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Article

Effect of Nr1d1 on Serpina3 mediated cell senescence by nuclear receptor Nr4a3 in mouse heart-derived Sca-1^{+}CD31^{-} cells

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Abstract: Aim: Sca-1^{+}CD31^{-} cells were shown to be endothelial stem/progenitor cells, found in many mammalian tissues, including heart, were able to differentiate into cardiomyocytes in vitro and in vivo. Our previous work indicated that heart derived Sca-1^{+}CD31^{-} cells increased Nr1d1 mRNA level and decreased cells plasticity with aging. However, little is known about how NR1D1 affects Sca-1^{+}CD31^{-} cells plasticity. Methods: Lentiviral vector was used to stably overexpress Nr1d1 in young Sca-1^{+}CD31^{-} cells and to knockdown Nr1d1 in aged Sca-1^{+}CD31^{-} cells. Cell differentiation, proliferation, apoptosis, and cell cycle were evaluated. Results and Conclusions: The overexpression of Nr1d1 in young Sca-1^{+}CD31^{-} cells inhibited cell proliferation and promoted apoptosis. Knockdown of Nr1d1 in aged Sca-1^{+}CD31^{-} cells promoted cell proliferation and inhibited apoptosis. Using mouse cardiac myocytes cell line, confirmed the effect of Nr1d1 and indicated that Nr1d1 induce Serpina3 expression via Nr1d1 interaction with Nr4a3. Nr1d1 may therefore be identified as a potent anti-aging receptor and be a therapeutic target for aging relative diseases.

Keywords: Sca-1^{+}CD31^{-} cells; Nr1d1; cell senescence; Serpina3; Nr4a3;

1. Introduction

Nr1d1 (also known as Rev-erbα) encodes a nuclear heme receptor, belongs to the nuclear receptor superfamily of ligand responsive transcription factors[1], and is involved in circadian rhythm, metabolic, inflammatory, and cardiovascular processes[2,3]. Nr1d1 exerts its control via interaction with DNA targets as a potent repressor of transcription[4]. Many researchers have found a wide variety of genes implicated in key cellular processes. For example, Rev-erbα directly represses expression of the positive clock components Bmal1 and Clock[5,6]; represses Ucp1, ApoA1 and ApoCIII, which regulates body temperature cycles and cholesterol metabolism[4,7]; represses PARP1, which is responsible for DNA repair of ROS-induced DNA damage in cancer cells[8]. Overexpression of Rev-erbα in C2C12 was shown to increase mitochondrial content and activity, responds to changes in energy availability[9].

The Sca-1^{+}CD31^{-} cells were shown to be endothelial stem/progenitor cells, found in many mammalian tissues, including heart, were able to differentiate into cardiomyocytes in vitro[10-12] and in vivo[13,14]. Sca-1^{+}CD31^{-} cells age-related changes include decreased differentiation ability and proliferation capacity and increased apoptosis. We have previously shown that mRNA abundance of Nr1d1 was increased with age, particularly in aged heart derived Sca-1^{+}CD31^{-} cells[15]. The agonist of Nr1d1 is considered to

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be an effective anti-tumor and anti-inflammatory strategy[16,17]. However, high expression of Nr1d1 in the hearts of elderly mice suggests that Nr1d1 plays an important role in heart aging. Currently, little is known about how Nr1d1 contributes to heart derived Sca-1^{+}CD31^{-} cells plasticity during aging and the regulatory mechanisms of Nr1d1 are needed to reveal.

2. Results

2.1 Nr1d1 mRNA expression during aging

The analysis of two independent datasets from the GEO database (GSE43556 and GSE7196), we found that the Nr1d1 mRNA level with significant upregulation in aged mouse heart tissue compared to young tissue (P<0.001). In order to reveal the relevance of Nr1d1 with aging, we detected Nr1d1 expression in heart, liver, bown marrow, brain and adipose tissues with different age's mice by qRT-PCR. The results showed a significantly expression increased especially in aged heart tissue when compared to young tissue. Other tissue samples were not statistically significant across ages (Fig. 1A). We previously published the mRNA expression profiles of heart derived Sca-1^{+}CD31^{-} cells at 2 and 20 months, indicated that mRNA level of Nr1d1 with significant upregulation (7.96 fold) in aged heart derived Sca-1^{+}CD31^{-} cells[15]. Notably, Nr1d1 was expressed predominantly in the aged heart derived Sca-1^{+}CD31^{-} cells.

