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Journal of Cellular Biochemistry 111:1619–1628 (2010)

Journal of Cellular Biochemistry

Mitogen-Activated Protein Kinase in Endothelin-1-Induced Cardiac Differentiation of Mouse Embryonic Stem Cells

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ABSTRACT

Endothelin-1(ET-1) is a potent vasoconstrictor involved in the development of cardiovascular diseases and is an important regulator of heart development. However, the role of ET-1 in cardiac differentiation of mouse embryonic stem cells (mESCs) and the underlying molecular mechanisms remain poorly understood. In the present study, we showed that ET-1 significantly up-regulated gene expression of the cardiac specific transcriptional factors Nkx2.5, GATA4, and conduction system specific marker CX40, with no affect on the gene expression of α-MHC and β-MHC in cardiac differentiation of mESCs. The percentage of beating embryoid bodies (EB) and the Troponin T (TnT) positive area in total EBs was unchanged following ET-1 treatment, while the percentage of spindle cells that stained positively with TnT was increased in the presence of ET-1. Further investigation indicated that the percentage of beating EBs and the TnT positive area were decreased by the extracellular signal-related kinases (ERK)-1/2 inhibitor U0126 and the p38 inhibitor SB203580, but not by the Jun amino-terminal kinases (JNK) inhibitor SP600125. Inhibition of ERK1/2, p38, and JNK pathways also blocked the up-regulation of Nkx2.5 and GATA4 by ET-1, however only inhibition of the ERK1/2 pathway had negatively effects on the increase in CX40 expression in response to ET-1. ET-1 induced an increase in the percentage of spindle cells was also inhibited by U0126. Our results suggest that ET-1 plays a significant role in the cardiac differentiation of mESCs, especially in those cells committed to the conduction system, with the ERK1/2 pathway playing a critical role in this process. J. Cell. Biochem. 111: 1619–1628, 2010.

KEY WORDS: EMBRYONIC STEM CELLS; ENDOTHELIN-1; MITOGEN-ACTIVATED PROTEIN KINASE; CARDIOMYOCYTE

Embryonic stem cells (ESCs) have the potential to differentiate into cardiomyocytes and represent different phenotypes, including sinus node, atrium, or ventricle of the heart [Maltsev et al., 1993, 1994]. Cardiomyocytes derived from ESCs are effective sources used in the study of cardiac development and provide the cell type used predominantly in regenerative medicine [Bongso et al., 2008; Murry and Keller, 2008]. Cardiomyocytes derived from ESCs have been transplanted into the myocardium following myocardial infarction and sick sinus syndrome [Kehat et al., 2004; Cai et al., 2007]. Therefore, it is essential to develop strategies for understanding the differentiation of cardiomyocytes from ESCs and the ability of lineage selection.

Heart development is mediated by a number of signaling molecules and transcriptional factors [Bruneau, 2002; Zaffran and Frasch, 2002; Chen et al., 2008]. Bone morphogenetic proteins (BMPs), Wnts, and fibroblast growth factors (FGFs) are the three critical families of signaling molecules essential in the development of the heart. BMPs and FGFs enrich the cardiac lineage by activating cardiac specific transcription factors, including Nkx2.5 and GATA4 [Ladd et al., 1998; Dell'Era et al., 2003]. These growth factors have been applied to enhance the differentiation of ESCs into cardiac progenitor cells [Behfar et al., 2002; Dell'Era et al., 2003]. In addition to these growth factors, several compounds have been used to induce stem cells into a specific lineage in vitro. Dimethylsulfoxide (DMSO) and retinoic acid were shown to facilitate the differentiation of stem cells into cardiac cells [Wobus et al., 1997; Ventura and Maioli, 2000]. Treatment with suramin resulted in enhanced formation of sinus node-like cells in the differentiation of ESCs into cardiomyocytes [Wiese et al., 2009].

Since its first description as a potent vasoconstrictor [Yanagisawa et al., 1988], endothelin-1 (ET-1) is considered as a paracrine/autocrine hormone that participates in the development of

Grant sponsor: National Natural Science Foundation of China; Grant number: 30971262; Grant sponsor: Natural Science Foundation of Guangdong Province; Grant number: 9451008901002399; Grant sponsor: Research Fund for the Doctoral Program of Higher Education of China; Grant number: 20090171120073.

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Received 13 June 2010; Accepted 21 September 2010 • DOI 10.1002/jcb.22895 • © 2010 Wiley-Liss, Inc.
Published online 4 November 2010 in Wiley Online Library (wileyonlinelibrary.com).

TABLE I. Primers for RT-PCR Analysis

Gene	Sense (5′–3′)	Antisense (5′–3′)	Product size (bp)
Nkx2.5	CGACGGAAGCCACGCGTGCT	CCGCTGTCGCTTGCACTTG	180
GATA4	CTCGATATGTTTGATGACTTCT	CGTTTTCTGGTTTGAATCCC	347
α-MHC	AGAAGCCCAGCGCTCCCTCA	GGGCGTTCTTGGCCTTGCCT	194
β-MHC	CTGATGCCCCGGCGGACAAA	GCGGATACCCTCCAGCACGC	230
CX40	CATGCTGGTCCTGGGCACCG	TGGGGACAGGGACTCCTGCG	477
HCN4	CGGAGGGCCTTCGAGACGGTT	GAGGGGCTGCTGGCGGGTGAA	443
β-actin	GAAATCGTGCGTGACATCAAAG	TGTAGTTTCATGGATGCCACAG	216

cardiovascular diseases, including heart failure, hypertension, and coronary heart disease. ET-1 also plays an important role in the maturation of the embryonic heart. Edn1$^{-/-}$ homozygote mice display cardiac malformations including ventricular septal defects with abnormalities of the outflow tract and an enlarged right ventricle. Moreover, vascular malformations including interrupted aortic arch, tubular hypoplasia of the aortic arch, and an aberrant right subclavian artery appear in homozygotes [Kurihara et al., 1995]. Exposure of cultured chicken embryonic cardiomyocytes to ET-1 induced the conversion of embryonic heart muscle cells into a Purkinje cell phenotype [Gourdie et al., 1998]. ET-1 also increased the percentage of pacemaker-like cells in the differentiation of ANP-EGFP expressing ESCs into cardiomyocytes [Gassanov et al., 2004]. ET-1 is a potent mitogen, which induced hypertrophy

