Inhibitor of DNA binding 1 regulates cell cycle progression of endothelial progenitor cells through induction of Wnt2 expression

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Abstract. Endothelial injury is a risk factor for atherosclerosis. Endothelial progenitor cell (EPC) proliferation contributes to vascular injury repair. Overexpression of inhibitor of DNA binding 1 (Id1) significantly promotes EPC proliferation; however, the underlying molecular mechanism remains to be fully elucidated. The present study investigated the role of Id1 in cell cycle regulation of EPCs, which is closely associated with proliferation. Overexpression of Id1 increased the proportion of EPCs in the S/G_2M phase and significantly increased cyclin D1 expression levels, while knockdown of Id1 arrested the cell cycle progression of EPCs in the G_1 phase and inhibited cyclin D1 expression levels. In addition, it was demonstrated that Id1 upregulated wingless-type mouse mammary tumor virus integration site family member 2 (Wnt2) expression levels and promoted β-catenin accumulation and nuclear translocation. Furthermore, Wnt2 knockdown counteracted the effects of Id1 on cell cycle progression of EPCs. In conclusion, the results of the present study indicate that Id1 promoted Wnt2 expression, which accelerated cell cycle progression from G_1 to S phase. This suggests that Id1 may promote cell cycle progression of EPCs, and that Wnt2 may be important in Id1 regulation of the cell cycle of EPCs.

Introduction

The World Health Organization has reported that coronary heart disease (CHD), which is primarily caused by atherosclerosis, remains the leading cause of mortality worldwide, resulting in 7.4 million deaths in 2012 (1). Current strategies, including risk factor control and percutaneous coronary intervention (PCI) are somewhat effective (2-4). However, lumen restenosis often occurs following PCI. Therefore, novel treatment strategies are required. Atherosclerosis, which is critical for the development of CHD, is often the result of vascular endothelial injury (5). Therefore, maintaining the integrity of the functional endothelial monolayer is crucial for the prevention of atherosclerosis initiation and the treatment of CHD (5). Previous studies have demonstrated that endothelial progenitor cells (EPCs), from origins including the spleen and bone marrow, homed to sites of vascular injury for re-endothelialization upon introduction into the circulation, suggesting a vascular restoration role for EPCs (6-9). However, EPCs proliferate slowly and the number of EPCs in peripheral blood is limited, thus restricting their clinical application (10). A previous study has demonstrated that inhibitor of DNA binding 1 (Id1) flips the angiogenic switch via the regulation of EPC migration from bone marrow (11). Furthermore, our previous research revealed that Id1 markedly promoted EPC proliferation (12). However, the mechanisms underlying Id1-mediated EPC proliferation remain to be elucidated.

Id1 is a member of the helix-loop-helix (HLH) family, which is involved in numerous developmental processes, including cellular proliferation, migration and differentiation, and functions as a heterodimer with other HLH proteins (13). Previous studies have demonstrated that Id1 is critical in the regulation of cell cycle progression of tumor cells by increasing cyclin D1 expression and promoting G_1/S transition (14-17). Therefore, Id1 may be involved in regulation of the cell cycle of EPCs.

Cyclin D1, a well-known positive cell regulator from G_1 to S phase, is also a classic target gene of the canonical wingless-type mouse mammary tumor virus integration site (Wnt)/β-catenin signaling pathway (17,18). Among the Wnt family, Wnt2 has been demonstrated to contribute to vascularization, and acts as a positive signal in the differentiation of stem cells into vascular endothelial cells (19-21). Furthermore, a previous study has revealed that Wnt2 was diminished in liver sinusoidal endothelial cells of Id1-deficient mice, and administration of exogenous Wnt2 restored hepato-vascular regeneration in Id1-deficient mice (22). However, the

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association between Id1 and Wnt2, and the effect of this on cell cycle regulation in EPCs remains to be elucidated.

In the present study, it was hypothesized that Id1 acts on the cell cycle progression of EPCs via the regulation of Wnt2 expression. Id1-overexpressed EPCs and Id1-knockdown EPCs were therefore used as models to investigate the role of Id1 on cell cycle regulation in EPCs and its possible underlying mechanisms. The *in vitro* data generated by the present study indicated that Id1 promoted cell cycle progression of EPCs from G_1 to S phase via a Wnt2-dependent mechanism.

Materials and methods

Culture and characterization of bone marrow-derived EPCs. All procedures were approved by the Care of Animal Experiment Committee of Third Military Medical University (Chongqing, China). A total of 150 C57BL/6J male mice (age, 6-8 week; weight, 22-30 g) were obtained from the Experimental Animal Center of Third Military Medical University. Mice were housed at 20-26˚C with 40-70% humitidy, under a 12-h light/dark cycle and with *ad libitum* access to food and water. The culture and characterization of bone marrow-derived EPCs were performed as described previously (23,24). Briefly, bone marrow-derived mononuclear cells were isolated from the tibia and femur of C57BL/6J mice by density gradient centrifugation using Histopaque®-1083 (Sigma-Aldrich, St. Louis, MO, USA). The mononuclear cells were cultured at 37˚C in Dulbecco's modified Eagle's medium (DMEM)/F-12 (GibcThermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS; HyClonGE Healthcare Life Sciences, Logan, UT, USA) in cell culture flasks coated with gelatin. After 24 h, non-adherent cells were separated and seeded into a new flask. Following an additional 48 h, non-adherent cells were removed and adherent cells were cultured continuously for *in vitro* experiments.