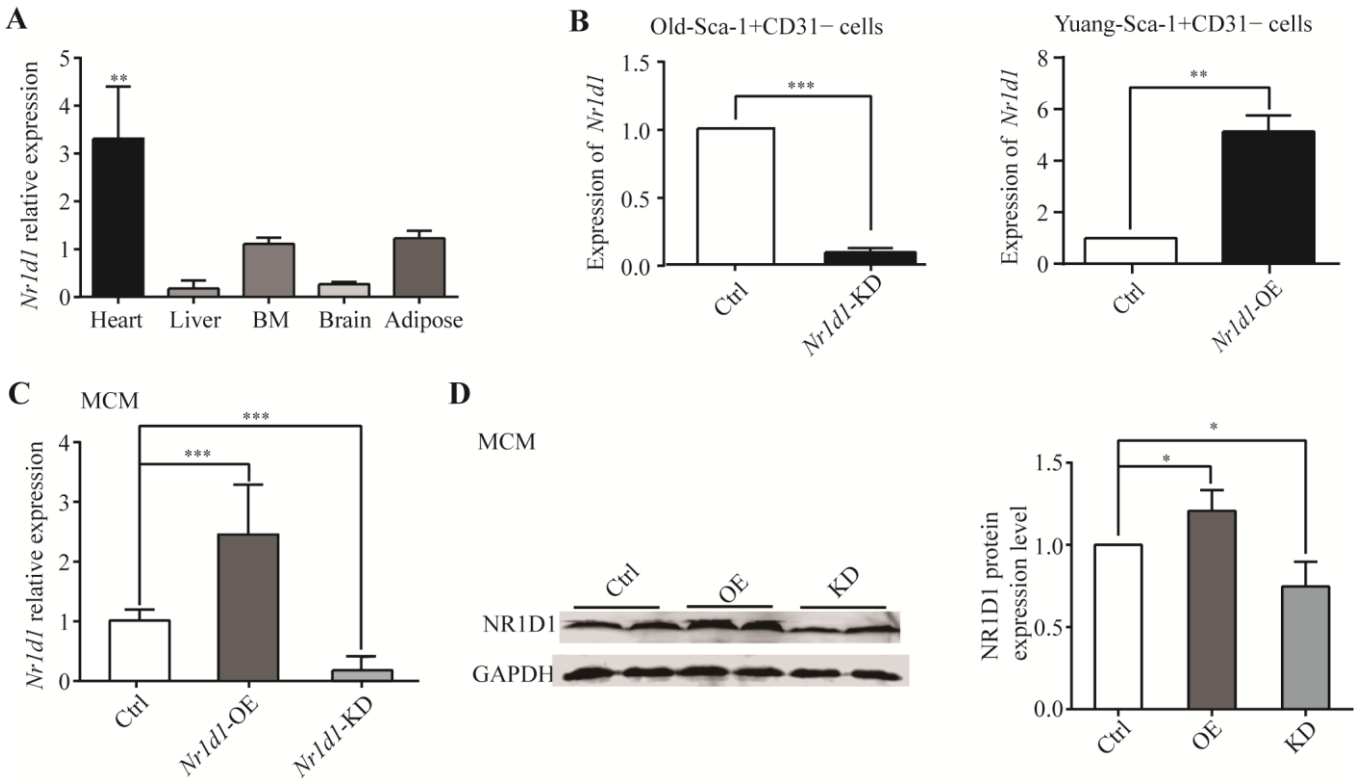

Figure 1. The expression of Nr1d1.(A): Nr1d1 mRNA expression signatures using real-time RT-PCR in aged organs. Each bar represents fold change of gene expression in aged vs. young mice(n=5). Expression levels were normalized by GAPDH and expression level in young mice was used as a calibrator to calculate fold change. (B): Nr1d1 mRNA expression for lentiviral transduction in Sca-1^{+}CD31^{-} cells by DD-PCR. Left lane represents old Sca-1^{+}CD31^{-} transduced with lentiviral particles of Nr1d1-shRNA(Nr1d1-knockdown), right lane represents young Sca-1^{+}CD31^{-} transduced with lentiviral particles of Nr1d1-cDNA(Nr1d1- overexpression), control was transduced with empty lentivector. (C): Nr1d1 mRNA expression for lentiviral transduction in MCM cells by real-time RT-PCR. (D): Immunoblotting of NR1D1 protein levels for lentiviral transduction in MCM cells. Overexpression represented MCM transducted with lentiviral particles of Nr1d1-cDNA, knockdown represented MCM transducted with lentiviral particles of Nr1d1-shRNA, the control was transducted with empty lentivector. Data were from three independent experiments and presented as mean ± SD. Statistical analysis was implemented by GraphPad Prism 5, *p < 0.05, **P < 0.01,and *** p < 0.001. Ctrl: control, KD: knockdown, OE: overexpression.

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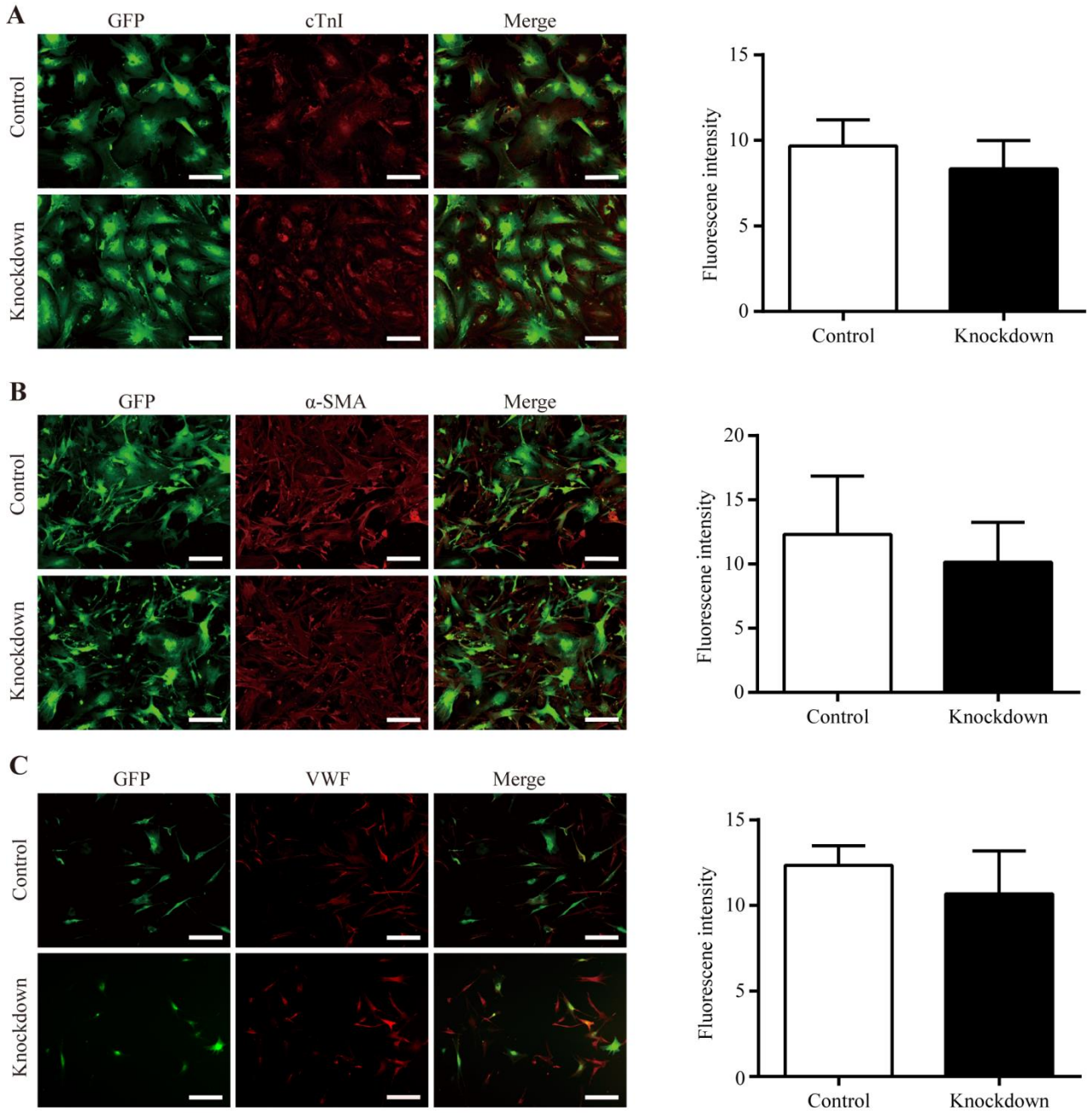

Figure 2. The effection of Nr1d1 knockdown for cells plasticity. (A-C) Old Sca-1⁺CD31⁻ cells differentiation after Nr1d1 knockdown compared to negative control group. The left panel shows representative immunofluorescence images, immunofluorescence staining (red) of cardiomyocyte-specific marker cTnI(A), smooth muscle marker α-SMA(B), and endothelial cell marker VWF(C). Sca-1⁺CD31⁻ cells were marked with GFP and were merged with DyLight® 550(red). Each experiment was repeated three times and each image represents a single result. Scale bars, 50 μm.. The bar graph on the right panel shows the grayscale values of DyLight® 550 within the GFP region using Imagej and Photoshop to determine

2.2 Nr1d1 was expressed predominantly in the aged heart derived Sca-1⁺CD31⁻ cells.

In order to assess the function of NR1D1, we used genetic targeting containing Nr1d1-shRNA lentiviral (knockdown) or Nr1d1-cDNA lentiviral(overexpression) vector, Nr1d1 expression was detected in heart derived Sca-1⁺CD31⁻ cells and MCM(Mouse Cardiac Myocytes) samples by qRT-PCR and western blotting. Nr1d1-shRNA lentiviral vector was used to knockdown Nr1d1 in O- Sca-1⁺CD31⁻ cells (old mouse heart derived Sca-1⁺CD31⁻ cells) and MCM cells, the data indicated a 90.9%(O- Sca-1⁺CD31⁻ cells) and 82.1% (MCM cells) reduction of Nr1d1 mRNA (Fig. 1B, C) and 35.0% reduction of NR1D1 protein when compared to control cells (Fig. 1D). Nr1d1-cDNA lentiviral vector was used to stably overexpress Nr1d1 in Y- Sca-1⁺CD31⁻ cells (Y mouse heart derived Sca-1⁺CD31⁻

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cells) and MCM cells, Nr1d1 mRNA increased 5.133-fold (P<0.001) in Y- Sca-1^{+}CD31^{-} cells and 2.45-fold in MCM cells as compared to negative control (Fig. 1B, C). And increased 1.25-fold increase of NR1D1 protein in MCM cells when compared to control cells (Fig. 1D). Sca-1^{+}CD31^{-} cells are too few to perform western blotting.