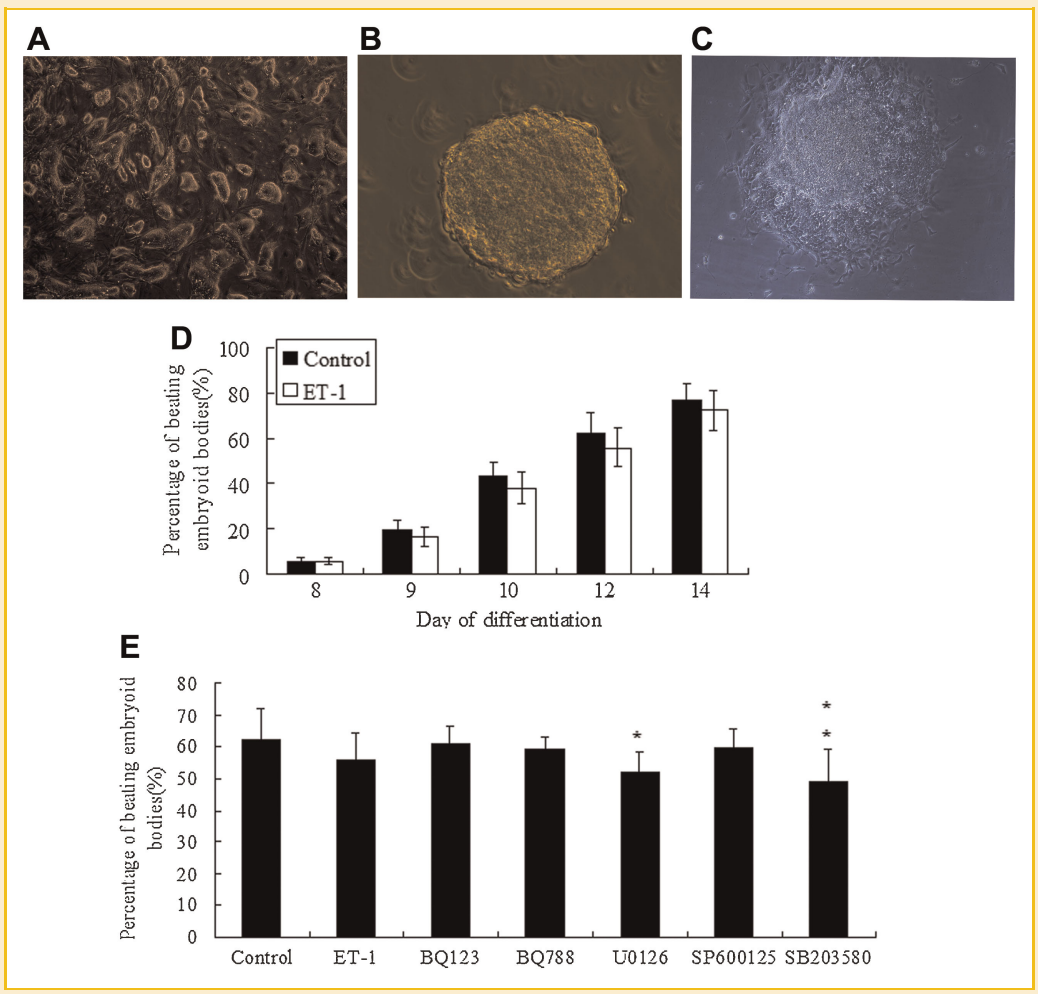

Fig. 1. Morphology of ESCs and EBs and the effects of pathways related drugs on the percentage of beating EBs. A: Undifferentiated mouse ESCs of line E14 (200×). B: Suspended EBs at day 5 (100×). C: Adherent EBs at day 12 (100×). D: EBs were treated with or without 10–7M ET-1 at the indicated time. More than 50 EBs per condition per time point were counted. ET-1 was prone to decrease the percentage of beating EBs compared with control, however the difference was not significant (n = 5). E: Effects of the ET-1 receptor antagonists (BQ123 and BQ788) and MAPK pathway inhibitors (U0126, SP600125, and SB203580) on the percentage of beating EBs at day 12. These results showed that U0126 and SB203580 decreased the percentage of beating EBs (n = 5). $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in cultured neonatal rat cardiomyocytes by activating the mitogen-activated protein kinase (MAPK) pathway [Bogoyevitch et al., 1994]. Recent studies have shown that MAPK pathways are involved in early heart development [Binetruy et al., 2007]. In the present study, we investigated the role of exogenous ET-1 on early cardiogenesis of mouse ESCs and the role of the MAPK pathway in this process.

MATERIALS AND METHODS

IN VITRO CELL CULTURE AND DIFFERENTIATION

E14 mouse ESCs (ATCC, Manassas, VA) were cultured on mitotically inactivated (mitomycin C) mouse embryonic fibroblasts (MEF) feeder layers in high glucose (4.5 g/L) Dulbecco's Modified Eagle's medium (DMEM; Gibco) supplemented with 15% fetal bovine serum (FBS; Gibco), 50 U/ml penicillin, 50 μg/ml streptomycin (Sigma–Aldrich), 10 μM β-mercaptoethanol (β-ME, Sigma–Aldrich), 0.1 mM nonessential amino acids (NEAAs, Sigma–Aldrich), and 1,000 U/ml leukemia inhibitory factor (LIF, Millipore).

For the differentiation of ESCs, the hanging drop culture method was used with minor modifications [Maltsev et al., 1993]. Approximately 400–600 ESCs in each drop of 20 μl in differentiation medium (4.5 g/L DMEM, 20% FBS, 50 U/ml penicillin, 50 μg/ml streptomysin, 10 μM β-ME, and 0.1 mM NEAAs) were plated on the lids of Petri dishes and cultured in hanging drops for 3 days, followed by another 2 days for suspension culture in the Petri dishes in order to form embryoid bodies (EBs). On day 5, suspended EBs were transferred onto gelatin-coated tissue culture plates in differentiation medium for terminal differentiation. The starting time of differentiation was recorded as day 0. To examine the effects of ET-1 on cardiac differentiation, ET-1 (Sigma–Aldrich) was added to the differentiation medium from day 3 to 8 at concentrations of 10^{-6} M, 10^{-7} M, 10^{-8} M, and 10^{-9} M. The ET-1 receptor antagonists, BQ123 (Sigma–Aldrich) and BQ788 (Sigma–Aldrich), were both used at a final concentration of 10^{-6} M. The extracellular signal-related kinases (ERK) 1/2 inhibitor U0126 (Sigma–Aldrich) was used at a final concentration of 10^{-5} M. The Jun amino-terminal kinases (JNK) inhibitor SP600125 (Sigma–Aldrich) and the p38 inhibitor SB203580 (Sigma–Aldrich) were used at final concentrations of 10^{-6} M.