For the characterization assay, cells were incubated at 37˚C with acetylated low density lipoprotein, labeled with 1,1'dioctadecyl-3,3,3', 3'-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL; Biomedical Technologies, Inc., Stoughton, MA, USA) for 4 h, fixed with 4% paraformaldehyde and incubated at 37˚C with fluorescein isothiocyanate-*Ulex europaeus* agglutinin-1 (FITC-UEA-l; Sigma-Aldrich) for 1 h. Cells were then incubated with DAPI for 5 min and observed under an immunofluorescence laser scanning confocal microscope (Leica TCS; Leica Microsystems GmbH, Wetzlar, Germany). DiI-Ac-LDL and FITC-UEA-l dual-stained cells were identified as EPCs. Additionally, flow cytometric analysis (FCM) was performed as described previously (12) with the following FITC-conjugated antibodies: Rat anti-mouse stem cell antigen-1 (Sca-1; 1 μg/10^5 cellcatalog no. ab25031; Abcam, Cambridge, UK), rabbit anti-mouse vascular endothelial growth factor receptor 2 (VEGFR-2; 2 μg/10^5 cell catalog no. ab11939; Abcam) and the corresponding rat IgG2a and rabbit IgG isotype control antibodies (2 μg/10^5 cell catalog nos. ab18446 and ab171870, respectively; Abcam).

Infection of recombinant adenoviral vectors expressing Id1. Recombinant adenoviruses expressing mouse Id1 (Ad-Id1) and negative control adenoviruses (Ad-vector) were synthesized by Hanbio Technology (Shanghai) Co., Ltd. (Shanghai, China). Infection of adenoviruses was performed according to the manufacturer's instructions. Following seven days of culture, EPCs were seeded onto gelatin-coated 6-well plates at a density of $7x10^5$ cells/well and incubated at 37˚C for 24 h. Prior to infection, cells were starved of serum overnight by incubation in DMEM/F-12 medium supplemented with 0.5% FBS. The culture medium was subsequently replaced with medium containing adenoviruses (at a multiplicity of infection of 300) for 2 h, followed by fresh medium for an additional 72-h culture at 37˚C. Infected EPCs were then harvested for subsequent experiments.

Transfection of small interfering RNA (siRNA). The mRNA sequences of mouse Id1 (NM_010495.3) and mouse Wnt2 (NM_023653.5) were acquired from the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov/). siRNA of mouse Id1 (si-Id1), mouse Wnt2 (si-Wnt2) and a non-silencing control sequence (si-con) were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences are presented in Table I. For siRNA transfection, EPCs were prepared as per adenovirus infection. The siRNA transfections were performed with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following transfection with siRNA, EPCs were cultured at 37˚C for an additional 72 h prior to harvesting for subsequent experiments.

Cell cycle analysis. Cell cycle analyses were performed using FCM to measure DNA content. Briefly, EPCs were harvested by centrifugation at 472 x g for 5 min at room temperature and washed with phosphate-buffered saline (PBS). Cells were fixed with 70% ethanol overnight at 4˚C, washed with PBS and stained using a Cell Cycle Analysis kit (Beyotime Institute of Biotechnology, Shanghai, China) at 37˚C for 30 min in the dark. Analysis was performed on ~20,000 cells/sample using a MoFlo™ XDP flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and ModFit LT™ software version 3.2 (Verity Software House, Inc. Topsham, ME, USA).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from EPCs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed to cDNA using a Reverse Transcription kit (Takara Bio, Inc., Otsu, Japan). RT-PCR was performed using gene specific primers and 2xEs Taq MasterMix (CWBio, Beijing, China) according to the manufacturer's instructions. PCR cycling conditions were as follows: Pre-denaturation at 94˚C for 2 min, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 62˚C for 30 sec and extension at 72˚C for 30 sec, with a final incubation at 72˚C for 2 min. Bands were quantified with Quantity One® version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The gene specific primers are presented in Table II.

Western blot analysis. EPCs were lysed in protease inhibitor-containing radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). Cytoplasmic and nuclear proteins were extracted with a Nuclear and

Table I. siRNA sequences.

siRNA	Forward	Reverse
si-Id1	5'-CUUGGUCUGUCGGAGCAAATT-3'	5'-UUUGCUCCGACAGACCAAGTT-3'
si-Wnt2	5'-ACACCCAGAUGUGAUGCGUGCCATT-3'	5'-UGGCACGCAUCACAUCUGGGUGUTT-3'
si-con	5'-UUCUCCGAACGUGUCACGUTT-3'	5'-ACGUGACACGUUCGGAGAATT-3'

siRNA, small interfering RNA; Id1, inhibitor of DNA binding 1; Wnt2, wingless-type mouse mammary tumor virus integration site family member 2; con, control.

Table II. Reverse transcription-polymerase chain reaction primer sequences.

Gene	Forward	Reverse	Product size, bp
Id1	5'-CGAGGTGGTACTTGGTCTGTC-3'	5'-GGTCCCTGATGTAGTCGAT-3'	218
Cyclin D1	5'-TGACTGCCGAGAAGTTGTGC-3'	5'-CTCATCCGCCTCTGGCATT-3'	164
Wnt2	5'-GTGATGTGTGACAATGTGCCA-3'	5'-GTTGCAGTTCCAGCGATGC-3'	150
β-actin	5'-CACTGTGCCCATCTACGA-3'	5'-CAGGATTCCATACCCAAG-3'	477

Id1, inhibitor of DNA binding 1; Wnt2, wingless-type mouse mammary tumor virus integration site family member 2.

Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology), used according to the manufacturer's instructions. Protein samples (20 μg) were separated on 12% SDS-PAGE gels (concentration, 80 V for 30 min; separation, 120 V for 70 min) and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked within 5% non-fat milk for 2 h followed by incubation with primary antibodies overnight at 4˚C. The primary antibodies were as follows: Rabbit anti-mouse Id1 (1:1,000; catalog no. ab134163; Abcam), rabbit anti-mouse cyclin D1 (1:2,000; catalog no. ab134175; Abcam), rabbit anti-mouse Wnt2 (1:500; catalog no. ab27794; Abcam), rabbit anti-mouse β-catenin (1:1,000; catalog no. ab6302; Abcam), rat anti-mouse β-actin (1:1,000; catalog no. aa128; Beyotime Institute of Biotechnology) and rabbit anti-mouse histone (1:1,000; catalog no. ab1791; Abcam). Membranes were then incubated with the corresponding secondary antibody conjugated to horseradish peroxidase, goat anti-rabbit IgG (catalog no. sc-2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or goat anti-rat IgG (catalog no. sc-2006; Santa Cruz Biotechnology, Inc.), diluted 1:5,000 for 1 h at 37˚C. The chemiluminescent signal was developed with enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) and detected by ImageQuant™ LAS 4000 mini (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The signal was then quantified with ImageQuant TL version 7.0 (GE Healthcare Bio-Sciences).

Statistical analysis. All experiments were performed at least three times independently. Data are presented as the mean ± standard deviation. Statistical analysis was performed with with SPSS software version 19.0 (IBM SPSS, Armonk, NY, USA) using one-way analysis of variance followed by Tukey's multiple comparison test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of bone marrow-derived EPCs. Following 4-7 days of culture, the adherent cells became spindle-shaped and grew in small colonies or linearly, which were considered to be EPCs (Fig. 1A). Immunofluorescence was performed to confirm the identity of EPCs. As presented in Fig. 1B, 91.4±2.0% of adherent cells were double-stained with DiI-Ac-LDL and FITC-UEA-1. The double-stained cells were confirmed as EPCs. In addition, cells were characterized by FCM to detect the expression of Sca-1 (a mouse stem cell marker) and VEGFR-2 (an endothelial cell marker). The percentage of positive cells was 84.5 and 80.2%, respectively (Fig. 1C).

EPCs were infected with adenoviruses to overexpress exogenous Id1, or transfected with siRNA to knockdown endogenous Id1. The efficiency was detected by RT-PCR and western blot analysis (Fig. 1D and E). The expression level of Id1 in Ad-Id1 EPCs was upregulated ~3-fold compared with wild type EPCs ($P=0.001$; n=3); no difference was observed between Ad-vector and wild type EPCs ($P=0.924$; n=3). The expression level of Id1 in si-Id1 EPCs was downregulated ~70% compared with wild type EPCs ($P=0.039$; n=3); no significant difference was observed between si-con and wild type EPCs ($P=0.645$; n=3).

Effects of Id1 on cell cycle progression and cyclin D1 expression levels in EPCs. Cell cycle progression is closely associated with proliferation. To investigate whether Id1 is involved in cell cycle progression of EPCs, FCM was performed to analyze the EPC cell cycle. The percentage of EPCs in G_1 phase decreased from 74.04±2.56 to 59.12±2.87% following Ad-Id1 transfection ($P=0.001$; n=3), and the percentage in S/G_2M phases increased from 25.96±2.56 to 40.88±2.87% ($P=0.001$; n=3; Fig. 2A). The percentage of EPCs in G_1 phase increased >10% following

