled as "passage 1", and the culture continued until "passage 4" (P_4). The MSCs (P_4) were identified with directly conjugated antibodies against anti-CD44, anti-CD29 and anti-CD34 (fluorescein isothiocyanate conjugated, FITC). The positive cells were counted per high-power field by fluorescence microscopy (Carl Zeiss Axiovert 200, Germany). Each sample was counted randomly in eight separate high-power fields.

The MSCs (P_4) were transfected with pAd-HIF-1α-Ala564-Ala803 and pAd-β-galactosidase (pAd-LacZ), and non-transfected MSCs were used as a control. The non-transfected and transfected MSCs, termed "MSCs", "HIF-1α-Ala564-Ala803-MSCs", and "LacZ-MSCs", were incubated for 24 h under standard tissue culture conditions (95% air and 5% CO_2) and then harvested. Analyses of immunofluorescence staining and Western blot were subsequently performed. Cell viability was assessed by counting the cells after staining with Annexin V and propidine iodine (PI).

Western blot

Cells were washed with cold PBS and lysed for 30 min on ice in modified RIPA buffer (20 mM Tris–HCl (pH 7.4), 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM EDTA and 1 mM Na_3VO_4) supplemented with freshly prepared proteinase inhibitors cocktail (10 μg/mL leupeptin, 10 μg/L aproptin, and 1 mM phenylmethylsulfonyl fluoride). The protein concentrations of cell lysates were determined according to the Bradford method (Bio-rad). Cell lysate was cleared by centrifugation at $10,000g$ for 10 min at 4°C and boiled for 5 min with 2-mercaptoethanol. The samples containing 100 μg protein per well were resolved on 10% SDS polyacrylamide gels under reducing conditions and electrotransferred to nitrocellulose membranes (Amersham, Germany). Membranes were blocked by 5% non-fat dry milk in TBS-T (20 mM Tris, pH 7.4, and 137 mM NaCl containing 0.1% Tween-20) for 1 h at room temperature and then incubated with the corresponding primary antibody. Membranes were blocked by 5% non-fat dry milk (HIF-1α, TGF-β1 (Abcam) at a dilution of 1:500) for 90 min. After three washes with TBS-T, the membranes were incubated with goat anti-mouse IgG horseradish peroxidase (HRP) secondary antibody at a dilution of 1: 5,000, and the immunoreactivity was detected by enhanced chemiluminescence (Pierce, USA) and captured on X-ray film. For the loading control, α-tubulin (Sigma, USA) was used at a dilution of 1:1,000.

MSC co-culture with neonatal rat ventricular myocytes

Primary ventricular myocytes were isolated from neonatal Sprague-Dawley rats (1–3 days old, obtained from the Experimental Animal Center of Sun Yat-sen University, Guangzhou, China) using trypsin digestion and a differential adherent technique as described previously [13]. The purity of CMCs cultured from neonatal rat ventricles was identified by use of cTnT antibody (Sigma). CMCs were subsequently cultured in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone) and 1% penicillin–streptomycin for 24 h. To inhibit the growth of non-muscular cells, the medium contained 0.1 mmol/L 5-bromo-2-deoxyuridine (Sigma, bromodeoxyuridine) for the first 48 h. MSCs were treated with 50 μg/mL 4,6-diamidino-2-phenylindol dihydrochloride (DAPI) for nuclear staining.

CMCs (1×10^4/well) were paved in 24-well plates; each well was added to a slide in advance. After three days of culture, MSCs treated with DAPI (0.5×10^4/well) were added to the cultured CMCs. MSCs and CMCs were co-cultured at a ratio of 1:2 in a chamber for 24 h. The cells were divided into three groups: 1 μL PBS was used for the positive control group; 1 μL pAd-LacZ (MOI of 100) was used for the contrast group; 1 μL pAd-HIF-1α-Ala564-Ala803 (MOI of 100) was used for the experimental group. For the negative control group, 1 μL pAd-HIF-1α-Ala564-Ala803(MOI of 100) was added into a single MSC culture. The cells were sequentially cultured for one week under standard tissue-culture conditions (5% CO_2) before immunofluorescence staining and RT-PCR.