2.3 The effect of Nr1d1 knockdown for cells plasticity

In order to investigate the relevance of Nr1d1 with aged Sca-1^{+}CD31^{-} cells plasticity and senescence, the potential for differentiation into cardiomyocyte, smooth muscle, and endothelial lineages was then evaluated in lentivirus Nr1d1-shRNA-transduced O-Sca-1^{+}CD31^{-} cells. There were no significant differences in cTn(Fig. 2A)，α-SMA(Fig. 2B), and VWF(Fig. 2C) cell numbers between the control and Nr1d1-shRNA-transfected cells. In summary, Nr1d1 knockdown did not affect the differentiation potential of O-Sca-1^{+}CD31^{-} cells.

2.4 The effect of Nr1d1 knockdown for cells senescence

Besides Nr1d1 irrelevant role in the differentiation potential, to further assess whether Nr1d1 participates in proliferation potential of O- Sca-1^{+}CD31^{-} cells. The results indicate cell proliferation rate was 26.4±0.91% in Nr1d1 knockdown O- Sca-1^{+}CD31^{-} cells and 21.93±1.19% in control samples (P<0.01, Fig. 3A). Nr1d1 knockdown in MCM has affirmed the function Nr1d1 in Sca-1^{+}CD31^{-} cells with promoted cell proliferation (P<0.001, Fig. 3B). Meanwhile, the apoptosis rate was 25.27±1.08% in Nr1d1 knockdown O-Sca-1^{+}CD31^{-} cells, 31.38±0.32% in control cells (P<0.05, Fig. 3C). Nr1d1 knockdown in MCM has affirmed the apoptosis was inhibited too (P<0.01, Fig. 3D). These results suggested Nr1d1 knockdown in O- Sca-1^{+}CD31^{-} cells and MCM could promote cell proliferation and inhibit apoptosis, this may be related to cellular aging.

Next, we assessed the cell senescence for Nr1d1 knockdown. Senescence-associated beta-galactosidase (SA-b-gal) activity displayed significantly lower counts in Nr1d1 knockdown group (P<0.05, Fig.3E). Cell cycle inhibition is interfered with cellular senescence, the proportion of cells in G0/G1 phase was 42±1.16% in knockdown O-Sca-1^{+}CD31^{-} cells and 62.44±2.31% in control samples (P<0.001). S phase proportions were 47.1±3.07% and 32.61±1.4% (P<0.01) and G2/M phase proportions were 10.89±1.91% and 4.94±2.46%, respectively (P<0.05) (Fig. 3F). Nr1d1 knockdown in MCM has affirmed to promote more cells to escape from the G0/G1 phase (Fig. 3G). These data revealed that downregulation of Nr1d1 reverses the O-Sca-1^{+}CD31^{-} cells aging and promotes more cells to escape from the G0/G1 phase and consequently increased the sizes of the S- and G2/M-phase fractions.

SR8278 is an antagonist of Nr1d1 and inhibits Nr1d1 expression(Supplementary Fig.1A). MCM cells treatment with Nr1d1 antagonist SR8278 has the similar affections with promoted cell proliferation, inhibited apoptosis , promoted more cells to escape from the G0/G1 phase and reversed cells aging(Supplementary Fig.1).

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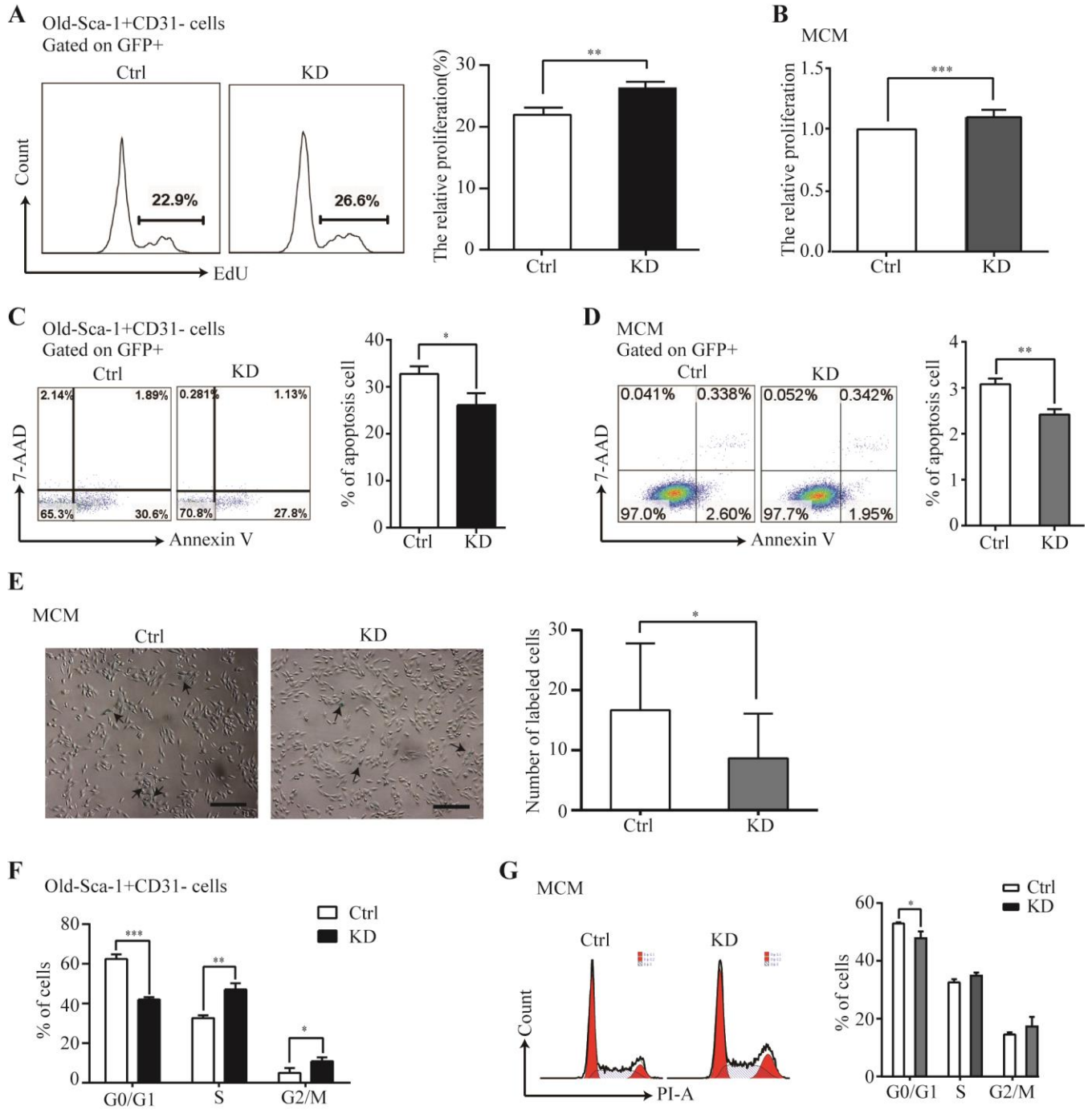

