E2A ablation enhances proportion of nodal-like cardiomyocytes in cardiac-specific differentiation of human embryonic stem cells

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ABSTRACT

Background: Human sinoatrial cardiomyocytes are essential building blocks for cell therapies of conduction system disorders. However, current differentiation protocols for deriving nodal cardiomyocytes from human pluripotent stem cells (hPSCs) are very inefficient.

Methods: By employing the hPSCs to cardiomyocyte (CM) in vitro differentiation system and generating E2A-knockout hESCs using CRISPR/Cas9 gene editing technology, we analyze the functions of E2A in CM differentiation.

Findings: We found that knockout of the transcription factor E2A substantially increased the proportion of nodal-like cells in hESC-derived CMs. The E2A ablated CMs displayed smaller cell size, increased beating rates, weaker contractile force, and other functional characteristics similar to sinoatrial node (SAN) cells. Transcriptomic analyses indicated that ion channel-encoding genes were up-regulated in E2A ablated CMs. E2A directly bounded to the promoters of genes key to SAN development via conserved E-box motif, and promoted their expression. Unexpected enhanced activity of NOTCH pathway after E2A ablation could also facilitate to induct ventricle workingtype CMs reprogramming into SAN-like cells.

Interpretation: Our study revealed a new role for E2A during directed cardiac differentiation of hESCs and may provide new clues for enhancing induction efficiency of SAN-like cardiomyocytes from hPSCs in the future.

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1. Introduction

With prolongation of human lifespans, the overall morbidity and mortality caused by cardiac conduction system (CCS) diseases and sinoatrial node (SAN) malfunction are increasing [1]. Biological pacemaker has been proved to be promising for the treatment of CCS diseases [2,3]. However, human pacemaker cells, for the sake of future clinical application, are very difficult to obtain. Although it is now possible to derive SAN-like cardiomyocytes (CMs) from human
Evidence before this study
As the classic bHLH protein, the role of E2A has been well demonstrated in lymphopoiesis. And a growing body of studies indicated E2A involves in regulating neural specification and differentiation in both embryonic mice and pluripotent cells. However, far less is known about its function in heart development. Cardiomyocytes differentiated from human pluripotent stem cells (hPSCs) provide a promising approach to reveal the regulatory mechanism in cardiac development. It is necessary to better understand the impacts of E2A in cardiac-specific differentiation of hPSCs.

Added value of this study
We found ablation of E2A expression markedly increased the ratio of nodal-like cardiomyocytes from original ~5% to as high as 40% in our defined 2D cardiac-specific differentiation system of hESCs. E2A could bound to the promoter region of TBX5, SHOX2, and TBX3, and regulated expression of these genes key to SAN node development. Furthermore, the activity of NOTCH signaling pathway was also affected by E2A during hESC cardiac differentiation. NOTCH inhibitor partially blocked the increasing nodal-like phenotypes of E2A deficient CMs.

Implications of all the available evidence
Our data revealed a new role of the transcription factor E2A in hESC cardiac differentiation and provided new insights into promoting induction efficiency of SAN-like CMs from cardiac-specific differentiation of hPSCs.

2. Methods
2.1. hESCs culture and differentiation
Human embryonic stem cells (hESCs) were maintained on culture plates coated with Matrigel (Corning, 356231) using mTeSR-1 medium (Stemcell Technologies, #85852), and passaged with Accutase (GIBCO, A1115001) at the ratio of 1:4 to 1:5 when confluence reached ~90%. Cardiomyocyte differentiation of hESCs were induced by chronologically treating with WNT agonist CHIR-99021 (Selleck, S2924) and antagonists IWR-1 (Sigma, I0161). In brief, RPMI 1640 (Corning)/B-27 minus insulin (Life Technologies) containing 12μM CHIR-99021 and IWR-1 were used to activate WNT signaling on Day 0 and 1. Medium was changed with RPMI/B-27 minus insulin on Day 2. Cells were treated with fresh medium containing 5μM IWR-1 for another 48h. From Day 5 onward, cells were cultured with CDM3 (RPMI1640; BSA; AscorbicAcid) medium, and changed every other day. Beating CMs appeared on day 7~8 after differentiation. To further explore the possibility of E2A on regulating nodal-like cells differentiation via Notch signaling, the NOTCH inhibitor RO4929097 (Selleck, S1575) was applied to the CDM3 culture medium at a final concentration of 30μM from Day 9 to 15 of differentiation. CMs of D30 after cardiomyocyte differentiation were utilized for downstream tests in this study.