Immunofluorescence staining of cells

For immunofluorescence staining, the cells were fixed for 10 min with 4% paraformaldehyde and were permeabilized for 30 min with 0.2% Triton X-100. The cells were blocked with PBS containing 10% fetal bovine serum overnight at 4°C and were then incubated with mouse monoclonal anti-HIF-1α (Abcam) at a dilution of 1:100 and cTnT or sarcomeric-α-actinin (Sigma) at a dilution of 1:50. After washing, the cells were incubated with rhodamine-conjugated goat anti-mouse IgG (Sigma) secondary antibodies at a dilution of 1:100 for 1 h at room temperature. All studies were performed in triplicate, using samples from different culture preparations. The proportion of MSCs differentiated into the myocardial lineage was measured by counting the average number of MSCs stained in the cytoplasm and nuclear per high-power field by fluorescence microscopy (Leica). Each sample was counted randomly in eight separate high-power fields.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cells using a RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Aliquots of 2.0 μg of total RNA were reverse-transcribed to cDNA using a Superscript First-Strand Synthesis System with an oligo-dT primer (Invitrogen). The primer sequences of HIF-1α, TGF-β1, Smad4, NKx2.5, GATA-4 and GADPH were designed as shown in Table 1. The PCR products were size-fractionated by 1.5% agarose gel electrophoresis. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as internal standard.

Table 1 Sequences of all primer sets used for PCR

Primer	Sequence
HIF-1α FP	5′-GACACAG AAGCAAAGAACCC-3′
RP	5′-TCAAAGCGACAGATAACACG-3′
TGF-β_1 FP	5′-CTGTCCAAACTAAGGCTCGC-3′
RP	5′-AGACAGCCACTCA GGCGTA-3′
Smad4 FP	5′-TGATCTCTCAGGATTACCAC-3′
RP	5′-GAACACCAATACTCAGGAGCAG-3′
NKx2.5 FP	5′-CAGTGGAGCTGGACAAAGCC-3′
RP	5′-TAGCGACGGTTC TGGAACCA-3′
GATA-4 FP	5′-CTGTCATCTCACTATGGGCA-3′
RP	5′-CCAAGTCCGAGCAGGAATTT-3′
GADPH FP	5′-GGCAAG TTCAATGGCACAGT-3′
RP	5′-AAGGTGG AGGAATGGGAG TT-3′

FP forward primer, *RF* reverse primer

Statistical analysis

Results are shown as means $\pm$ SD. They were analyzed by one-way analysis of variance and Student's t test. Statistical analysis were performed with SPSS (version 11.0), and a value of $p < 0.05$ was considered statistically significant.

Results

Ad-vector-transfected MSCs

The MSCs (P_4) expressed CD44 ($93.98 \pm 4.92\%$), CD29 ($92.53 \pm 5.42\%$), and CD34 ($3.86 \pm 0.08\%$). The MSCs (P_4) transfected with pAd-HIF-1a-Ala564-Ala803 and pAd-LacZ (MOI of 100) were cultured for 24 h under standard tissue-culture conditions (95% air and 5% CO_2). HIF-1α-Ala564-Ala803-MSCs showed that HIF-1α was not only distributed in the cytoplasm of the MSCs but was also concentrated in the peri-nucleus. The MSCs and LacZ-MSCs showed no HIF-1α expression in the cytoplasm and peri-nucleus. The transfection efficiency was $90 \pm 4.2\%$, as determined by Ad-vector-mediated double-mutant HIF-1α expression in cultured MSCs (Fig. 1a). The viability of the transfected MSCs was found to be more than 90% by Annexin V/PI staining. Overall, the survival rate was similar to that of non-transfected and Ad-transfected MSCs ($93 \pm 5.42\%$ vs. $91 \pm 6.87\%$, $p > 0.05$), indicating that adenoviral transduction did not affect cell survival.

To determine whether HIF-1α-Ala564-Ala803 altered gene expression in MSCs, MSCs, HIF-1α-Ala564-Ala803-MSCs, and LacZ-MSCs were harvested and RT-PCR and western blotting were performed. RT-PCR showed that the HIF-1α-Ala564-Ala803-MSCs exhibited high expression of HIF-1α and TGF-β1 mRNA (a target gene of HIF-1α) (Fig. 1b), whereas LacZ-MSCs and MSCs only exhibited a week expression of TGF-β1 mRNA. In addition, western blot analysis also showed that the HIF-1α-Ala564-Ala803-MSCs exhibited high expression of HIF-1α and TGF-β1 protein. The LacZ-MSCs and MSCs only exhibited a low expression of TGF-β1 protein. Compared with the LacZ-MSCs or MSCs, the HIF-1α-Ala564-Ala803-MSCs showed higher TGF-β1 protein expression ($p < 0.01$) (Fig. 1c and d). These data suggest that Ad-mediated HIF-1α-Ala564-Ala803 can effectively promote MSCs to express HIF-1α and its downstream target genes.