Figure 3. The effection of Nr1d1 knockdown for cells senescence.(A):The proliferation of Old Sca-1⁺CD31⁻ cells transfected with Nr1d1-shRNA lentivirus is indicated by the embedding of EdU. The flow cytometric analysis of representative Edu embedding is shown on the left, and the bar graph on the right is a statistical plot of EdU embedding. (B): The proliferation of MCM transducted with lentiviral Nr1d1-shRNA by MTT. (C, D):Apoptosis rate in Old Sca-1⁺CD31⁻ cells(C) and MCM(D) transducted with lentiviral Nr1d1-shRNA by FCM with annexin V-PE/7-AAD. On the left is a representative flow cytometric scatter plot, and on the right is a bar graph of apoptosis statistics. (E): Senescence-associated beta-galactosidase staining of MCM transducted with lentiviral Nr1d1-shRNA. Control was transduced with empty lentivector. The image on the left is a representative field of view, scale bars: 50 μm. The bar graph on the right shows the number of cells that were stained blue in each random field of view. Each experiment was repeated 3 times, and 10 random fields of view were counted each time. (F, G): Cell cycle analysis in Old Sca-1⁺CD31⁻ cells(F) and MCM(G) transducted with lentiviral Nr1d1-shRNA by FCM. Statistical analysis using GraphPad Prism 5. Data were collected from more than three independent experiments, and presented as mean ± SD.*P < 0.05, **P < 0.01, and ***P < 0.001 compared to control. Ctrl: control, KD: knockdown.

2.5 The effect of Nr1d1 overexpression for cells plasticity and senescence

The potential of Y- Sca-1⁺CD31⁻ cells to differentiate into cardiomyocyte, smooth muscle, and endothelial lineages was assessed upon transduction with Nr1d1-cDNA or

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control vector. There were no significant differences in cTn(Fig. 4A), α-SMA(Fig. 4B), and VWF (Fig. 4C) cell numbers between the control and Nr1d1-cDNA transfected cells.

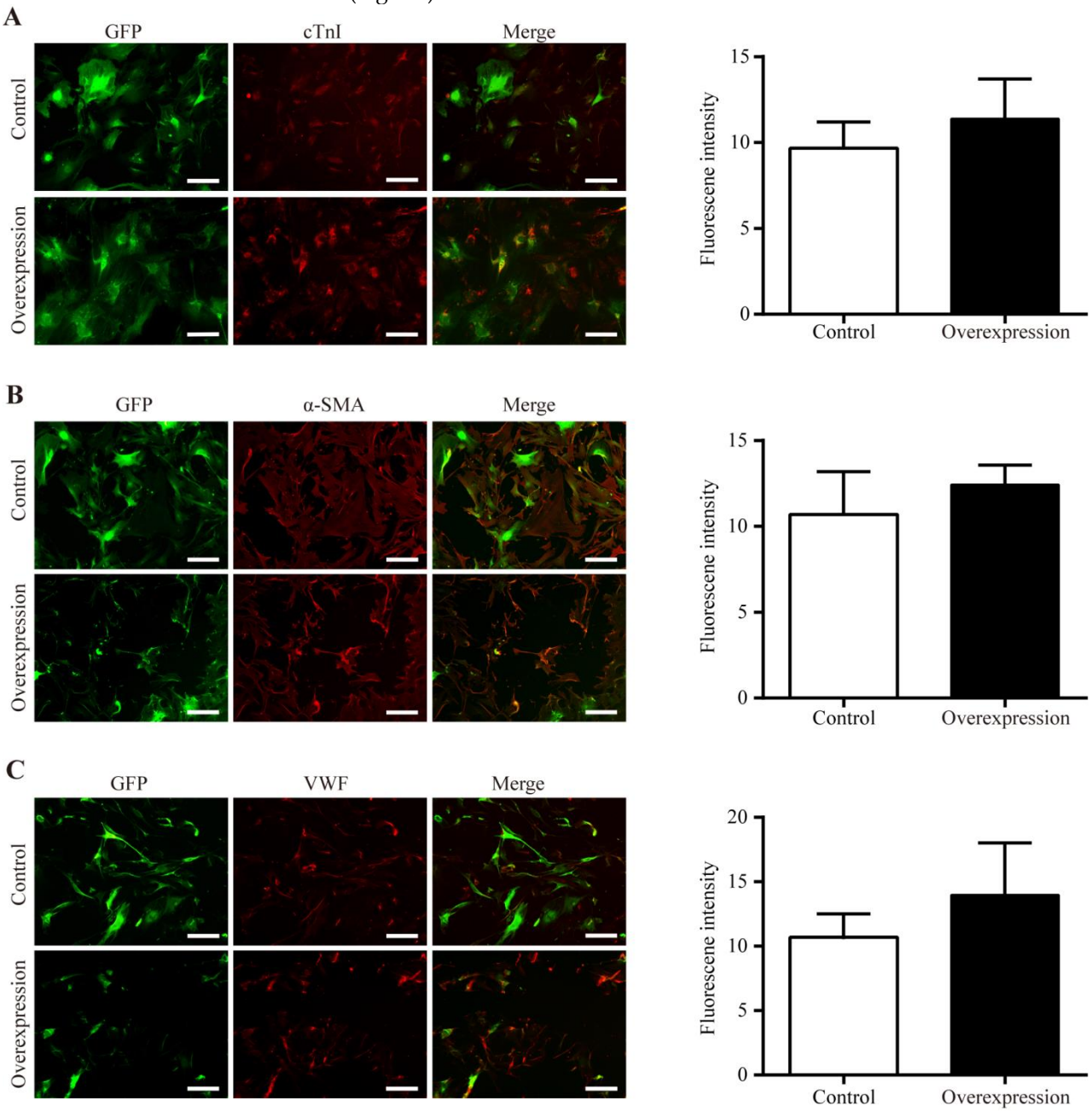

Figure 4. The effection of Nr1d1 overexpression for cells plasticity. (A-C)Young Sca-1^{+}CD31^{-} cells differentiation after Nr1d1 overexpression compared to negative control group. The left panel shows representative immunofluorescence images, immunofluorescence staining (red) of cardiomyocyte-specific marker cTnI(A), smooth muscle marker α-SMA(B), and endothelial cell marker VWF(C). Sca-1^{+}CD31^{-} cells were marked with GFP and were merged with DyLight® 550(red). Each experiment was repeated three times and each image represents a single result. Scale bars, 50 μm.. The bar graph on the right panel shows the grayscale values of DyLight® 550 within the GFP region using Imagej and Photoshop to determine。