2.2. Generation of E2A knockout hESC lines
To generate E2A null hESCs lines, we utilized CRISPR/CAS9 genome editing system. sgRNAs were designed targeting on exon 3 of the human E2A gene (http://crispr.mit.edu/) and cloned into the D10A-mutant nickase version of Cas9 (pX462 from Addgene), px462-sgRNAa and px462-sgRNAb were transfected into hESCs by electroporation (Neon Transfection System, ThermoFisher Scientific), E2A-null clones were picked and screened by Western blot and further confirmed by genomic sequencing after selected with puromycin for one week.

2.3. Quantitative Real-time PCR
Total RNA was extracted using Trizol reagent (Invitrogen, 15596018) according to the manufacturer's instructions. 1μg RNA was reverse-transcribed into cDNA by using ReverTra Ace qPCR RT Kit (TOYOBO). Real-time PCR was done using the Hieff qPCR SYBR Green Master Mix (YEASEN, 11202ES08) based on the manufacturers' protocol on a Light-Cycler 96 System (Roche). Gapdh were used as an internal control. Primer sequences were listed in Supplementary Table 1.

2.4. Flow cytometry
CMs were treated with Collagenase I (Sigma) for 20 min ~1 h, and dissociated into single cells with 0.25% Trypsin (GIBCO). CMs were fixed and permeabilized with permeabilize solution (BD Biosciences), immunostained with the anti-cTnT primary antibody (Abcam) or IgG control antibody for 2 h at 4°C, washed 2-3 times, and then stained with PE-labeled secondary antibodies for 1 h at 4°C. Stained-cells were resuspended and detected with a FACScalibur (BD Biosciences)
and analysed by the FlowJo software. Information of the used antibodies in this study are listed in Supplementary Table 2.

2.5. Alkaline phosphatase staining

hESCs were dissociated and plated on slides for 2–3 days culture. Cells were then stained with the FRV, Naphthol and H2O (1:1:1) mixture for 15 min at room temperature avoiding light. Images were taken by a Leica Dmi8 microscope (Leica).

2.6. Immunofluorescence staining

The cells were rinsed with PBS and fixed with 4% paraformaldehyde for 5 min. The cells were permeabilized with 0.05% Triton X-100 at room temperature for 15 min, and then incubated in 4% goat serum for 30 min. The cells were stained by primary antibodies overnight at 4 degrees. The next day, the cells were incubated with Alexa Fluor conjugated secondary antibodies at 37°C for 1 h, and then counterstained with DAPI (Abcam) at room temperature for 5 min, observed under a Leica Dmi8 fluorescence microscope (Leica). To assess pluripotency, hESC colonies were stained with antibody against SOX2 (Abcam), OCT4 (Santa cruz), SSEA4 (Abcam) and NANOG (Santa cruz). To assess differentiated cells, stained with antibody against cTNT2 (Abcam), α-actin (Abcam), α-smooth muscle actin (Proteintech), Secondary antibodies labeled with Alexa Fluor 488 or 594 were applied (Proteintech). Finally, cells were stained with DAPI (Abcam) and Cell area was further quantified by the ImageJ software (National Institutes of Health) as previously described [12]. Information of the used antibodies in this study are listed in Supplementary Table 2.

2.7. In vivo teratoma formation assays

4-5 × 10⁶ WT or E2A mutant hESCs were resuspended in 100 μl matrigel (Corning, 356231) and then subcutaneously injected to six NOD-SCID mice. All mice were housed in an animal facility with a 12h light/dark cycle and free access to food and water. All procedures were performed in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of Fudan University (Protocol: 20140226-056). Eight weeks later, teratomas were dissected, and fixed with 4% PFA. Hematoxylin-eosin staining was performed to detect whether three germ layers formed.

2.8. Western blot

Total protein was extracted from cultured cells lysed in RIPA lysis buffer (Beyotime, P0013C) with protease inhibitors (Bimake, B14001). 30 μg protein was electrophoresed and separated in SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 2 h. The membranes were then treated with primary antibodies of human E2A (Santa Cruz) or β-actin (Abcam) overnight at 4°C. Membranes were gently washed three times with PBS-T buffer for 10 minutes at a time, then secondary antibodies (Proteintech) were applied for 60 min at room temperature. Membranes were washed again in PBS-T, the method is the same as above. Immunoblots were visualized by chemiluminescent imaging system (Tanon 5200) and quantified by the ImageJ software (National Institutes of Health). Information of the used antibodies in this study are listed in Supplementary Table 3.