SEMI-QUANTITATIVE RT-PCR

Total RNA was extracted from differentiating ESCs at days 6, 8, 10, 12, and 14 using TRIzol reagent (Invitrogen) and the cDNA was synthesized from 2 μg of RNA using reverse transcriptase (Takara) according to the manufacturer's instructions. Primers were designed using Primer 5.0 software and are shown in Table I. PCR reactions

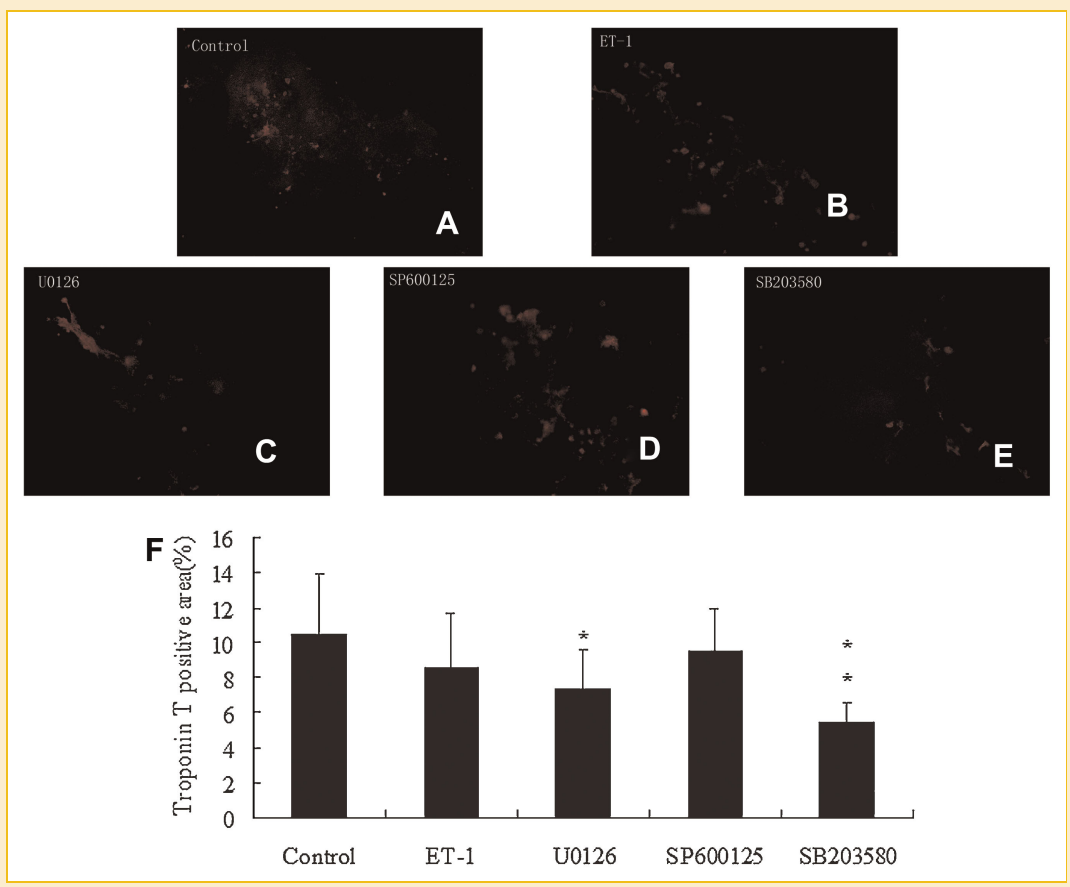

Fig. 2. The effects of ET-1 and MAPK pathway inhibitors on TnT expression in corresponding EBs. A–E: Adherent EBs were stained with TnT at day 12 (100×). F: ET-1 and SP600125 did not change the TnT positive area in corresponding EBs compared with the control group while U0126 and SB203580 decreased the TnT positive area in corresponding EBs ($n = 10$). $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

were carried out according to the following conditions: denaturation at 94 °C for 30 s, annealing at 56–65 °C for 30 s, and extension for 60 s at 72 °C (30 cycles). Products were subjected to electrophoresis on 2% agarose gels and the fluorescent densities of the resulting bands were determined using of Quantity One software (Bio-Rad) and normalized according to expression of β-actin.

IMMUNOCYTOCHEMISTRY

The adherent EBs on gelatin-coated tissue culture plates at day 12 were fixed in 4% polyoxymethylene for 15 min, washed three times with phosphate-buffered saline (PBS; 3 × 5 min), permeabilized with 0.2% Triton for 20 min and blocked with 1% bovine serum albumin (BSA) for 30 min at room temperature. The samples were incubated with anti-Troponin T (TnT) primary antibody (Abcam) at a dilution of 1:500 for 2 h at 37 °C and washed three times with PBS. Following incubation with the Cy3-conjugated secondary antibody (Sigma–Aldrich) for 1 h at 37 °C and washed three times with PBS, the samples were visualized using fluorescence microscopy (Leica systems) to detect TnT localization. Some adherent EBs were digested into single cells and cultured for another 2 days for immunocytochemistry detection. Nuclei were stained with 1 μg/ml Hoechst 33258 (Sigma–Aldrich). Quantification of the TnT positive area in corresponding EBs was performed using the Image-Pro Plus 6.0 software.

STATISTICAL ANALYSIS

All results are presented as means ± SD of at least three independent experiments. Significant differences were determined using one-way ANOVA with SPSS 13.0 software. A value of $P < 0.05$ was considered statistically significant.