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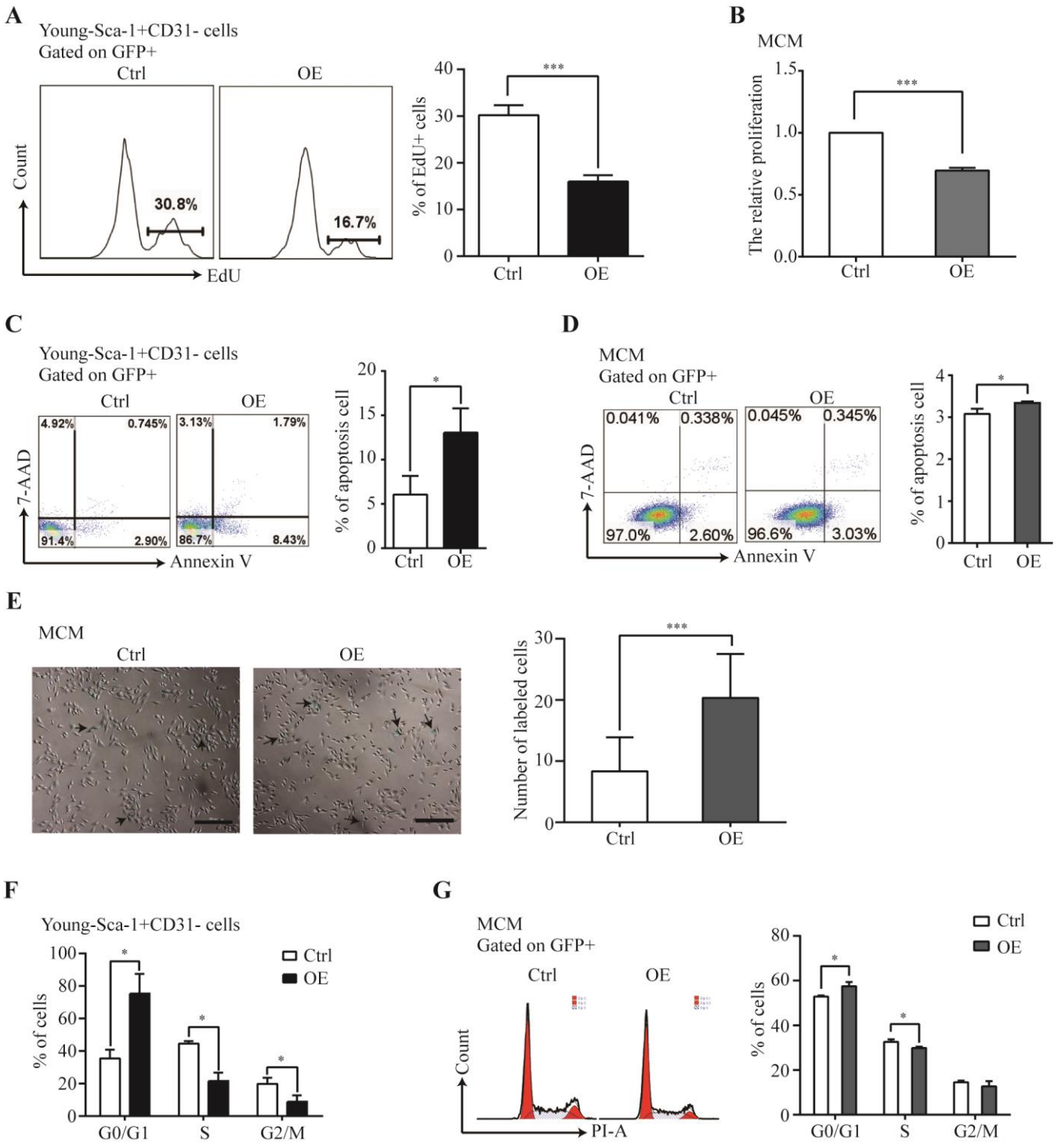

Figure 5. The effection of Nr1d1 overexpression for cells senescence.(A): The proliferation of Young Sca-1^{+}CD31^{-} cells transducted with Nr1d1-cDNA lentiviral is indicated by the embedding of EdU. The flow cytometric analysis of representative Edu embedding is shown on the left, and the bar graph on the right is a statistical plot of Edu embedding. (B): The proliferation of MCM transducted with lentiviral Nr1d1-cDNA by MTT. (C, D):Apoptosis rate in Young Sca-1^{+}CD31^{-} cells(C) and MCM(D) transducted with lentiviral Nr1d1-cDNA by FCM with annexin V-PE/7-AAD. On the left is a representative flow cytometric scatter plot, and on the right is a bar graph of apoptosis statistics. (E): Senescence-associated beta-galactosidase staining of MCM transducted with lentiviral Nr1d1-cDNA. Control was transduced with empty lentivector. The image on the left is a representative field of view, scale bars: 50 μm. The bar graph on the right shows the number of cells that were stained blue in each random field of view. Each experiment was repeated 3 times, and 10 random fields of view were counted each time. (F, G): Cell cycle analysis in Young Sca-1+CD31− cells(F) and MCM(G) transducted with lentiviral Nr1d1-cDNA by FCM. Statistical analysis using GraphPad Prism 5. Data were collected from more than three independent experiments, and presented as mean ± SD.*P < 0.05, **P < 0.01, and ***P < 0.001 compared to control. Ctrl: control, OE: overexpression.

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Similarly, we examined the effect of Nr1d1 overexpression on Y-Sca-1^{+}CD31^{-} and MCM senescence-related factors. Cell proliferation rates were 16.03±1.33% with Nr1d1 overexpression Y-Sca-1^{+}CD31^{-} cells and 30.2±2.16% in control cells (P<0.001, Fig. 5A). Upregulation of Nr1d1 in Y- Sca-1^{+}CD31^{-} cells inhibited proliferation relative to the control group. Nr1d1 overexpression in MCM has the similar affections with inhibited cell proliferation(P<0.001, Fig. 5B). Cell apoptosis was 13.17±2.73% in Nr1d1 overexpression Y-Sca-1^{+}CD31^{-} cells, and 6.07±2.01% in control cells (P<0.05, Fig. 5C). Nr1d1 overexpression in Senescence-associated beta-galactosidase (SA-b-gal) activity also displayed significantly higher counts in Nr1d1 overexpression. (P<0.001, Fig. 5E). The percentage of cells in the G0/G1 phase was 75.38±12.08% in overexpression Y-Sca-1^{+}CD31^{-} cells and 35.43±5.43% in control cells (P<0.05). S phase percentages were 21.51±5.17% and 44.57±1.58% (P<0.05) and G2/M phase percentages were 8.78±4.03% and 19.82±3.71% (P<0.05), respectively(Fig. 5F). These results indicated that Nr1d1 overexpression in Y-Sca-1^{+}CD31^{-} cells arrests cells in the G0/G1 phase, consequently inhibiting cells to the S and G2/M phases when compared with the control group. Cell cycle of Nr1d1 overexpression in MCM with similar arrested cells in the G0/G1 phase (Fig. 5G).

In addition, we also treated MEM cells with GSK4112 an agonist of NR1D1. It was found that the cell proliferation of MEM cells was inhibited. At the same time, the proportion of apoptotic cells increased, cell cycle entry was slowed and the proportion of senescent cells increased(Supplementary Fig.1).

Overall, the above results indicate that the higher expression of Nr1d1 mRNA in Sca-1^{+}CD31^{-} cells is closely related to aging and the decrease of cell potential.