2.9. Cell size measurement

Cardiomyocyte size was determined in cardiomyocytes stained with cTNT antibody (Abcam) and anti-rabbit Coralite 594 (Proteintech). At least 60 cells from randomly selected fields were analyzed using the ImageJ software (NIH) following online instruction. Briefly, scale was set to present measurement results in calibrated units. Then cells surface was measured by using the “Freehand selections” button. The measurements for each cell area were displayed in the “Results” window. To exclude observational errors and personal influences, we improved precision by averaging the test values of repeated measurement.

2.10. Cardiomyocyte contractility measurement

Single cardiomyocyte cells were seeded on matrigel-coated conical dishes (Nalge Nunc International). After spontaneously beating, the contractility was detected by video-based motion edge detection system (Zeiss CFM500 inversion fluorescence microscope). And real-time contractile data were recorded by the FelixGX 4.2.2 software (FelixGX, PTI). Voltage of y axes indirectly reflected the relative contractility of beating cardiomyocytes and the number of peaks in 30 s indicated the relative contracting rate indirectly.

2.11. Whole cell patch clamp recordings

Cardiomyocytes of day30 were mechanically and enzymatically dissociated to obtain single cells, and were then seeded on Matrigel-coated glass coverslips (Warner Instruments). Cells with spontaneous beatings were selected and action potentials were recorded using an EPC-10 patch clamp amplifier (HEKA) as previously described [13]. Continuous extracellular solution perfusion was achieved using a rapid solution exchanger (Bio-logic Science Instruments). Data were acquired using PatchMaster software (HEKA) and digitized at 1 kHz. Data analyses were performed using Igor Pro (Wavemetrics) and Prism (Graphpad). A TC-344B heating system (Warner Instruments) was used to maintain the temperature at 35.5–37°C. Tyrodes solution was used as the external solution containing 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 10 mM glucose, 1.8 mM CaCl₂ and 10 mM HEPES (pH 7.4 with NaOH at 25°C). The internal solution contained 120 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 3 mM Mg-ATP, and 10 mM EGTA (pH 7.2 with KOH at 25°C).

2.12. Multi-electrode array (MEA) recordings

The multi-electrode array (MEA) system (Multi-Channel Systems, MEA-2100, Germany) was used to record the extracellular field potential. Beating CMs were dissociated and plated on matrigel-coated MEAs dishes for 2–3 days to achieve attachment to the electrodes. Electric signals were collected by a MCS rack system (Multi-channel Systems) and analyzed using the Spike2 7.05 software (Cambridge Electronic Design, UK) [14,15]. β-adrenergic (1 μM isoproterenol; Sigma-Aldrich) and its antagonist metoprolol (1 μM carbachol; Sigma-Aldrich) were applied to assess the adrenergic response.

2.13. Dual-Luciferase reporter assays

From public ATAC-seq and Dnase-seq data, the promoters of TFs, including TBX5, TBX3, SHOX2 and TBX18 were predicted to interact the relative contractility of beating cardiomyocytes and the number of peaks in 30 s indicated the relative contracting rate indirectly. The promoters were then inserted into the multiple cloning site (MCS) located in the dual-luciferase reporter vector pGL3 respectively. 293T cells were co-transfected with each constructed reporter vector plus the E2A-pCMV-C-β-galactosidase plasmid by DNA Transfection Reagent (Bimake) for 24 h. Cells were then harvested using a Dual-Luciferase Reporter Gene Assay Kit (Beyotime, RG027) according to the manufacturer’s protocol. The relative luciferase activity was normalized to Renilla luciferase activity. The pGL3-basic and PCMV-C-HA was simultaneously transfected as an internal control.
2.14. RNA extraction, Illumina library preparation, and sequencing