RESULTS

EFFECTS OF ET-1 ON CARDIAC DIFFERENTIATION OF ESCS IN VITRO

Mouse ESCs were maintained as undifferentiated on feeder layers and formed tight, compact, and rounded colonies (Fig. 1A). Cardiac differentiation was achieved by forming EBs (Fig. 1B). On day 5, suspended EBs were transferred to culture plates, with most EBs attaching to the plates within 24 h. Adherent EBs became flattened and formed thin multilayered structures (Fig. 1C). Spontaneously contracting EBs began to appear at day 8. The percentage of beating EBs in culture plates was recorded at days 8, 9, 10, 12, 14. Approximately 5.4% of EBs were beating at day 8, with the percentage increasing to 43.2% at day 10 and 76.6% at day 14. At days 9, 10, 12, 14, the percentage of beating EBs was lower in the presence of ET-1, however, this effect was not significant (Fig. 1D).

The effects of ET-1 on the cardiac differentiation in ESCs were further confirmed by immunostaining. Beating EBs treated with or without ET-1 were stained with TnT at day 12 (Fig. 2A,B). The relative positive area of the cardiac specific marker TnT in the ET-1 group was consistent with the control group (Fig. 2F). Adherent-beating EBs digested into single cells were stained positively with TnT in some cells and the positive cells were spindle, round, and multiangular shaped (Fig. 3A–C). ET-1 significantly increased the percentage of spindle cells from 12.9% to 26.6% ($P < 0.01$), with

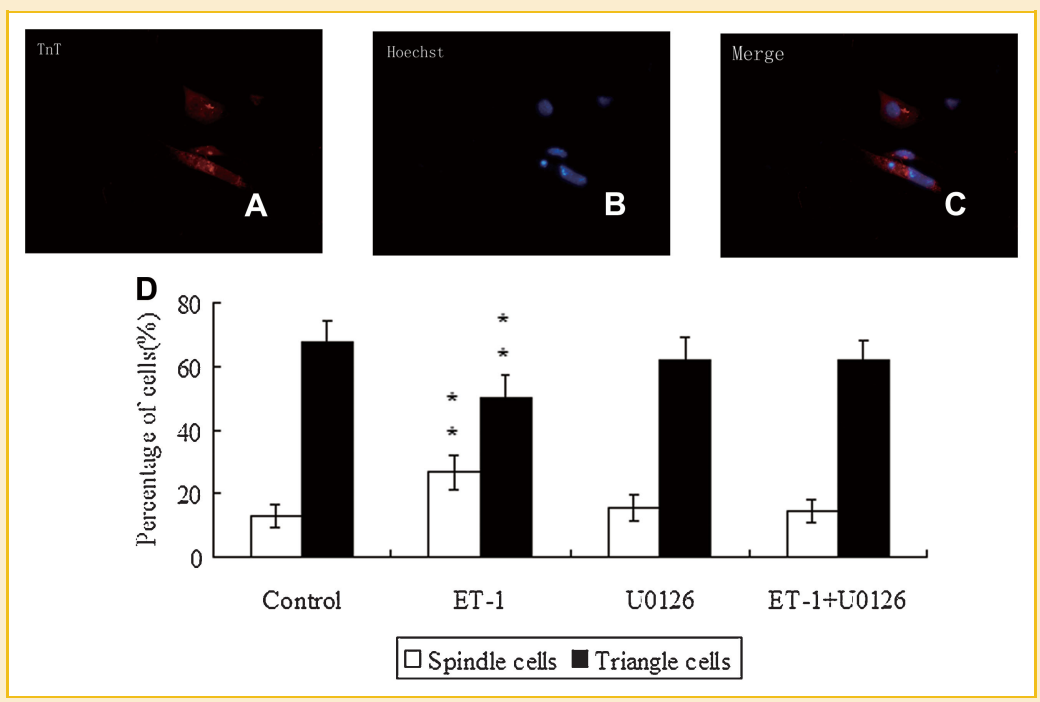

Fig. 3. The effects of ET-1 and U0126 on the percentage of spindle cells. Single cells digested from beating EBs were stained with TnT (A) or Hoechst33285 (B). C: Merged figure A and B (400×). The positive cells were spindle, round, and multiangular shaped. D: ET-1 increased the percentage of spindle cells with decreasing the percentage of multiangle cells compared with control group and the role of ET-1 was inhibited by U0126 (n = 10). $^{**}P < 0.01$ vs. control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

decreasing the percentage of multiangular cells from 67.7% to 49.9% ($P < 0.01$) in the TnT positive cells (Fig. 3D).

Three types of cardiac differentiation-related genes were detected by RT-PCR: (1) the cardiac specific transcription factors, Nkx2.5 and GATA4; (2) the cardiac contractile proteins, α-MHC and β-MHC; (3) and the conduction system specific markers, CX40 and HCN4. ET-1 significantly up-regulated mRNA expression of the cardiac-specific transcriptional factors Nkx2.5 and GATA4 at day 8 in a dose-dependent manner, with the maximal effect at a concentration of 10^{-7} M (Fig. 4). Expression levels of Nkx2.5 decreased with cardiac differentiation from days 6 to 14 in the control and ET-1 groups, however, ET-1 expression was higher than the control group at several time points (Fig. 5A). Expression of the cardiac transcription factor GATA4 increased from days 6 to 14. At days 6, 8, 9, 10, and 12, GATA4 expression was higher in the ET-1 group compared with the control group (Fig. 5A). Expression patterns of α-MHC and β-MHC were detected subsequently. The results showed that both α-MHC and β-MHC were expressed at detectable levels from day 8, with the expression increasing according to cardiac differentiation of EBs (Fig. 5B). Nevertheless, these two contractile proteins remained unchanged in the ET-1 group compared with the control group. Likewise the transcriptional level of the sinus node specific marker HCN4 remained constant in the ET-1 group (Fig. 5C). However, another conduction system marker, CX40, was up-regulated in the ET-1 group from day 9 and persisted to day 14 (Fig. 5C). These results suggest that ET-1 is not prone to enhance the differentiation of cardiomyocytes derived from ESCs, but promotes sublineage commitment of the conduction system via specific transcriptional factors.