2.6 The mechanism of Nr1d1

To investigate the mechanism of Nr1d1 is involved in Sca-1^{+}CD31^{-} cells plasticity, gene expression data were obtained using a microarray comparing Nr1d1-cDNA-infected and negative control Sca-1^{+}CD31^{-} cells. A total of 22 differentially expressed genes were identified, with a false discovery rate < 0.05 and fold change ≥ 2. Hierarchical clustering of differential genes was performed (Fig.6A), Nr1d1 expression level was similar between microarray and qPCR, with significant upregulation in Nr1d1-cDNA-infected Sca-1^{+}CD31^{-} cells. The interaction relationship among differentially expressed genes revealed that Nr1d1 directly interaction with Nr4a3 and indirectly regulates gene expression patterns in Sca-1^{+}CD31^{-} cells (Fig. 6B). The expression of differentially expressed genes after Nr1d1 knockdown and overexpression in Sca-1^{+}CD31^{-} and MCM cells were assessed by real-time PCR. It was found that the Nr1d1 knockdown in O-Sca-1^{+}CD31^{-} cells decreased the expression of Dpt (-1.926±0.21% P<0.001) and Serpina3 (-1.951±0.09% P<0.001), increased the expression of Nr4a3 (1.363±0.46% P<0.01), Il11 (1.219±0.009% P<0.001), and Polr2k (1.344±0.19% P<0.05), and did not affect the expression levels of other differentially expressed components (Fig. 6C). Overexpression of Nr1d1 in Y- Sca-1^{+}CD31^{-} cells increased the expression of dermatopontin (Dpt, 3.931±2.46% P<0.01) and Serpina3(1.897±0.77% P<0.01), decreased the expression of Nr4a3 (-3.028±1.57% P<0.01), Il11 (-5.856±1.32% P<0.01), and Polr2k (-2.173±0.27% P<0.01), and did not affect the expression levels of other differentially expressed components (Fig. 6D). The expression of genes related to Nr1d1 knockdown or overexpression in MCM cells was then examined. Among the genes we investigated, only Nr4a3 was 1.84±0.47 fold significantly up-regulated with Nr1d1 shRNA(P<0.01 Fig. 6E) and 52.15% down-regulated with Nr1d1 cDNA when compared to control(P<0.01 Fig. 6F); meanwhile, Serpina3 was 50.05% down-regulated in MCM cells transfected with Nr1d1 shRNA(P<0.05 Fig. 6E) and 1.94±0.56 fold up-regulated in MCM cells transfected with Nr1d1 cDNA when compared to control(P<0.05 Fig. 6F). Since the knockdown of Nr1d1 caused significant up-regulation of Nr4a3 mRNA. Meanwhile, we used the GCBI cloud platform to predict the molecular interaction proteins of NR1D1 and found that Nr4a3 was closely related to NR1D1(Fig. 6B). we further investigated whether Nr1d1 directly regulates the expression of Nr4a3. By performing luciferase reporter assay, we examined the binding of Nr1d1 to the promoter region of Nr4a3. MCM cells were transfected with Nr4a3 luciferase reporter plasmid for 24 hours, and incubated with or without the Nr1d1

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luciferase reporter plasmid, Nr4a3 promoter was enriched 2.24±0.46-fold (P<0.001) in the sample(Fig. 6G). On the other hand, expression of Serpina3 promoter was enriched by Nr4a3 promoter(P<0.001 Fig. 6H). Thus, we performed that Nr1d1 indeed binds to the promoter of Nr4a3 and represses the expression of Nr4a3, and then Nr4a3 binds to the promoter of Serpina3 and represses the expression of Serpina3.