Total RNA were extracted from WT and E2A knockout hESCs at day 0-15 or day 30 post directed differentiation using Trizol (Invitrogen, 15596018). The mRNAs containing Poly-A tail were purified by poly-T oligo-linked magnetic beads from 1μg total RNA and then digested into small fragments using divalent cations. The fragmented mRNAs were synthesized on the first-strand cDNA and the second strand cDNAs were then synthesized using DNA Polymerases I and RNase H (Invitrogen). After an end repair process by adding a single “A” base and ligation of the adapters, these products were then purified and enriched with PCR to create the final cDNA library. The concentration of cDNA was then determined by the Qubit® RNA Assay Kit in Qubit® 3.0. The clustering of the index-coded samples was performed on a cBot cluster generation system using HiSeq PE Cluster Kit v4-cBot-HS (Illumina) according to the manufacturer’s instructions. After cluster generation, the libraries were sequenced on an Illumina sequencing platform (Illumina Hiseq™ 4000, San Francisco, USA).

2.15. Statistical analysis

Data were represented as mean ± SEM. Two-tailed Student’s t-test was used to compare the statistical differences between two independent groups. Analysis of variance (ANOVA) tests were used to compare statistical differences among more than two groups. Significant differences were considered when the P-value was less than 0.05.

3. Results

3.1. Expression of E2A during cardiac-specific differentiation of hESCs

The 2D cardiac-specific differentiation protocol for hPSCs has been well-established and is a good model for studying human cardiac development and differentiation [16,17]. Here we utilized this protocol and dissected E2A expression and function during this process (Fig. 1a). We observed spontaneous beating CMs at day7 post differentiation. The expression patterns of pluripotency marker (NANOG), early mesoderm markers (T, EOMES), cardiac mesoderm markers...
(MESP1), cardiac progenitor cell (CPC) markers (NKX2-5, ISL1), and CMs markers (TNNT2, MYH6, MYL7) during cardiac differentiation are consistent with previous reports from other groups [Fig. 1b] [18]. Increasing ratios of MYH7/MYH6 and MYL2/MYL7 expression also suggested a time-dependent maturation for these hESC-derived CMs (Fig. 1c). These results indicated that our hESC cardiac differentiation system was successful. We next examined E2A mRNA levels during the hESC cardiac differentiation process and found that, unlike the stage-specific expression of many cardiogenic transcription factors, E2A was constantly expressed throughout the differentiation process (Fig. 1d).

3.2. E2A knockout did not affect pluripotency but altered cardiac-specific differentiation of hESCs

To evaluate the role of E2A in human heart development and cardiac-specific differentiation, we established E2A knockout hESC cell lines by CRISPR/Cas9 mediated genomic editing. The guide RNAs were designed to target exon 3 of the E2A gene (Supplementary Fig. 1a). Successful interruption of E2A expression by frameshifting indels in single H7 and H9 hESC clones were confirmed by sequencing and Western blotting (Supplementary Fig. 1b and Fig. 2a). The morphology and growth of E2A knockout hESCs were similar to those of the

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**Fig. 2.** E2A deficiency altered cardiac-specific differentiation of hESCs.

(a) Representative western blots showing E2A levels in H7 and H9 knockout homozygous clones. (b) Expression pattern of cardiogenic mesoderm markers (MESP1); cardiac progenitor cells markers (NKX2-5, ISL1) and cardiomyocytes structural genes (TNNT2 and MYL7, except for MYH6) were down-regulated in E2A knockout differentiated cells. Data are shown as the mean ± SEM from at least three independent experiments. * P value < 0.05, ** P value < 0.01, *** P value < 0.001, # P value < 0.0001 (Two-tailed Student’s t-test).

(c) Quantification of cTnT positive cardiomyocytes in E2A knockout CMs by flow cytometry. Gray shapes represented the blank control. (d) IF analysis of differentiated cardiomyocytes generated from H7 and H9 hESCs, showing attenuated cTnT relative intensity. White arrows pointed at small size CMs. (e) Quantitation of cTnT intensity was weakened in E2A knockout CMs. (f) Quantitation of CMs cell area was reduced in E2A knockout CMs. (g) Contraction traces of wildtype and E2A knockout CMs. (h) Rising beating rates were observed in CMs derived from E2A knockout CMs. (i) E2A knockout CMs showed attenuated contractile force, n > 100. * P value < 0.05, ** P value < 0.01, *** P value < 0.001, # P value < 0.0001 (Two-tailed Student’s t-test).
wildtype (WT) hESCs. Immunofluorescence staining of pluripotency markers OCT4 and NANOG also showed similar nuclear staining pattern (Supplementary Fig. 2a). The mRNA levels of certain pluripotency markers were slightly up-regulated in E2A knockout hESCs (Supplementary Fig. 2b). We also injected E2A knockout hESCs into NOD/SCID immunodeficient mice for teratoma development. Three embryonic germ layers were found in both E2A knockout and WT teratomas (Supplementary Fig. 2c). These data indicated that E2A deficiency did not compromise the pluripotency of hESCs.