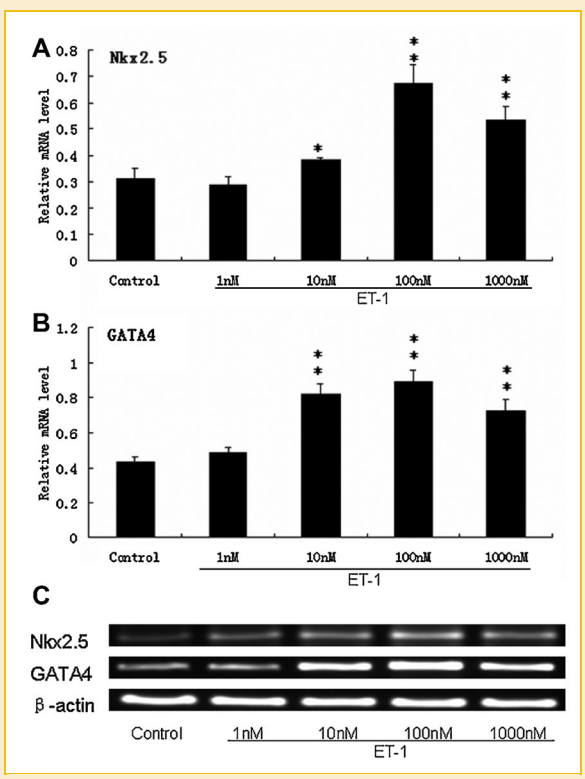

Fig. 4. ET-1 increased Nkx2.5 and GATA4 gene expression in a dose-dependent manner and the maximal effect of ET-1 presented at a concentration of 10-7M. RNA samples extracted from EBs were detected at day 8. A,B: Densitometric analysis showed the ratio of PCR products of Nkx2.5 and GATA4 to that of β-actin ($n = 5$). $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control. C: PCR products isolated using agarose gel electrophoresis.

EFFECTS OF ET-1 ARE MEDIATED BY BINDING WITH THE ET_A AND ET_B RECEPTORS

ET_A (ET_AR) and ET_B receptors (ET_BR) are two main receptors of ET-1 and are both expressed in the embryonic heart [Kanzawa et al., 2002]. To investigate which receptor was involved in ET-1 effects on ESCs, the ET_AR and ET_BR specific antagonists BQ123and BQ788 were administered. These results indicated that neither BQ123 nor BQ788 changed the percentage of beating EBs (Fig. 1E) and the gene expression of Nkx2.5, GATA4, and CX40 (Fig. 6). A significant decrease in the expression levels of Nkx2.5, GATA4, and CX40 were observed in ET-1-treated cultures plus BQ123 or BQ788 compared with ET-1 alone (Fig. 6). Nevertheless, there was no significant difference between the control group and the ET-1 plus BQ123 or BQ788 groups. These results indicated that the effects of ET-1 on ESCs are mediated through ET_AR and ET_BR signaling in a receptor-dependent manner.

INVOLVEMENT OF MAPK IN ET-1-INDUCED ESC DIFFERENTIATION

Three MAPK pathway inhibitors were utilized in these experiments. The ERK1/2 inhibitor U0126 and the p38 inhibitor SB203580 were able to reduce the percentage of beating EBs compared with control (Fig. 1E). The TnT positive area of these two groups diminished correspondingly (Fig. 2C–F). Gene expression of Nkx2.5 and GATA4 were down-regulated in the U0126 group and the SB203580 group compared with the control group. Notably, the JNK inhibitor SP600125 did not impair cardiomyogenesis and transcription factor expression. However, all these three drugs inhibited the effects of ET-1 on Nkx2.5 and GATA4 expression (Fig. 7A). Increased CX40 in the ET-1 group was significantly inhibited in the presence of U0126, as opposed to SP600125 or SB203580 (Fig. 7B). Immunocytochemistry experiments showed that the ET-1 induced increase in the percentage of spindle cells was also inhibited by U0126 (Fig. 3D). These results suggest that the ERK1/2 and p38 pathways play an important role in cardiac differentiation with the effects of ET-1 on cardiac sublineage commitment primarily mediated through the ERK1/2 pathway.

DISCUSSION

ET-1 is highly expressed in the endocardium of the outflow tract of the heart, endothelium of the arch arteries, and dorsal aorta in 10.0-day-old mice and is apparent in the conotrunal region in 11.5-day-old mice during embryonic heart development [Kurihara et al., 1995]. ET-1 knockout mice exhibit several types of cardiovascular malformations, with those treated with the ET_A receptor antagonist BQ123 showing increased odds and extent of malformation [Kurihara et al., 1995]. These results suggest that the function of ET-1 on the development of the embryonic heart and these biological responses are associated with its receptors.