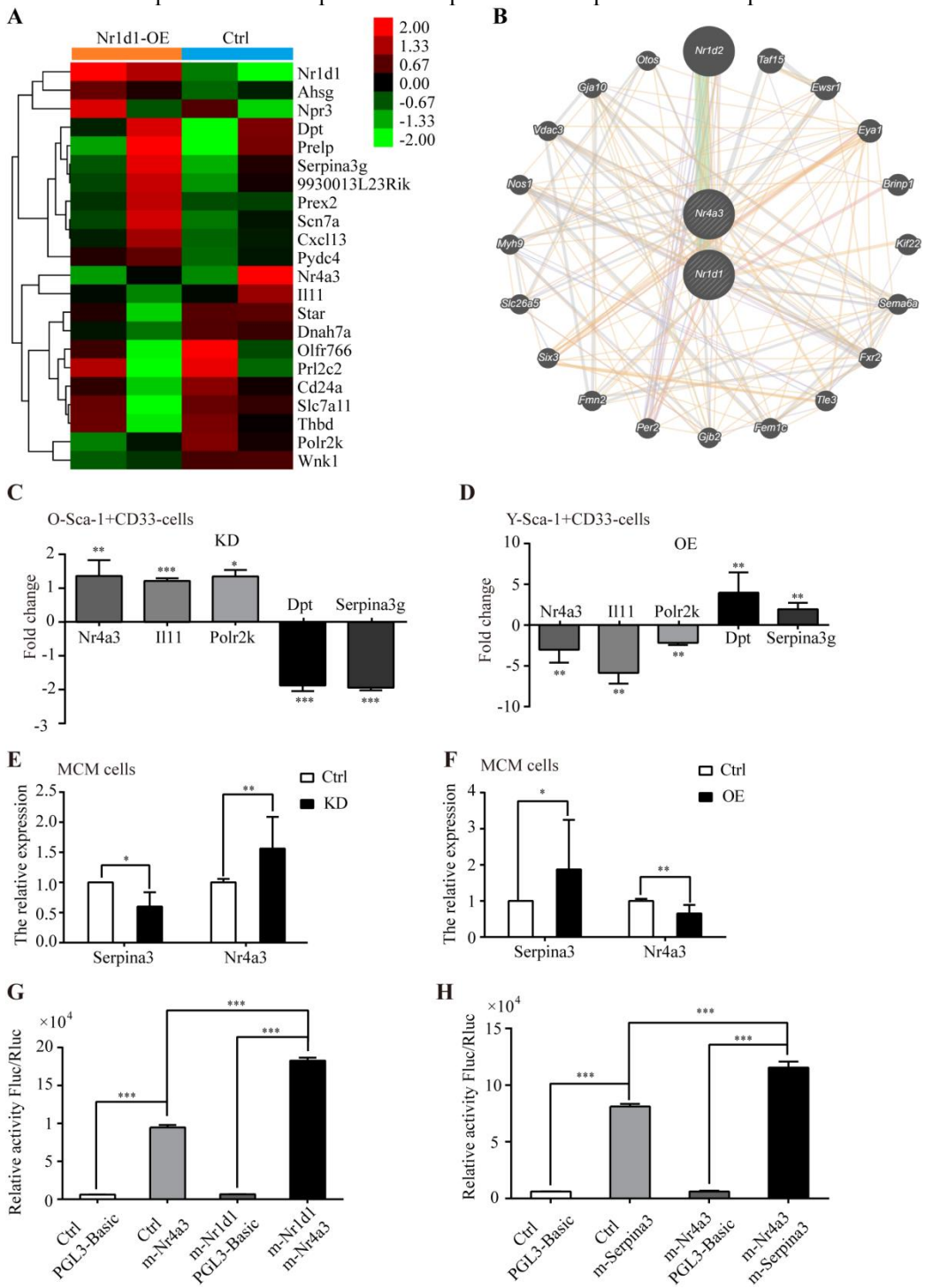

Figure 6: Differentially expressed genes and luciferase reporter assay. (A): The differentially expressed genes identified by microarray compared to Young Sca-1^{+}CD31^{-} cells with Nr1d1-cDNA-infected and negative control.The tree was based on log2 transformation of normalized probe signal intensity using hierarchical clustering. Red: upregulated gene expression; Green: downregulated gene expression. Every sample was tested twice. A total of 22 differentially expressed genes were identified via pairwise comparisons. (B): Interaction relation of differentially expressed genes by generadar (https: //www. gcbi.com. cn/gcanalyze/html/generadar/index). (C, D):Differentially expressed genes detected by real-time RT-PCR after Nr1d1 knockdown(C) and overexpression(D) in Sca-1^{+}CD31^{-} cells. (E, F):Differentially expressed genes detected by real-time RT-PCR after Nr1d1 knockdown(E) and overexpression(F) in MCM. Expression levels were normalized by GAPDH and expression levels in negative control were used as calibrator to calculate fold change. Calculated difference change based on a mean of three biological replicates. The ΔΔCT values

were subjected to unpaired Student's t-test implemented using Prism software. Bars above and below the x-axis show genes that are up- or downregulated, respectively. (G, H): Effect of Nr4a3 and Serpina3 on Nr1d1 transcription by Dual-Luciferase reporter system. MCM cells were transfected with Nr4a3 luciferase reporter plasmid for 24 hours, and incubated with or without the plasmid of Nr1d1 (G) or MCM cells were transfected with Serpina3 luciferase reporter plasmid for 24 hours, and incubated with or without the plasmid of Nr4a3(H). Cells were lysed with lysis buffer, and the lysates were subjected to luciferase activity assay. Data were collected from more than three independent experiments, and asterisks indicate samples with statistically significant values, *P < 0.05, **P < 0.01, ***P < 0.001. Ctrl: control, KD: knockdown, OE: overexpression.

3. Discussion

Sca-1$^+$CD31$^-$ cells age-related changes include decreased differentiation ability and proliferation capacity and increased apoptosis[15]. Elevated levels of Nr1d1 in Y- Sca-1$^+$CD31$^-$ cells did not affect the differentiation potential but triggered cell apoptosis, regulated cell cycle to arrest cells in the G0/G1 phase, and then inhibited proliferation. On the other hand, decreased levels of Nr1d1 in O- Sca-1$^+$CD31$^-$ cells inhibited cell apoptosis, regulated cell cycle stages to escape from the G0/G1 phase, and consequently increased the sizes of S- and G2/M-phases and then promoted proliferation. However, no effect on the differentiation potential was present. With advancing age, the fundamental properties of O- Sca-1$^+$CD31$^-$ cells were weaker than of Young[15]. Due to the different cellular microenvironments, the effects of Nr1d1 expression change are also different. Other studies have shown that Nr1d1 can promote BMSC(Bone marrow mesenchymal stem cell) aging with inhibited cell proliferation and osteogenesis[18]. Our results indicate that Nr1d1 promotes heart derived Sca-1$^+$CD31$^-$ aging by arresting cells in the G0/G1 phase, inhibits Sca-1$^+$CD31$^-$ cells from entering the cell cycle, and inhibits cell proliferation. At the same time Nr1d1 can increase cell apoptosis. However, it has no significant effect on the differentiation of aged Sca-1$^+$CD31$^-$ cells. The main effect of Nr1d1 may be related to the self-renewal ability of the heart.

The effect of Nr1d1 overexpression on Sca-1$^+$CD31$^-$ cells was analyzed using a microarray and compared Nr1d1-cDNA-infected and negative control. Samples were processed in parallel from dissection to array hybridization, making technical differences unlikely. A total of 22 differentially expressed genes were identified, where Nr1d1 expression level was significantly upregulated in Nr1d1-cDNA-infected Sca-1$^+$CD31$^-$ cells, with a top1 fold change. Nr1d1 exerts its control as a transcription factor via interaction with DNA targets[4]. Many identified genes have not been previously identified as Nr1d1-responsive. Nr1d1 plays a key role in circadian rhythm[2], however, the microarray data indicated that Nr1d1 did not regulate the circadian components in Sca-1$^+$CD31$^-$ cells. It may also indicate that there are many other unknown regulatory genes by Nr1d1 and Nr1d1-targeted regulatory genes in different tissues or cells are specific. Among the genes we investigated, only Nr4a3 and Serpina3 were reported directly or indirectly related to Nr1d1.

Serpina3 is a member of a serine proteinase inhibitor gene family, is highly expressed in hematopoietic stem cells (HSCs)[19], and has been shown to be involved in anti-adipogenesis[20] and anti-inflammation and associated with a number of human diseases[21]. Nr4a3 (also known as Nor1) belongs to the Nr4a subfamily of nuclear receptors and is a constitutively active transcription factor regulating cellular proliferation, differentiation, inflammation, and apoptosis[22,23]. Nor1 overexpression limited the VSMC apoptosis[24] and promoted cell proliferation with cyclin D1 and D2 as NOR1 target genes[23,25]. Nor1 deletion in HSCs accelerated the atherosclerosis formation[22]. A recent study confirmed that the downstream apoptosis trigger was independent of p53 by REV-ERB agonists[16]. The Nr1d1 overexpression in Young Sca-1$^+$CD31$^-$ cells had the characteristics of aged cells characteristic. Expression analysis indicated that Nr1d1 increased the gene expression of Serpina3 and decreased Nr4a3. Other research identified Serpina3 as an ApoA4-regulated gene transcriptionally via nuclear receptors Nr4a3 and Nr1d1 in hepatocytes[21]. The increased expression of Serpina3 and decreased expression of Nr4a3 may interact with Nr1d1 and participate in apoptosis promotion and cell proliferation inhibition in aged Sca-1$^+$CD31$^-$ cells.

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On the other hand, Serpina3 and Nr4a3 may interact with Nr1d1 and participate in apoptosis promotion and cell proliferation inhibition in aged cells. Our observations indicate that Nr1d1 can directly induce and silence gene expression in different cells in a gene-specific manner. Additional studies on the mechanisms of Nr1d1 effects on Sca-1^{+}CD31^{-} cells aging are needed. Although some studies suggested that Rev-erb agonists are specifically lethal to cancer cells and have no effect on the viability of normal cells or tissues[16], Rev-erb agonist SR9009 treatment inhibited post-myocardial infarction mortality and improved cardiac function by modulating inflammation and remodeling processes[26]. However, our data showed that increased Nr1d1 expression in heart Sca-1^{+}CD31^{-} cells may contribute to reduced cells repair capacity. The intrinsic determinants of cellular senescence with higher Nr1d1 expression promote cell death and impair cell growth, compromising myocyte loss increases and cardiac function with age. These results strongly indicated that the effect on the heart must be taken into account when the pharmacological modulation of circadian machinery is used as a cancer treatment.