To see whether E2A is important in the specification and differentiation of cardiomyocytes, we next analyzed expressions of crucial cardiac-related markers in E2A knockout hESCs during differentiation. The mRNA levels of cardiac mesoderm genes MESP1, CPC marker genes ISL1 and NKX2-5, as well as CM structural genes TNNT2 and MYL7 (except for MYH6) were all down-regulated at various degrees in E2A knockout hESCs compared with those in WT (Fig. 2b). Both H7 and H9 E2A knockout hESC clones were able to differentiate into monolayer beating cardiomyocyte sheets. Flowcytometry analysis showed that the percentage of cTnT-positive CMs were similar in WT and E2A knockout hESC differentiation, with E2A knockout cells showing a decreased cTnT expression intensity (Fig. 2c). Immunostaining of E2A knockout hESC-derived CMs further showed a reduced content of contractile protein cTnT (Fig. 2d and 2e). Remarkably, we also noticed a significantly increased proportion of smaller and more round shaped cardiomyocytes in E2A knockout group indicated with white arrows in Fig. 2d. And quantification of average cell area showed a substantial decrease in E2A knockout group (Fig. 2f). To investigate the influence of E2A knockout on cardiomyocyte contractility, we next measured the relative contraction force of dissociated single beating cardiomyocytes. From day15 post differentiation, E2A knockout hESCs derived cardiomyocytes gradually beat much faster than WT hESC-CMs (Fig. 2g and 2h; Supplementary video 1-4) and exhibited relative attenuated contraction force. The mRNA levels of cardiogenic mesoderm genes MESP1, CPC, TNNC1, MYOM1 and ACTN2, were down-regulated, while many ion channel encoding genes, such as CACNA1G, KCNJ3, and KCNA5, were up-regulated in E2A knockout cardiomyocytes (Fig. 3d). Of note, GO analysis also showed that up-regulated genes on day3 and day7 post differentiation were highly random with multiple initiation sites (Fig. 4c and Supplementary Fig. 4c). These results indicated that hESC cardiomyocytes differentiation was affected to some degree by E2A gene knockout.

3.3. Global transcriptomic analysis of E2A knockout hESC-CMs

To further examine the phenotypes of E2A knockout cardiomyocytes derived from hESCs and better determine the genome-wide impact of E2A deficiency, we performed whole genome RNA sequencing analyses and compared WT and E2A knockout cells at day 3, 7 and 15 post differentiation. As shown in Fig. 3a and 3b, there are 1099 up-regulated genes and 790 down-regulated genes in E2A ablated hESC-CMs (cutoff = 2.0, P value < 0.05) relative to WT at day15 post differentiation. GO analysis indicated that up-regulated genes were associated with ion transport, cell fate commitment, and mesenchyme development etc. Muscle contraction, actin filament-based process, and muscle structure development were among the most affected biological processes in down-regulated genes (Fig. 3c). In detail, genes associated with cardiomyocyte contractile structure, myofibril assembly, and cardiomyocyte development, such as MYH7, TNNC1, MYOM1 and ACTN2, were down-regulated, while many ion channel encoding genes, such as CACNA1G, KCNJ3, and KCNA5, were up-regulated in E2A knockout cardiomyocytes (Fig. 3d). Of note, GO analysis also showed that up-regulated genes on day3 and day7 post differentiation, which is the time of cardiac mesoderm and the initial formation of CMs from hESCs, were enriched in neuron development and differentiation, while down-regulated genes were enriched in categories such as cardiovascular system development and muscle contraction (Supplementary Fig. 3). Gene set enrichment analysis (GSEA) confirmed the concordant down-regulated change of myofibril structural genes in both early cardiomyocytes (day7) and late cardiomyocytes (day15) (Fig. 3d and 3f). While ion channel encoding genes were only up-regulated in day15 cardiomyocytes (Fig. 3d and 3g). We further validated these differential gene expressions by quantitative PCR in day30 E2A knockout hESC-CMs. Expressions of T-type calcium channels CACNA1G, CACNA1I and CACNA1H, were all up-regulated, while expressions of L-type calcium channels, such as CACNA1C and CACNA1D were decreased (Fig. 3h). These results suggested that E2A deficiency drives cardiac differentiation of hESCs toward an uncommon direction, with many channel genes up-regulated after day15 post differentiation.