Differentiation of MSCs to CMCs

To determine whether MSC differentiation was affected by pAd-HIF-1α-Ala564-Ala803, MSCs (P_4) were co-cultured with CMCs at a ratio of 1:2 and MSC-derived CMCs were characterized by immunofluorescent staining. As shown in Fig. 2, all CMCs were positive for cTnT (red cytoplasm) and α-actinin (green cytoplasm). The MSCs were negative for cTnT and α-actinin and exhibited nuclei with multiple nucleoli (blue nucleus). When MSCs were co-cultured with CMCs for seven days, the MSCs differentiated into cardiac phenotypes (pink or light blue nucleus and red or green cytoplasm). cTnT expression increased significantly in the pAd-HIF-1α-Ala564-Ala803 group compared with the control or pAd-LacZ groups (Table 2). Consistent with the cTnT changes, α-actinin also increased significantly in the pAd-HIF-1α-Ala564-Ala803 group compared with the control or pAd-LacZ groups (Table 2). Although the MSCs expressed CD44 and CD29, they did not express cTnT in the single MSC culture treated with pAd-HIF-1α-Ala564-Ala803 (Fig. 2). These data suggest that pAd-HIF-1α-Ala564-Ala803 promotes MSC differentiation in the environment provided by cardiac cells.

RT-PCR showed that MSCs expressed HIF-1α, TGF-β_1, and Smad4 but did not express the cardiac transcription factors NKx2.5 and GATA4 in the single MSC culture treated with pAd-HIF-1α-Ala564-Ala803. The cells of the co-culture groups not only expressed HIF-1α, TGF-β_1, and Smad4 but also expressed the cardiac transcription factors NKx2.5 and GATA4. All factors were augmented in the co-culture group treated with pAd-HIF-1α-Ala564-Ala803, compared with the co-culture group treated with pAd-LacZ or control (Fig. 3).

Discussion

We employed PCR-based site-specific mutagenesis with the template of pShuttle2-HIF-1α plasmids to construct pShuttle2-HIF-1α-Ala564-Ala803 double-mutant plasmids