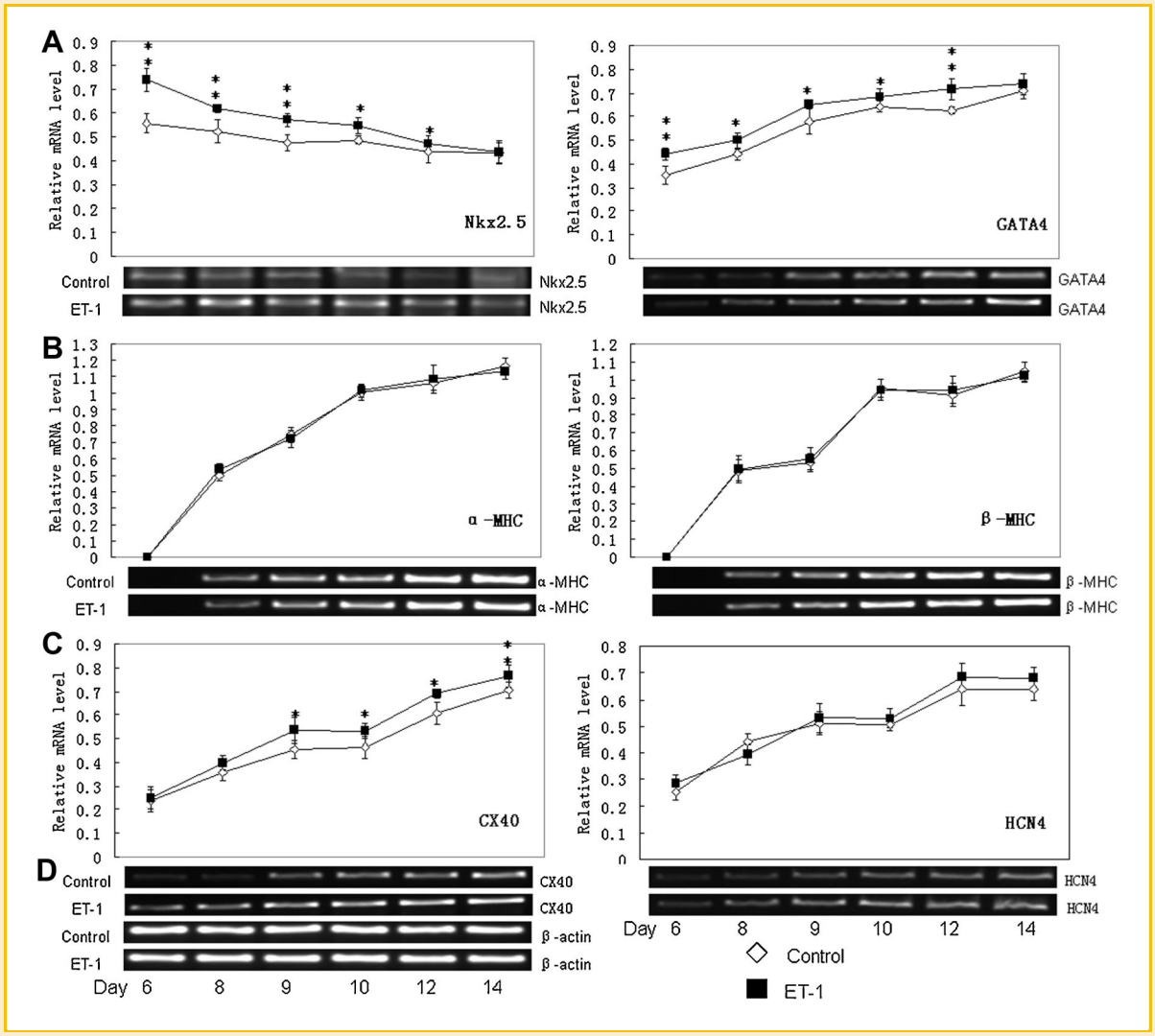

Fig. 5. RT-PCR analysis of the transcription of six genes at the given time points from the control and ET-1 groups. Nkx2.5, GATA4, and CX40 gene expression were up-regulated by ET-1 at the indicated time points. A: Relative mRNA levels of cardiac specific transcription factors, Nkx2.5, and GATA4 ($n = 5$). B: Relative mRNA levels of cardiac contractile proteins, α-MHC, and β-MHC ($n = 5$). C: Relative mRNA levels of conduction system specific genes, CX40 and HCN4 ($n = 5$). $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control. D: Expression of the housekeeping gene β-actin.

In vivo and in vitro experiments have demonstrated that ET-1 converts the contractile myocyte phenotype into the Purkinje fiber cell phenotype in a receptor-dependent manner, during which ET converting enzyme-1 is required [Gourdie et al., 1998; Takebayashi-Suzuki et al., 2000; Kanzawa et al., 2002; Patel and Kos, 2005]. Effects of ET-1 on heart development imply that ET-1 may act as a cytokine to determine ESCs lineage selection. Previous investigations indicate that the percentage of spindle-shaped cells, which exhibit a higher spontaneous beating rate, faster hyperpolarization-activated cyclic nucleotide-gated current (I_f current) activation, and larger I_f current densities, are increased when ANP-EGFP expressing ESCs differentiate into cardimyocytes exposed to ET-1 [Gassanov et al., 2004]. In the present study, we show that exogenous ET-1 plays a significant role in the cardiac differentiation of mESCs and MAPKs are involved in this process.

In our experiments, six representative genes were detected. Nkx2.5 is a homeobox transcriptional factor manipulating cardiac commitment and differentiation [Lints et al., 1993]. It is expressed several hours prior to cardiac α-actin and β-MHC activation. Nkx2.5 knock-out mice are unable to initiate looping morphogenesis [Lyons et al., 1995]. GATA4, another cardiac specific transcription factor, is detected very early in cardiogenesis and persists later in the developing heart [Kelley et al., 1993]. Nkx2.5 and Gata4 are mutual cofactors exerting the functions of inductive signals during specification, patterning, and differentiation of heart [Durocher et al., 1997; Zaffran and Frasch, 2002; Brown et al., 2004]. Accumulated evidence implies that Nkx2.5 and GATA4 are also involved in conduction system differentiation [Takebayashi-Suzuki et al., 2001; Patel and Kos, 2005; Harris et al., 2006]. In vivo studies show that Purkinje fibers express significantly higher levels of Nkx2.5 and GATA4 mRNA compared with ordinary heart muscle