3.4. E2A knockout significantly up-regulated the ratio of nodal-like CMs in hESC cardiac-specific differentiation

Considering the fact that T-type calcium channels are enriched in conductive cardiomyocytes and L-type calcium channels in working cardiomyocytes, the increased expression of T-type calcium channels and some other channel genes in E2A knockout cardiomyocytes further suggested that the phenotype may associate with an increase in the amount of nodal-like cardiomyocytes. Examination of action potential (AP) profiles for single cardiomyocyte is a standard approach to distinguish the heterogeneous cardiomyocyte subtypes [19]. To test our hypothesis, we next recorded APs of day30 single cardiomyocytes derived from both WT and E2A knockout hESCs using the single-cell patch clamp technique. By comparing key AP parameters, including maximum diastolic potential (MDP), overshoot, AP amplitude (APA), AP duration (APD), AP duration at 50% repolarization (APD50), AP duration at 70% repolarization (APD70), AP duration at 90% repolarization (APD90), maximal upstroke velocity (Vmax), and beating rates, we classified WT and E2A knockout cardiomyocytes into three different subtypes. We found that, after E2A knockout in H7 hESCs, the percentage of nodal-like (N-like) CMs increased from 2.7% to 41.3%, while that of ventricular-like (V-like) CMs decreased from 75.7% to 39.1% and atrial-like (A-like) CMs decreased from 21.6% to 19.6% (Fig. 4a and Table 1). Similarly, we further confirmed that the number of nodal-like cardiomyocytes also significantly increased to >28% in H9 E2A knockout hESCs (Supplementary Fig. 4a and Table 2).

We next assessed the electric activity of E2A knockout cardiomyocytes by multi-electrode arrays (MEAs). WT or E2A knockout cardiomyocytes were plated in MEA probes in a beating monolayer as shown in Fig. 4b and supplementary Fig. 4b. As it is known, the synchronized contraction of heart is controlled by the spread of electrical signal from cardiacmyocyte to cardiacmyocyte. The assessment of the conduction velocity of the cardiac impulse can be reflected by electrophysiological recordings of the 60 electrodes in the MEA probe. After determination of local activation time at each electrode, we obtained the detailed activation maps depicting the development of synchronized action potential propagation from earliest activation (red) towards latest activation (blue). We observed that the initiation site of electric activity was stably localized in a specific site in both H7 and H9 WT cardiomyocytes monolayer. In contrast, the electric activity of both H7 and H9 E2A knockout cardiomyocytes monolayers were highly random with multiple initiation sites (Fig. 4c and Supplementary Fig. 4c), suggesting there were increased nodal cell-generated pacing sites. E2A deficient cardiomyocytes monolayer beat faster than WT and had prolonged field potential duration (FPD, WT, 0.30 ± 0.04s; KO, 0.42 ± 0.02s; P value < 0.0001), shorter inter-spike interval (ISI, WT, 0.92 ± 0.05s; KO, 0.78 ± 0.05s; P value < 0.0001) and increased cFPD (ratio of FPD/ISI, WT, 0.32 ± 0.05; KO, 0.55 ± 0.05; P value < 0.0001) (Fig. 4d-4e, and Supplementary Fig. 4d), which indicated that E2A deficiency led to shorter periods of electric activity.

Nodal-like cardiomyocytes could be generated from TBX18+ progenitors, which is a prominent factor in SAN formation [20]. We further stained WT and E2A knockout cardiomyocytes with TBX18 to estimate the percentage of nodal-like cells. It showed that the percentage of TBX18+ cells in E2A knockout cardiomyocytes was markedly increased, while that of MLC-2v positive ventricular-like
cells was significantly decreased, consistent with our whole-cell patch clamp data (Fig. 4f-4h and Supplementary Fig. 4e). Flowcytometry analyses further confirmed that the percentage of cTnT and MLC-2v double positive ventricular-like cardiomyocytes was significantly decreased from >90% to >40-45% after E2A knockout (Supplementary Fig. 5). Collectively, these data indicated that E2A ablation led to the proportional increase of nodal-like cells and decrease of ventricular-like cells in hESC-derived cardiomyocytes.