Fig. 1 Adenovirus vector-transfected MSCs. The MSCs and LacZ-MSCs exhibited no HIF-1α expression. The HIF-1α-Ala564-Ala803-MSCs showed HIF-1α expression distributed in the cytoplasm and the peri-nucleus (**a**). RT-PCR showed mRNA expression of HIF-1α and TGF-β_1 (**b**); western blotting showed protein expression of HIF-1α and TGF-β_1 (**c**). The intensity of the western blot, determined by use of a densitometer, was calculated and plotted. Each value represents the mean ± SE from three independent experiments. *$p < 0.01$ versus the control or pAd-LacZ group (**d**). (1) MSCs transfected with pAd-HIF-1α-Ala564-Ala803; (2) MSCs transfected with pAd-LacZ; (3) non-transfected MSCs. *M*: ladder marker
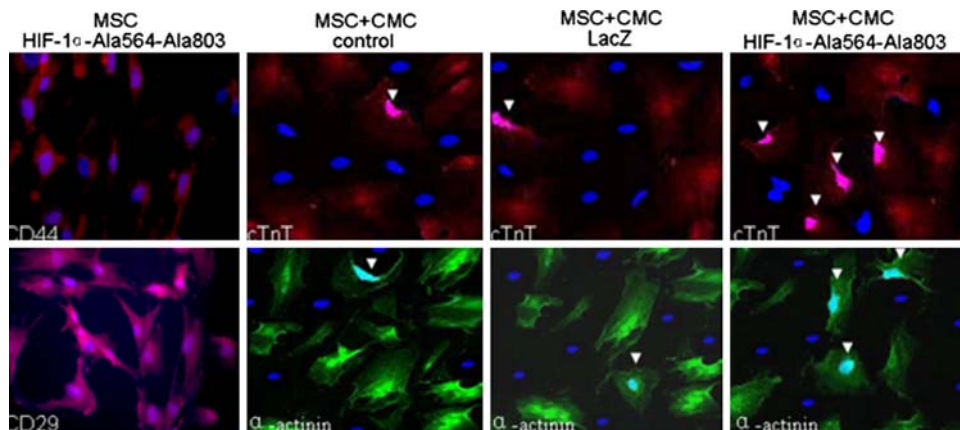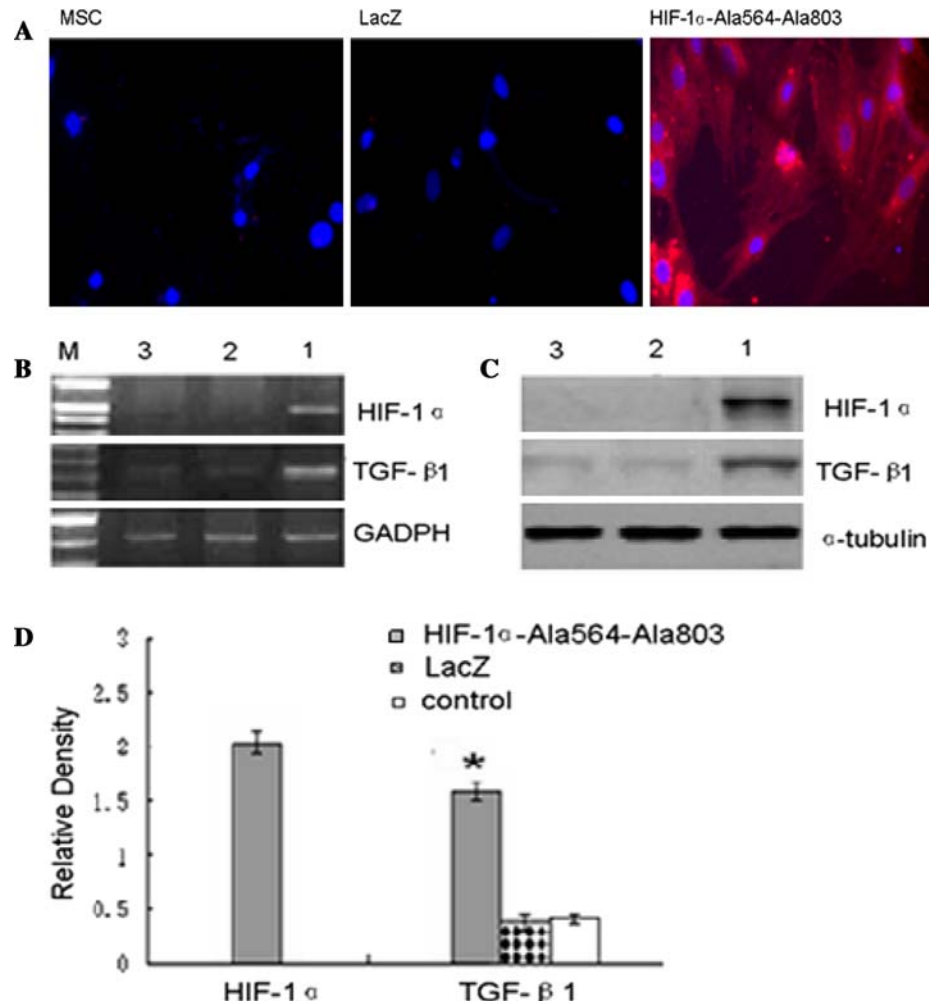

Fig. 2 Differentiation identified by immunofluorescence staining in vitro. DAPI was used for staining MSC nuclei (*blue* nuclei). MSCs labeled with DAPI were co-cultured with CMCs. The CMCs were stained with rhodamine-conjugated cTnT (*red* cytoplasm) or FITC-conjugated α-actinin antibodies (*green* cytoplasm). *Arrows* show MSCs that have differentiated into cardiac phenotypes (*pink* or *light blue* nucleus and *red* or *green* cytoplasm). The MSCs had CD44 and CD29 expression and no expression of cTnT and α-actinin in the single MSC culture treated with pAd-HIF-1α-Ala564-Ala803

to produce an active form of HIF-1α under normoxia. The double-mutant was introduced in an adenovirus vector to construct Ad-expressing double-mutant HIF-1α plasmids

(pAd-HIF-1α-Ala564-Ala803). PCR and sequencing confirmed the size and direction of the recombinant plasmids, and construction of the expression vector was successful.