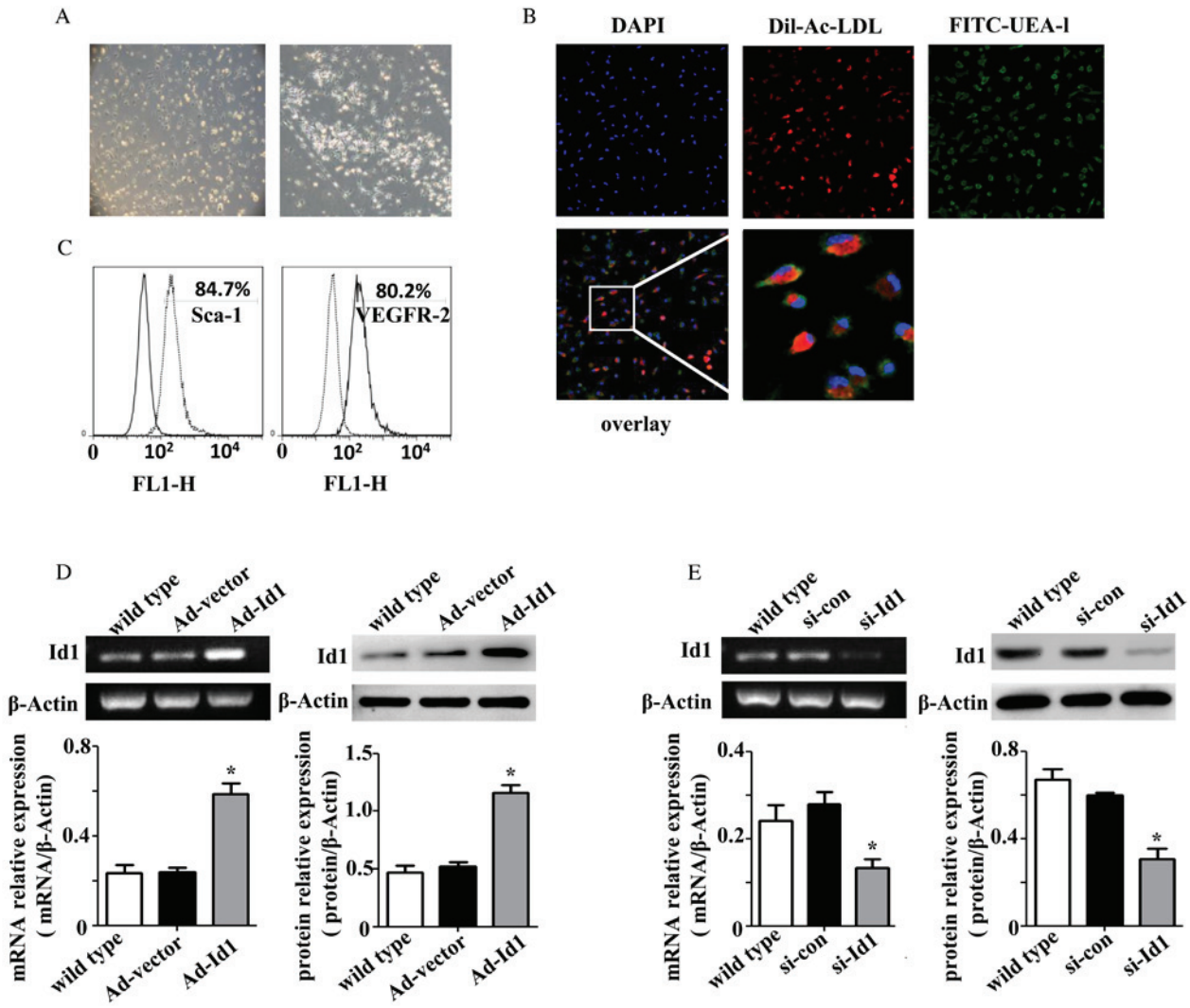

Figure 1. Characterization of bone marrow-derived EPCs. (A) Following 4-7 days culture, bone marrow-derived EPCs became spindle-shaped, elliptical or triangular, and grew in small colonies or linearly (magnification, x200). (B) EPC uptake of DiI-Ac-LDL (red) and binding of FITC-UEA-l (green) was determined by fluorescence microscopy (magnification, x200; magnification of the final image, x800). The majority of cells were double stained and therefore confirmed as EPCs. (C) Cells were incubated with fluorescent antibodies recognizing Sca-1 and VEGFR-2 (right peaks), or the corresponding negative controls (left peaks). The lines denote the positive gate and the numbers indicate the percentage of cells within this positive gate. The expression levels of Id1 mRNA and protein from EPCs treated with (D) Ad-Id1 or (E) si-Id1 were detected using reverse transcription-polymerase chain reaction and western blot analysis, respectively. Overexpression of Id1 using Ad-Id1 increased Id1 mRNA and protein expression levels, while knockdown of Id1 with si-Id1 decreased Id1 mRNA and protein expression levels. The expression level was analyzed relative to β-actin (n=3). *P<0.05 vs. wild type. EPCs, endothelial progenitor cellDiI-Ac-LDL, acetylated low density lipoprotein, labeled with 1,1'dioctadecyl-3,3,3', 3'-tetramethylindocarbocyanine perchloratFITC-UEA-1, fluorescein isothiocyanate-*Ulex europaeus* agglutinin-1; Sca-1, stem cell antigen-1; VEGFR-2, vascular endothelial growth factor receptor 2; Ad, adenoviruId1, inhibitor of DNA binding 1; si, small interfering; con, control.

transfection with si-Id1 (P<0.001; n=3) and the percentage in S/G_2M phases decreased significantly compared with wild type EPCs (P<0.001; n=3; Fig. 2B). These results demonstrate that Id1 may regulate cell cycle progression of EPCs.

To further investigate the role of Id1 on cell cycle regulation of EPCs, the expression level of cyclin D1 was detected by RT-PCR and western blot analysis. Id1 overexpression significantly increased cyclin D1 mRNA (P=0.007; n=3) and protein (P<0.001; n=3) expression levels compared with wild type EPCs (Fig. 2C). In addition, Id1 knockdown significantly decreased cyclin D1 mRNA (P=0.031; n=3) and protein (P<0.001; n=3) expression levels (Fig. 2D). These results suggest that Id1 may promote cell cycle progression from G_1 to S phase and regulate cyclin D1 expression levels in EPCs.