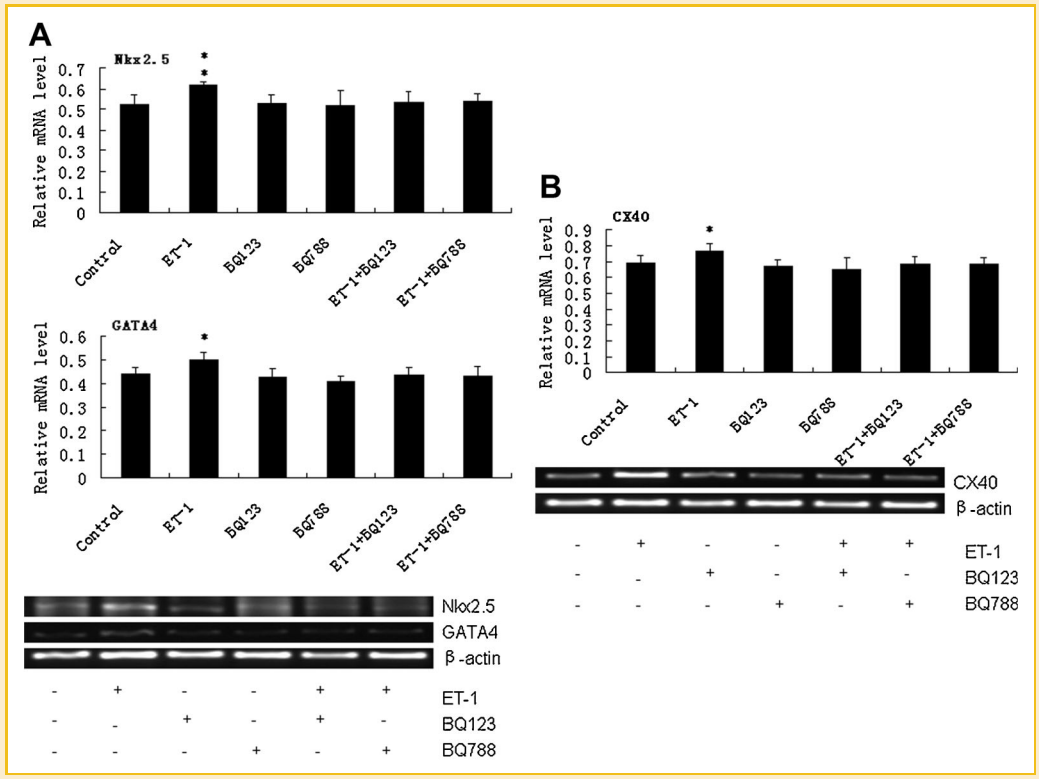

Fig. 6. Effects of ET-1 on Nkx2.5, GATA4, and CX40 were inhibited by co-treatment with the ET-1 receptor specific antagonists BQ123and BQ788. A: RNA samples extracted from control, ET-1, BQ123, BQ788, ET-1+BQ123, and ET-1+BQ788 groups were detected by RT-PCR of Nkx2.5 and GATA4 at day 8 ($n = 5$). B: RNA samples from control, ET-1, BQ123, BQ788, ET-1+BQ123, and ET-1+BQ788 group were detected by RT-PCR of CX40 at day 12 ($n = 5$). $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control.

cells. In vitro experiments indicate that ET-1 coverts embryonic cardiomyocytes into conduction system cells accompanied with up-regulation of Nkx2.5 and GATA4 gene expression. Our results suggest that Nkx2.5 and GATA4 expression was significantly up-regulated in the ET-1 group in the early and intermediate stages of cardiac differentiation (Fig. 5A). These results were in agreement with the findings in murine embryonic cardiomyocytes in vitro [Patel and Kos, 2005]. Expression levels of Nkx2.5 were much higher than those of controls at days 6–9, but not higher at days 10–12, while GATA4 was consistently higher than controls over all days with the exception of day 14 (Fig. 5A). Expression of ET_AR and ET_BR is developmental regulated [Kanzawa et al., 2002] and the declined effects of ET-1 on Nkx2.5 may be due to down-regulation of ET_AR and ET_BR during development. Although these two genes had different patterns, the effects of ET-1 on both of them was absent at day 14. We considered that regulation of Nkx2.5 and GATA4 by ET-1 may utilize different pathways, but the underlying mechanisms need to be further elucidated. Although the two main cardiac specific transcriptional factors were up-regulated by ET-1, cardiomyogenesis was unchanged. The percentage of beating EBs did not increase correspondingly (Fig. 1D). The positive area of beating EBs stained with TnT remained constant in ET-1 group (Fig. 2A,B,F). Immunocytochemistry experiments suggest that ET-1 increased the percentage of spindle cells. Although ANP-EGFP expressing spindle cells all have a pacemaker-like phenotype [Gassanov et al., 2004], TnT-stained spindle cells may have different electrophysiological properties. The electrophysiological properties of TnT-EGFP-expressing spindle cells can be investigated in the next experiment. Two myosin heavy chain isoforms, α-MHC and β-MHC, were not up-regulated at the transcriptional level (Fig. 5B), notwithstanding that GATA4 is considered as an upstream transcription factor of α-MHC [Molkentin et al., 1994]. These results were quite understandable since Nkx2.5 and GATA4 knock-out experiments indicated that either of these two genes play a partial role in heart development and other transcription factors involve in cardiac differentiation [Lyons et al., 1995; Narita et al., 1997]. The conduction system specific marker, CX40, was significantly up-regulated in the presence of ET-1 (Fig. 5C). The consequence was consistent with the results of previous studies [Gassanov et al., 2004; Patel and Kos, 2005]. Electrophoretic mobility shift assays (EMSA) show that the transcriptional factors Nkx2.5, GATA4, and Tbx5 act together to modulate CX40 transcription [Linhares et al., 2004]. Nkx2.5 and GATA4 are able to activate the promoter of CX40 while Tbx5 exerts its depressive effect. In our experiments, CX40 was up-regulated following Nkx2.5 and GATA4 up-regulation from day 9. These results suggest that transcription factors Nkx2.5 and GATA4 modulate conduction system differentiation via their downstream gene CX40. HCN4 is a member of the hyperpolarization-activated cyclic nucleotide-gated channels family, which is prominently