Table 2 The positive rate of cardiac markers in MSCs of each group (%, means ± SD)

	MSC + CMC (control)	MSC + MSC (pAd-LacZ)	MSC + CMC (pAd-HIF-1α-Ala564-Ala803)
CTnT	8.25 ± 2.54	9.58 ± 2.86	30.06 ± 5.68*
a-Actinine	9.24 ± 2.84	10.36 ± 3.16	32.08 ± 6.25*

* $p < 0.01$ versus the control or pAd-LacZ group
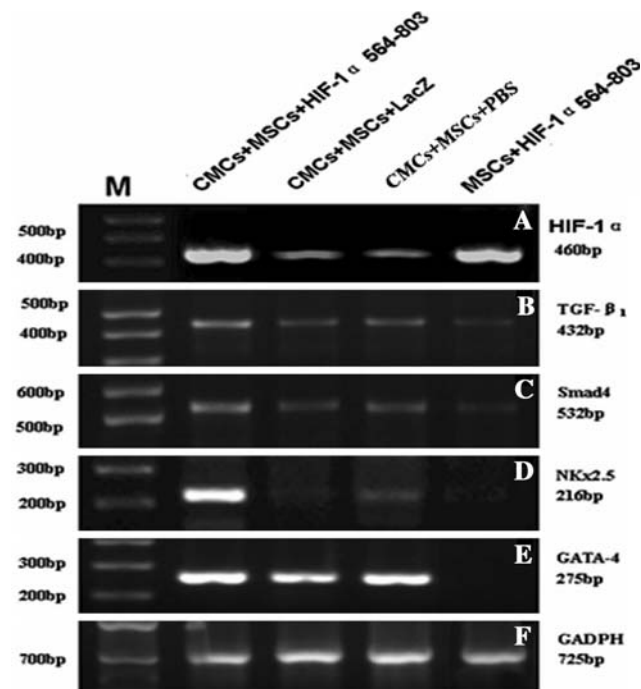
Fig. 3 RT-PCR showed that the cells expressed HIF-1α and its downstream factors. MSCs treated with pAd-HIF-1α-Ala564-Ala803 expressed HIF-1α, TGF-β1 and Smad4 but did not express NKx2.5 or GATA-4. The MSC–CMC co-culture treated with pAd-LacZ, PBS, and pAd-HIF-1α-Ala564-Ala803 expressed HIF-1α and its downstream factors. All factors were increased in the MSC–CMC co-culture treated with pAd-HIF-1α-Ala564-Ala803

Retrovirus and lentiviral vectors have been used for differentiation procedures of stem cells by exogenous gene delivery [14, 15]. These procedures may not be suitable for therapeutic applications, because the genes were continuously expressed, even after cell differentiation. Ad vectors have proven useful in differentiating stem cells into functional cells, because of their transient expression [16]. Thus, we used an Ad vector to transduce the HIF-1α double-mutant gene into stem cells. This study showed that transient double-mutant HIF-1α transduction into MSCs by using the Ad vector could effectively transduce MSCs and express HIF-1α, which was distributed in the cytosol and peri-nucleus. RT-PCR and western blot analysis showed

that HIF-1α-Ala564-Ala803-MSCs not only exhibited stable HIF-1α mRNA and protein expression but also expressed TGF-β_1 mRNA and protein, a target gene of HIF-1α. In the MSC–CMC co-culture, RT-PCR showed that HIF-1α mRNA remained highly expressed seven days after pAd-HIF-1α-Ala564-Ala803 was transduced (Fig. 1). These data indicate that the newly constructed pAd-HIF-1α-Ala564-Ala803 plasmids exhibit stable biological functioning at least one week after transfection.

When pAd-HIF-1α-Ala564-Ala803 was transduced into the MSC–CMC co-culture for seven days, the Ad vector enhanced cardiac differentiation of MSCs, suggesting that the combination of pAd-HIF1α-Ala564-Ala803 transduction and treatment in the MSC–CMC co-culture was important in attaining efficient cardiac differentiation of MSCs. The MSC–CMC co-culture induced MSC differentiation to CMCs [2–4], which was enhanced by hypoxia [5, 6]. However, these studies did not elucidate the manner in which hypoxia promoted MSC differentiation to CMCs. Hypoxia can increase the level of HIF-1α mRNA and protein expression. Can high HIF-1α expression promote MSC differentiation to CMCs in the co-culture system?