Id1 regulates Wnt2 expression levels and β-catenin nuclear translocation in EPCs. To investigate the underlying mechanism of cell cycle regulation by Id1, the activation of the Wnt signaling pathway, which is upstream of cyclin D1 (18), was examined. As Wnt2 is required for expanding the vascular progenitor population (21), and β-catenin is central to the Wnt canonical signaling pathway (25), the Wnt2 expression level and β-catenin nuclear translocation in EPCs were analyzed. The mRNA (P<0.001; n=3) and protein (P<0.001; n=3) expression levels of Wnt2 were significantly increased in Ad-Id1 EPCs compared with wild type EPCs (Fig. 3A and B). In addition, Id1 significantly increased the cytoplasmic (P=0.001; n=3) and nuclear β-catenin protein (P=0.001; n=3) expression levels (Fig. 3B and C). Id1 knockdown by si-Id1 led to a reduction in Wnt2 mRNA (P<0.001; n=3) and protein (P=0.001;

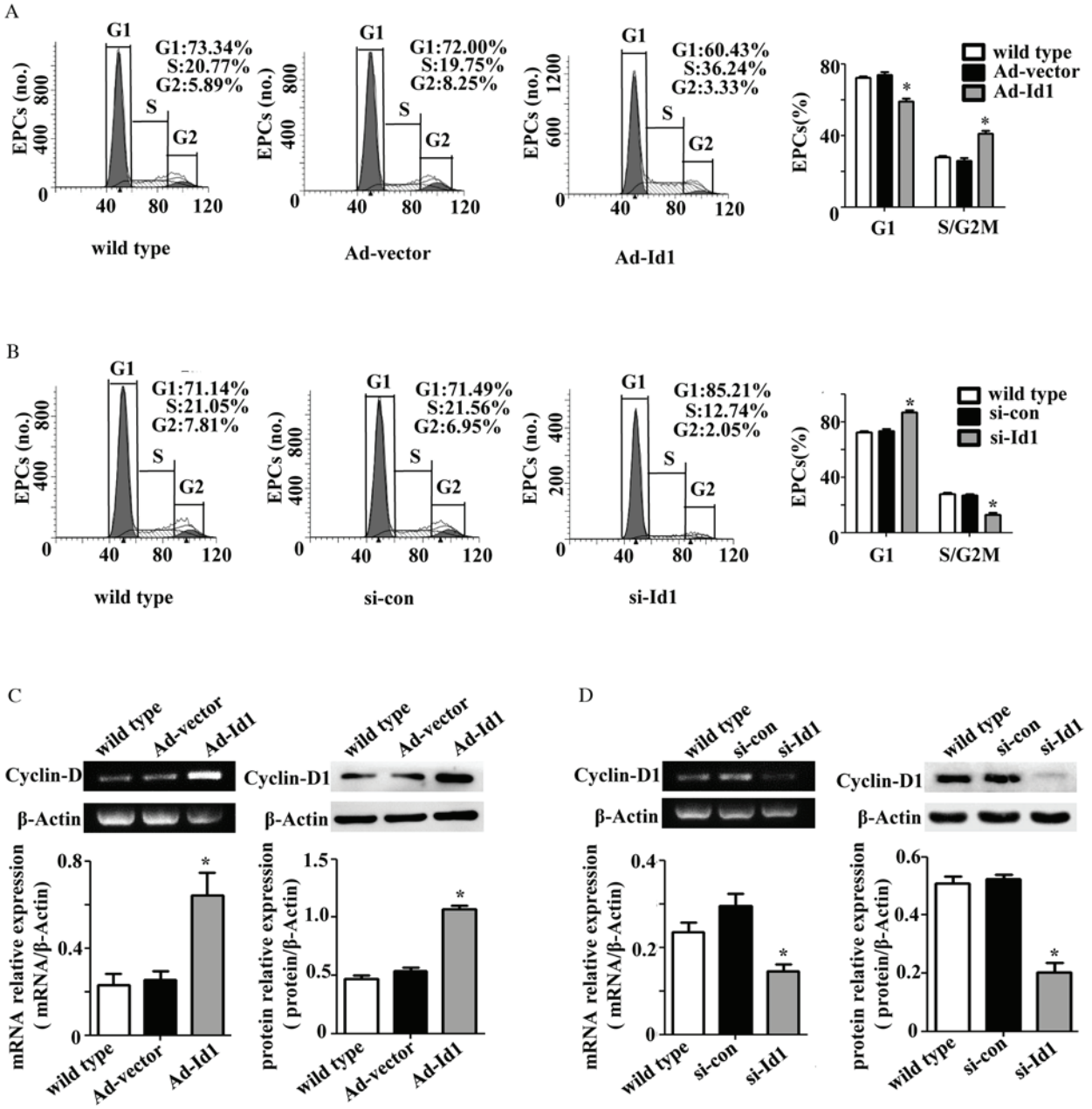

Figure 2. Effects of Id1 on cell cycle progression of EPCs. The cell cycle distribution of EPCs, transfected with (A) Ad-Id1 or (B) si-Id1, was analyzed by flow cytometry. Ad-Id1 transfection decreased the percentage of EPCs in G_1 phase and increased the percentage in S/G_2M phases, while si-Id1 transfection induced the opposite effect. Cyclin D1 mRNA and protein expression levels from EPCs treated with (C) Ad-Id1 or (D) si-Id1 were detected using reverse transcription-polymerase chain reaction and western blot analysis, respectively. Ad-Id1 transfection increased, and si-Id1 transfection decreased, cyclin D1 mRNA and protein expression levels. The expression level was analyzed relative to β-actin (n=3). *P<0.05 vs. wild type. EPCs, endothelial progenitor cellAd, adenoviruId1, inhibitor of DNA binding 1; si, small interfering; con, control.

n=3) expression levels compared with wild type EPCs (Fig. 3D and E). In addition, Id1 knockdown significantly decreased the β-catenin protein expression levels in the cytoplasm (P<0.001; n=3) and nucleus (P<0.001; n=3; Fig. 3E and F). These results demonstrate that Id1 may upregulate Wnt2 expression levels, and promote β-catenin accumulation in the cytoplasm and translocation to the nucleus of EPCs.