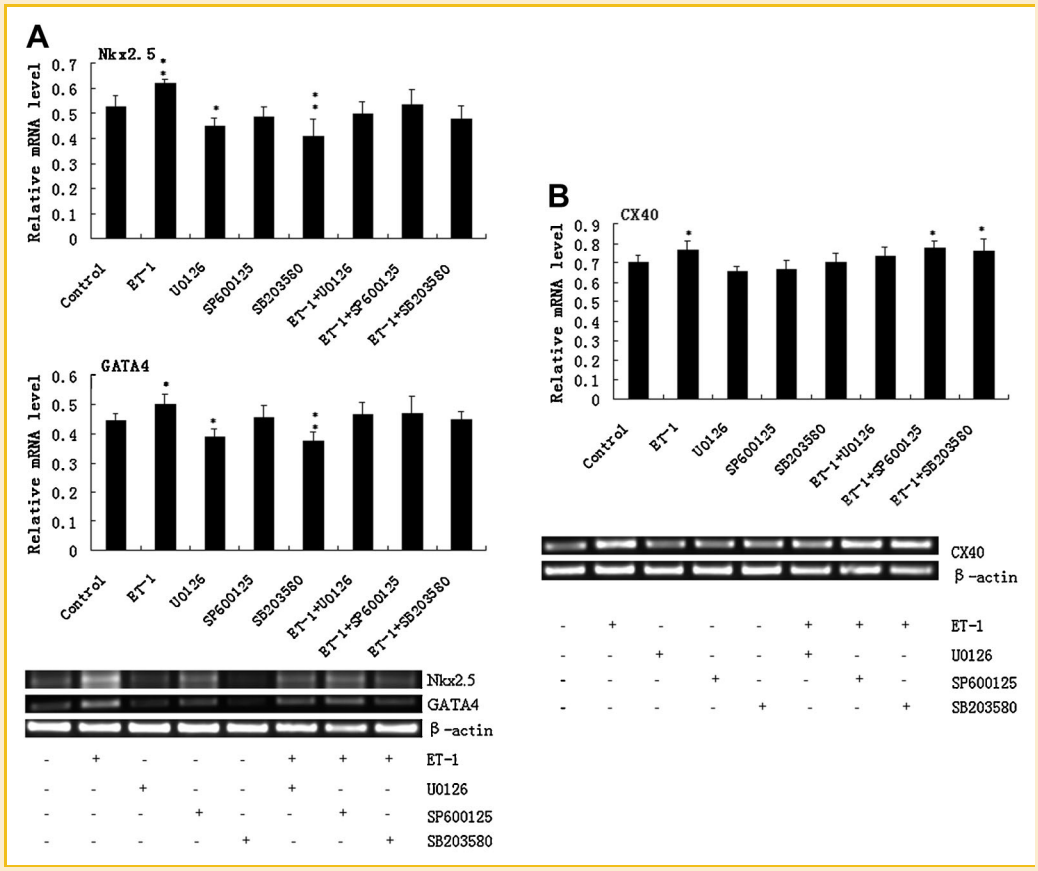

Fig. 7. Involvement of the MAPK pathways in ET-1-induced up-regulation of Nkx2.5, GATA4 (A), and CX40 (B). RT-PCR analysis showed that the ERK1/2 inhibitor U0126, the p38 inhibitor SB203580, and the JNK inhibitor SP600125 could inhibit effects of ET-1 induced up-regulation of Nkx2.5, GATA4 at day 8 while only U0126 inhibited the effects of ET-1 induced up-regulation of CX40 at day 12 ($n = 5$). $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control.

expressed in the sinoatrial node. Our results indicate that HCN4 expression remained consistent in the ET-1 group compared with the control group (Fig. 5C). Our results demonstrated that ET-1 exerts its distinct effects on mouse ESC differentiation unlike the other signaling molecules BMPs, Wnts, and FGFs [Behfar et al., 2002; Dell'Era et al., 2003; Naito et al., 2006].

ET-1 signaling is triggered by binding of ET-1 to its G protein-coupled receptors, ET_AR and ET_BR [Arai et al., 1990]. ET_AR and ET_BR have been identified in embryonic heart showing distinct expression patterns [Kanzawa et al., 2002]. ET_AR is expressed extensively in the embryonic heart, while ET_BR is expressed at higher levels in the atrium and left ventricle than in the right ventricle. The expression pattern of these two isotypes implies that ET_AR and ET_BR may have different contributions to heart development. In our experiments, ET_AR and ET_BR specific antagonists BQ123and BQ788 were added to the medium in the presence of ET-1. BQ123 or BQ788 alone did not change cardiac differentiation patterns, however, both of them could block the effects of ET-1 (Fig. 6). These results demonstrate that the effects of ET-1 on cardiac differentiation were mediated by both ET_AR and ET_BR.

MAPKs are important signal-transduction enzymes, which are involved in many facets of cellular regulation including ESC lineage commitment [Binetruy et al., 2007]. The MAPK family comprises four groups of proteins: ERK1/2, JNK1/2/3, p38a/b/g/d, and ERK5. The p38 pathway is considered to be essential to cardiac differentiation, whereas the ERK pathway is partly involved in this process [Eriksson and Leppa, 2002]. ET-1 is a potent mitogen, which is able to activate ERK, JNK, and p38 pathways exerting its hypertrophy effects on cultured neonatal rat cardiomyocytes [Choukroun et al., 1998; Irukayama-Tomobe et al., 2004]. In the present studies, we hypothesized that the MAPK pathways were involved in the effects of ET-1 on ESCs cardiac differentiation. The results indicate both inhibition of the p38 pathway with SB203580 and inhibition of the ERK pathway with U0126 were capable of decreasing cardiac differentiation which showed a reduction in the percentage of beating EBs (Fig. 1E), reducing the positive area of TnT (Fig. 2F), and down-regulation of Nkx2.5 and GATA4. SB203580 appeared to have more notable effects, while inhibition of the JNK pathway with SP600125 had no effect on cardiomyogenesis. These results illustrate that the ERK1/2 and p38 pathways are essential to heart development, and the p38 pathway plays a more important role in this process. Expression of Nkx2.5 and GATA4 were impaired by U0126 and SB203580, while increased expression of the transcriptional factors Nkx2.5 and GATA4 in the ET-1 group were inhibited by these three inhibitors, indicating that the JNK pathway only plays a role in the presence of ET-1 (Fig. 7A). These three inhibitors alone did not change the expression pattern of CX40, and up-regulation of CX40 by ET-1 was only inhibited by

U0126 (Fig. 7B), suggesting that only the ERK1/2 pathway was critical to the effects of ET-1 on CX40 expression. ET-1 induced increase in the percentage of spindle cells was also inhibited by U0126. These results suggest that ERK1/2 pathway may play an important role in the course of converting cardiomyocytes into conduction system cells by ET-1.

In summary, ET-1 plays a significant role in cardiac differentiation of mESCs, particularly in the conduction system, and the ERK1/2 pathway was critical to this process. Our results suggest that ET-1 might be a strategy for efficient development of conduction system cells, which is a valuable step in pacemaker cell selection for regenerative medicine. However, the best method to purify the pacemaker cells from agglomerated EBs and how to transplant these cells to patients such as those with sick sinus syndrome required further study.

ACKNOWLEDGMENTS

This study was supported by a grant from the National Natural Science Foundation of China to Jingfeng Wang (No. 30971262), a grant from the Natural Science Foundation of Guangdong Province to Shuanglun Xie (No. 9451008901002399), and a grant from the Research Fund for the Doctoral Program of Higher Education of China to Shuang-lun Xie (No. 20090171120073).

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