This study demonstrated that the MSC–CMC co-culture treated with pAd-HIF1α-Ala564-Ala803 for seven days had high HIF-1α mRNA expression with higher expression of cTnT and α-actinin in MSCs under normoxic conditions, which suggests that high HIF-1α expression may promote MSC differentiation to CMCs in the co-culture system. On the basis of our results and previous studies, we infer that HIF-1α may play a significant role in enhancing cardiac differentiation of MSCs under hypoxic conditions in the co-culture system. Confirmation is needed, with a study demonstrating that HIF-1α knock-down prevents or blunts the hypoxic effect on cardiac differentiation of MSCs. Our study also showed that MSCs of a single MSC culture treated with pAd-HIF1α-Ala564-Ala803 for seven days exhibited high HIF-1α expression but did not express cTnT and α-actinin. This suggests that the cardiac cells provided an environment in which MSCs could differentiate into the cardiac lineage, and this differentiation was enhanced by pAd-HIF-1α-Ala564-Ala803.

The cardiac host environment is sufficient to commit stem cells with a high repertoire of potential fates toward a very specific cell lineage, the myocardial lineage [1–4]. Growth and differentiation factors determine the fate of stem cells [17]. TGF-β released by cardiac myocytes is required to commit stem cells to the myocardial lineage in the cardiac host environment, because disrupters of TGF-β receptor-mediated signaling (e.g., LAP, noggin and ΔKTGFβRII) can prevent stem cell differentiation in vitro or in vivo [18, 19]. Our study showed that the MSC–CMC co-culture treated with pAd-HIF1α-Ala564-Ala803 exhibited high HIF-1α mRNA expression with increased

TGF-β_1, Smad4, NKx2.5, and GATA4 mRNA expression. The MSCs of the single MSC culture treated with pAd-HIF1α-Ala564-Ala803 showed high HIF-1α mRNA expression with increased TGF-β_1 and Smad4 mRNA expression but no NKx2.5 or GATA4 mRNA expression. Expression of NKx2.5 and GATA4 mRNA existed in CMCs of a single neonatal rat CMC culture, but there were no significant increase in the expression of NKx2.5 and GATA4 mRNA when they were treated with pAd-HIF1α-Ala564-Ala803 for seven days (data not shown). These data suggest that HIF-1α can trigger silent gene expression (e.g., NKx2.5 and GATA4) in stem cells only under special conditions, such as the cardiac host environment.

In this study, RT-PCR was used to demonstrate that the change in TGF-β_1 mRNA expression was, in general, consistent with Smad4 mRNA expression in the three co-culture groups, because Smad4 is downstream gene of TGF-β_1. TGF-β1/Smad4 can activate the cardiac-specific transcription factors NKx-2.5 and GATA-4. However, in the CMC differentiation of P19CL6 cells, the induction of either Csx/Nkx2.5 or GATA-4 was not sufficient for initiating differentiation of this cell line; the induction of both Csx/Nkx-2.5 and GATA-4 was sufficient for BMP-mediated differentiation into CMCs in the presence of DMSO [20]. The MSC–CMC co-culture treated with pAd-HIF1α-Ala564-Ala803 consistently exhibited high expression of Nkx-2.5 and GATA-4 with more than 30% differentiation, and the MSC–CMC co-culture without treatment showed a high expression of GATA-4 and a low expression of Nkx-2.5, with less than 10% differentiation. This cooperative effect of Nkx-2.5 and GATA-4 may increase myocardial differentiation of MSCs in the co-culture system, and the increased expression of Nkx-2.5 and GATA-4 synchronized by pAd-HIF1α-Ala564-Ala803 may aid the cooperative effect. Nkx-2.5 and GATA-4 can interact to activate downstream target genes because the GATA-binding domain is one of two independent cardiac-specific enhancers of Nkx-2.5 [21]. Nkx-2.5 and GATA-4 can form a stable protein complex, which is important for their cooperative effect on activation [22].

In conclusion, Adenovirus-mediated hypoxia-inducible factor 1α double-mutant, pAd-HIF-1α-Ala564-Ala803, can stably express an active form of HIF-1α and enhance the effect of MSC differentiation into cardiac lineage provided by cardiac cells, by augmenting its activated genes and their downstream cardiac transcription factors.

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