Wnt2 knockdown partially reverses the effects of Id1 on cell cycle regulation in EPCs. To further investigate the hypothesis that Id1 regulates the cell cycle progression of EPCs via regulation of Wnt2 expression, Ad-Id1 EPCs were transfected with Wnt2 siRNA (Ad-Id1/si-Wnt2). The G_1 phase population of the Ad-Id1/si-Wnt2 EPCs significantly increased (P=0.025; n=3) and the S/G_2M phase population significantly decreased (P=0.025; n=3) compared with Ad-Id1/si-con EPCs. However, the percentage of G_1 phase Ad-Id1/si-Wnt2 EPCs was reduced (P=0.038; n=3) and the percentage of the S/G_2M phase population was increased (P=0.038; n=3) compared with Ad-vector/si-con EPCs (Fig. 4A). These results were consistent with the change of cyclin D1 expression levels. The cyclin D1 mRNA (P=0.008; n=3; Fig. 4B) and protein (P=0.002; n=3; Fig. 4C) expression levels were significantly reduced in Ad-Id1/si-Wnt2 EPCs compared with those of the Ad-Id1/si-con

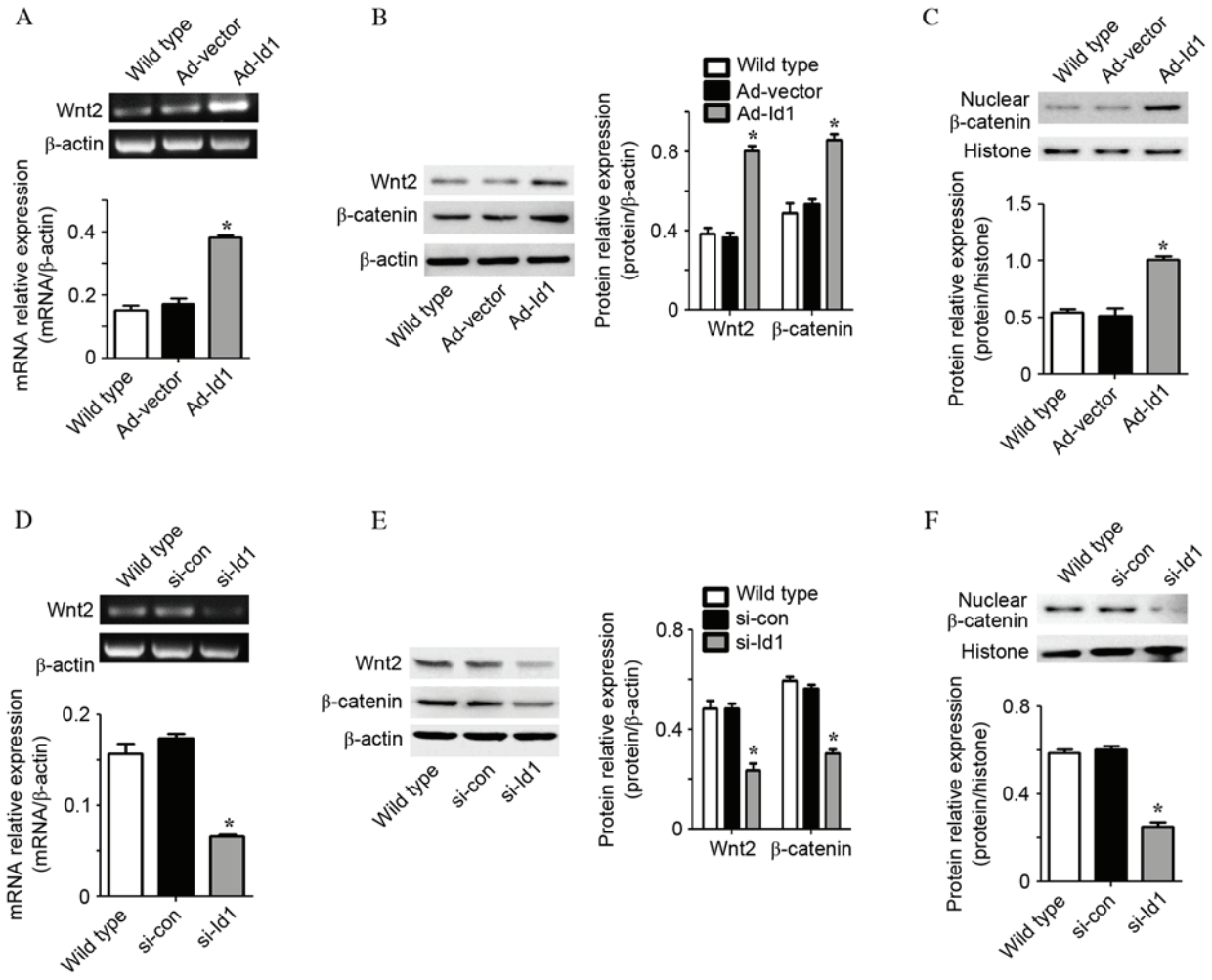

Figure 3. Id1 regulates Wnt2 expression levels and β-catenin nuclear translocation in EPCs. (A) Wnt2 mRNA expression levels following treatment of EPCs with Ad-Id1, as detected by RT-PCR. (B) The protein expression levels of Wnt2 and cytoplasmic β-catenin in EPCs treated with Ad-Id1 were detected by western blot analysis. The expression level was analyzed relative to β-actin. (C) Nuclear β-catenin protein expression levels in Ad-Id1 EPCs were detected by western blot analysis and the expression level was analyzed relative to histone. Ad-Id1 transfection increased Wnt2 mRNA and protein expression levels, and β-catenin protein expression levels. (D) Wnt2 mRNA expression levels following treatment of EPCs with si-Id1, as detected by RT-PCR. (E) The protein expression levels of Wnt2 and cytoplasmic β-catenin in EPCs treated with si-Id1 were detected by western blot analysis and analyzed relative to β-actin. (F) Nuclear β-catenin protein expression levels in si-Id1 EPCs were detected by western blot analysis and the expression level was analyzed relative to histone (n=3). si-Id1 transfection