4. Materials and Methods

4.1 Experimental animals and cell culture

Mice were purchased from SLAC Ltd(SCXK(Xiang)2011-0003). Animals were maintained in the Guangxi Normal University Laboratory Animal Center and handled in accordance with the institution's guidelines. All experimental protocols were approved by the Guangxi Animal Management Committee of Guangxi S&T Department (Approval Number: 20190313-003). FACS-sorted Lin^{-}CD45^{-}Sca-1^{+}CD31^{-} were used for experiments. Young(Y, 2months old) and old (O, 22months old) mice Sca-1^{+}CD31^{-} cells respectively were prepared and cultured as previously described[11,15].

The Muse Crdiac Mocytes (MCM) cell line was purchased from BeNa Culture Collection was cultured in DMEM containing 10% FBS, 100 μg/ml streptomycin and 100U/ml penicillin at 37°C under 5% CO2. The MCM cells were passaged after 3days. To study the effects of Nr1d1 agonists, MCM cells were incubated in DMEM without FBS for 16 h to deplete intracellular heme concentration and then switched to DMEM supplemented with either DMSO or Nr1d1 agonist GSK4112(10μM) or antagonist SR8278 (10μM) for 6 h.

4.2 Nr1d1lentivirus vectors constructs and stable transfection

For Nr1d1 overexpression, full-length murine Nr1d1 cDNAs were obtained by PCR using the primers 5'-tatcgaattc (EcoRI) ATGACGACCCTGGACTCCA-3' (forward) and 5'-GGATCCGCGG CCGCTTCTAGAtatcggatcc(BamHI)-3'(reverse). The amplified Nr1d1 cDNAs fragments were digested at EcoRI/BamHI and then cloned into CMV-MCS-IRES-EGFP lentiviral cDNA vector (Hanbio, Shanghai, China).

For Nr1d1 knockdown, oligonucleotides (GCAAGGCAACACCAAGAA TGT) targeting Nr1d1 were used to clone short hairpin (sh)RNA into the hU6-MCS-PGK-EGFP lentiviral RNAi vector (Hanbio). The Recombinant overexpression and knockdown lentiviral vectors were produced by co-transfection of 293T cells with the pSPAX2, pMD2G, and pHBLV plasmids using LipoFiter reagent (Hanbio). Lentivirus-containing supernatant was collected 48 h after transfection and passed through a 0.45-μm cellulose acetate filter (Millipore, Billerica, MA, USA). Recombinant lentiviruses were concentrated by ultracentrifugation for 2 h at 72,000 × g.

MCM cells were transfected with the lentiviral Nr1d1cDNA vector, lentiviral Nr1d1 RNAi vector, or empty vector encoding EGFP, respectively, using polybrene (5 μg/ml, Hanbio). Selection was initiated in medium containing 2μg/ml puromycin (Invitrogen).After selection, a stable Nr1d1 transfectant overexpression, knockdown and the control cell line were established.

4.3 Cell differentiation and immunofluorescence analysis

Starting 2 days after the viral infection, lentivirus-infected and negative control Sca-1^{+}CD31^{-} cells were cultured in cardiomyocyte, smooth muscle cell, and endothelial

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differentiation induction medium for 14 days, then examined for the expression of cardiac lineage-specific markers by immunocytochemistry, the experiments detail as previously described[11,15].

4.4 Analysis of cell cycle, proliferation, and apoptosis

Y-/O-Sca-1^{+}CD31^{-} cells were transduced with Nr1d1-shRNA, Nr1d1 cDNA, and negative control lentiviral particles. After 48 h, cell apoptosis was detected by flow cytometry using an Annexin V-phycoerythrin (PE) Apoptosis Detection kit (Invitrogen, Carlsbad, CA, USA). The percentage of apoptotic cells was defined as the sum of annexin V-PE single-positive and annexin V-PE/7-aminoactinomycin D (7-AAD) double-positive cells. The 5-ethynyl-2'-deoxyuridine (EdU) assay was performed to assess cell proliferation 48h after lentiviral infection by incubating the cells in a 10 μmol/L EdU solution (RiboBio, Guangzhou, China) for 2 h followed by flow cytometry analysis. Cell cycle analysis was performed using the 7-AAD Flow Cytometry Assay kit (Ebioscience, San Diego, CA, USA) according to the manufacturer's instructions. These methods have been described in detail in previous work[11,15].Cell apoptosis and cell cycle are performed on MEM cells as in Sca-1^{+}CD31^{-} cells. Proliferation of MEM cells was detected by MTT assay.

4.4 Microarray gene expression data

The Affymetrix Mouse Genome 2.0 Microarray (Santa Clara, CA, USA) was utilized to analyze Gene expression of samples by high-throughput technologies. The experiments detail and data access were described in previously published[27,28]. Corresponding CEL files are publicly available on the Gene Expression Omnibus database (accession numbers GSE43556, GSM1024592-94, and GSE7196).

4.5 Quantitative real-time RT-PCR and Droplet digital PCR

Total RNA and cDNA were prepared using a kit (Promega) according to manufacturer's instructions. The details of real-time RT-PCR and Droplet digital PCR(ddPCR)were described previously[29].The primers were listed in supplementary table s1.

4.6 Luciferase assay

The promoter sequence (2000 bp) upstream of the transcriptional start site of mouse Nr4a3 and Serpina3 was cloned into luciferase reporter plasmid and verified with direct sequence, respectively as m-Nr4a3-pro+pRL-TK and m-Serpina3-pro+pRL-TK.The MCM cells (2×105cells/well in 24-well plates) were transiently transfected with pGL3 or Nr1d1 plasmids(m-Nr1d1) and pRL-TK plasmid (Promega) using lipofectamine 3000. Cells were lysed 24h after transfection and assayed for firefly and Renilla luciferase activity using the Dual-Luciferase reporter system (Promega). The data are expressed as the ratios of firefly to Renilla activity.

4.7 Statistical analysis

Data analysis was performed using GraphPad Prism 6 software (GraphPad software, CA, USA). Unpaired t-test was used for two-group comparisons. Two-sided p-values were calculated and $P<0.05$ was considered statistically significant.

5. Conclusions

This study clarified the role and molecular mechanism of Nr1d1 in cardiac senes-cence. High expression of Nr1D1 in cardiac-derived Sca-1+CD31+ cells and MEM causes cell cycle arrest in the G0/G1 phase, inhibits proliferation, promotes apoptosis and se-nescence, and thus promotes cardiac senescence. Knockdown of Nr1D1 expression in Sca-1+CD31+ cells and MEM promoted cell proliferation and reduced cellular senescence. Nr1d1 induce Serpina3 expression via Nr1d1 interaction with Nr4a3 promoting cell senescence. The results will establish the functional relationship between cardiac senescence and Nr1d1 abnormal expression, reveal the molecular pathway of Nr1d1 regulat-

ing cardiac senescence and its functional degeneration, and provide new targets and strategies for the diagnosis and treatment of age-related heart diseases.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Effect of inhibition of Nr1d1 expression on cellular senescence; Figure S2: Effect of promoting Nr1d1 expression on cellular senescence; Table S1: Sequences of primers.

Author Contributions: S.P. analyzed data and directed the conduct of experiments; Q. Wa. performed all experiments except cell differentiation; Q.L.performed cell differentiation; H.Z. reviewed and interpreted data; Z.Z.designed the study, reviewed and interpreted data; Q.Wu. designed the study, interpreted data, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All experiments were performed in accordance with the Guide for the Animal Care and Use Committee of Guangxi Normal University. The ethical review acceptance number is 20190313-003(Mar. 13, 2019).

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