decreased Wnt2 mRNA and protein expression levels, and β-catenin protein expression levels. *P<0.05 vs. wild type. EPCs, endothelial progenitor cellWnt2, wingless-type mouse mammary tumor virus integration site family member 2; Ad, adenoviruId1, inhibitor of DNA binding 1; si, small interfering; con, control; RT-PCR, reverse transcription-polymerase chain reaction.

EPChowever, the cyclin D1 mRNA (P=0.016; n=3; Fig. 4B) and protein (P=0.003; n=3; Fig. 4C) expression levels were significantly increased compared with the Ad-vector/si-con EPCs group. Therefore, Wnt2 knockdown partially reversed cell cycle progression and the increase of cyclin D1 expression in Ad-Id1 EPCs. No significant difference was identified in Id1 mRNA (P=0.811; n=3) and protein (P=0.707; n=3) expression levels between Ad-Id1/si-Wnt2 EPCs and Ad-Id1/si-con EPCs (Fig. 4B and C). These results suggest that Id1 promoted cell cycle progression in EPCs via regulation of Wnt2 expression.

Discussion

EPC proliferation is regarded as one of the crucial underlying mechanisms of endothelial repair (9). Previous studies have demonstrated that silencing of Id1 in bone marrow results in a significant reduction in the EPC population in the peripheral blood of tumor-bearing mice (26-28). In addition, our previous study revealed that overexpression of Id1 markedly promoted EPC proliferation *in vitro* (12). However, the underlying mechanism responsible for Id1-induced EPC proliferation remains to be elucidated.

Previous studies have suggested that Id1 is essential for cell cycle regulation, which is closely associated with proliferation, in various cell lines (14-16). Thus, the present study investigated whether Id1 regulates proliferation of EPCs by promoting cell cycle progression. The results from the present *in vitro* studies demonstrate that exogenous Id1 promoted cell cycle progression of EPCs from G_1 to S phase and increased cyclin D1 expression levels. A decrease in endogenous Id1 expression in EPCs via siRNA prevented the cell cycle progression of G_1 phase EPCs and inhibited cyclin D1 expression.

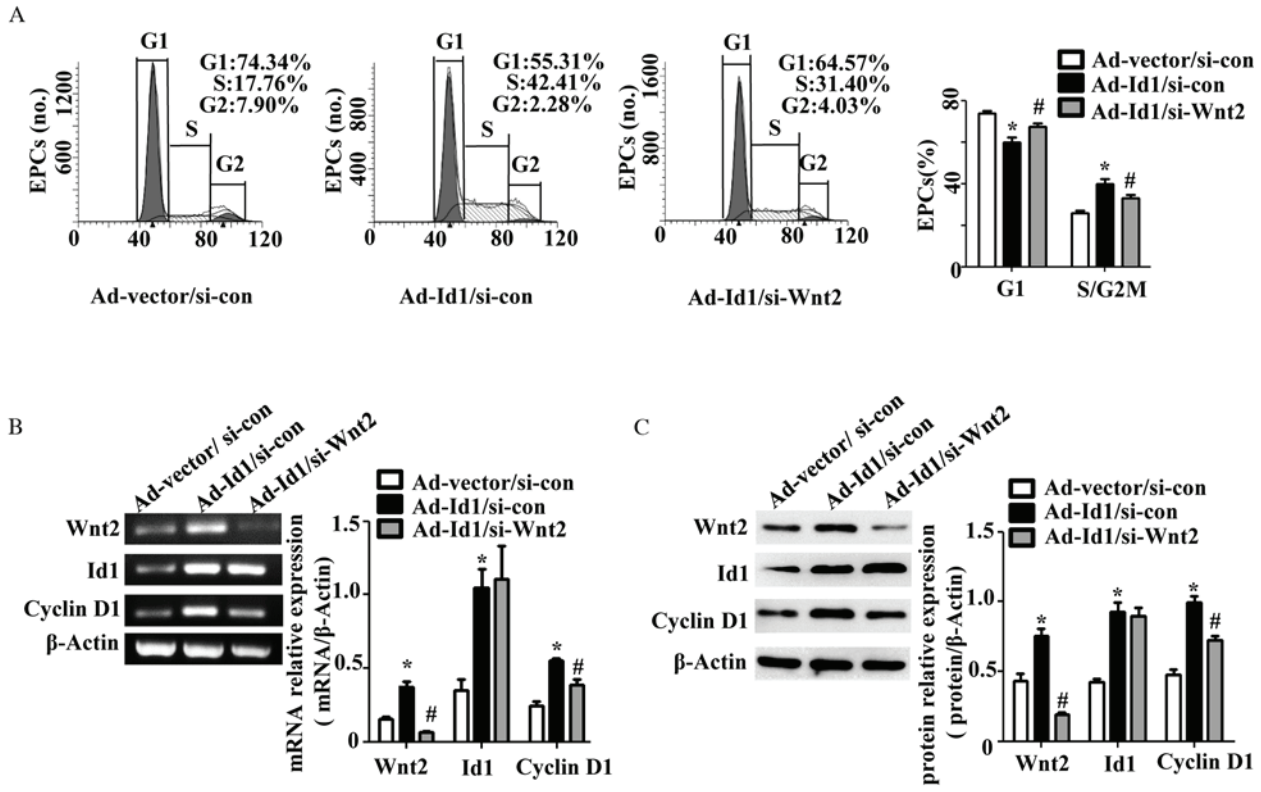

Figure 4. Wnt2 knockdown attenuates the effects of Id1 on cell cycle progression in EPCs. (A) The cell cycle distribution of Ad-Id1/si-Wnt2 EPCs was analyzed by flow cytometry. Transfection of Ad-Id1 and si-Wnt2 increased the percentage of EPCs in G_1 phase and decreased the percentage in S/G_2M phases, compared with Ad-Id1/si-con. (B) The mRNA expression levels of Wnt2, Id1 and cyclin D1 in EPCs were detected by reverse transcription-polymerase chain reaction. (C) The protein expression levels of Wnt2, Id1 and cyclin D1 in EPCs were detected by western blot analysis. The expression levels were analyzed relative to β-actin (n=3). Cyclin D1 mRNA and protein levels were decreased in Ad-Id1/si-Wnt2 EPCs compared with EPCs transfected with Ad-Id1/si-con. *P<0.05 vs. Ad-vector/si-con; #P<0.05 vs. Ad-Id1/si-con. Wnt2, wingless-type mouse mammary tumor virus integration site family member 2; EPCs, endothelial progenitor cellAd, adenoviruId1, inhibitor of DNA binding 1; si, small interfering; con, control.

Previous studies have reported that Wnt2-deficient mice exhibit vascular abnormalities (19,20) and that Wnt2 may expand the vascular progenitor population during embryonic differentiation (21). In addition, the Wnt signaling pathway may result in the accumulation and nuclear translocation of β-catenin, leading to the transcription of target genes, including cyclin D1; the absence of Wnts promotes the phosphorylation and, therefore, the degradation of β-catenin (29). The present study demonstrated that overexpression of Id1 increased Wnt2 expression levels, and enhanced β-catenin accumulation and nuclear translocation. Silencing of Id1 reduced Wnt2 expression levels, and decreased β-catenin expression levels in the cytoplasm and nucleus. Therefore, the underlying mechanism of Id1 enhancement of cell cycle progression in EPCs may involve regulation of Wnt2 expression. In addition, Id1 upregulated Wnt2 expression, leading to enhanced β-catenin stability and nuclear translocation. β-catenin accumulation in the nucleus may activate cyclin D1 expression to accelerate cell cycle progression of EPCs from G_1 to S phase. To support this hypothesis, Wnt2 expression was knocked down by siRNA in Ad-Id1 EPCs, abrogating the effects of Id1 on cell cycle regulation. The expression levels of cyclin D1 and the proportion of EPCs in G_2M/S phases were reduced. These findings suggested that Id1 induced the expression of Wnt2, leading to cell cycle progression of EPCs from G_1 to S phase. In addition, >50 target genes of the Wnt/β-catenin signaling pathway have been identified (20), of which certain genes are involved in the cell cycle of EPCs, including VEGF, connexin 43, c-Myc and cyclooxygenase-2 (30-33). This suggests that Id1 may additionally regulate these genes to affect cell cycle progression of EPCs via Wnt2 expression.

A previous study demonstrated that Id1 inhibited the expression of p21, a cell cycle associated protein, in EPCs (34). This may explain why si-Wnt2 did not fully reverse the effect of Id1 on cell cycle regulation of EPCs. In addition, Id1 has been reported to regulate certain other cell cycle-associated factors in numerous cell lines, including p16, p27 and fibroblast growth factor-2 (34,35). Therefore, additional mechanisms may underlie Id1 regulation of the cell cycle of EPCs.

It has been demonstrated by previous studies that EPCs have functions other than incorporation into injured vascular endothelium. Paracrine signals derived from EPCs promote the adhesion and proliferation of neighboring endothelial cells (36-38). Wnt2 is a secreted extracellular signaling molecule that binds to Frizzled receptors to transfer signals (20), and Wnt2 has been confirmed to promote proliferation of various mature differentiated endothelial cells (39). These findings suggest that Id1 regulates the cell cycle to promote EPC proliferation and induces the release of EPC-derived paracrine signals to enhance neighboring endothelial cell viability. These two mechanisms may underlie the repair of endothelial injury by Id1-overexpressed EPCs.

Although the results of the present study clearly demonstrate that Id1 regulated the expression of Wnt2 to promote cell cycle progression of EPCs *in vitro*, it remains to be demonstrated whether Id1 has the same function in EPCs *in vivo*. Further studies in animal models of vascular injury are therefore required.

In conclusion, the results of the present study demonstrate that Id1 acts as a positive regulator of Wnt2 to activate the expression of cyclin D1 and promote the cell cycle progression of EPCs. These results suggest that the Wnt2/cyclin D1 signaling pathway may be an important mechanism underlying Id1-induced EPC proliferation. The present study provides a novel strategy by which to investigate the proliferation of EPC and endothelial repair.

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