3.5. E2A directly regulates transcription factors associated with the development of conduction cells

SAN is composed of a heterogeneous cell population and its development is controlled by several transcription factors, mainly including TBX18, TBX3, TBX5, and SHOX2 [21]. Our RNA-seq data showed that expression of TBX18, TBX5, and SHOX2 were all up-regulated in E2A knockout hESC-CMs at day15 after differentiation (Fig. 5a). The
mRNA abundance of TBX18 and SHOX2 persisted to increase at day 30 after differentiation (Fig. 5b). And we observed that the expression of the other vital transcription factor TBX3 was also significantly upregulated (Fig. 5b). But the TBX5 mRNA level did not show statistical significance between WT and E2A knockout cardiomyocytes at day 30 (Fig. 5b). Since TBX5 acts as the activator of TBX3 and SHOX2, there could be a fine balance between the expression level of TBX5 and TBX3 during cardiac specification. To investigate whether the above-mentioned transcription factors are directly regulated by E2A, we analyzed public ATAC-seq and DNase-seq data from hESCs, cardiac progenitors (CP), cardiomyocytes (CMs), and fetal heart tissues to locate the open chromatin regions of these genes. Considering
Table 1

| H7-WT & H7-KO single cell Patch-Clamp recording Statistics | MDP (mV) | Overshoot (mV) | APA (mV) | APD (ms) | APD90 (ms) | APD70 (ms) | APD50 (ms) | Vmax-D (V/s) | Beating rates (per minute) | SD of Interspike Interval | % Total |
|----------------------------------------------------------|-----------|---------------|--------|---------|-----------|-----------|-----------|------------|-----------------------------|-------------------------|---------|
| KO (n=19) -54.3 | 0.8 | 47.6 | 100 | 1280 | 2456 | 1272 | 1214 | 1214 | 1112 | 1374 | 146 | 92.5 | 6.6 |
| KO (n=9) -49.8 | 1.1 | 43.8 | 3.3 | 100.9 | 23.2 | 293.6 | 183.3 | 26.1 | 143.6 | 14.3 | 37.7 | 10.0 | 92.5 | 3.2 |
| KO (n=18) -59.7 | 1.7 | 94.1 | 2.5 | 293.6 | 10.7 | 192.2 | 192.2 | 5.9 | 81.7 | 118.9 | 3.9 | 41.3 | 25.7 |
the differentiation direction of E2A knockout cardiomyocytes back toward wildtype.

4. Discussion

In this study, we found that the bHLH family transcription factor E2A participated in regulating differentiation of distinct cardiomyocyte subtypes from hESCs. E2A knockout cardiomyocytes displayed characteristic transcriptional expression pattern, increased beating rates, smaller cell size, attenuated sarcomere structure, and action potentials close to the unique features of SAN pacemaker cells and distinct from those of WT hESC-CMs. The patch clamp study also revealed E2A ablation dramatically promoted the proportion of nodal-like cells from previous <7-8% to as high as ~40% in hESCs-derived cardiomyocytes, while significantly suppressed the number of ventricular-like cardiomyocytes. We further found that spatiotemporal inhibition of Notch signaling at day 9-15 of differentiation diminished the enrichment of nodal-like cells derived from E2A knockout hESCs. Together, these results pointed to a new role of transcription factor E2A in the derivation of nodal-like cells from hESCs.

It is undeniable that identification of nodal-like cells from working type cardiomyocytes is still a big challenge since the SAN itself...
consists of a heterogeneous population of cardiomyocytes, let alone in an in vitro differentiation system without anatomic landmarks. Thus, it is better to use a combination of special physiological, functional, and molecular characteristics as a whole to screen nodal-like cells from working type cardiomyocytes. And, it should be noted to distinguish the role of E2A deficiency on elevating the population of the nodal-like cells rather than suppressing cardiomyocytes maturation.

There are several evidences present in our study showing that a substantial increasing number of E2A knockout hESC-CMs were nodal-like cells. First, the parameters used to identify action potential of separate subtype cardiomyocytes derived from hESCs, which is a

Fig. 6. E2A partially relies on regulating the activity of NOTCH signal pathway for involving in CMs differentiation process and its inhibitor reversed the phenotypes of E2A knockout CMs.

(a) mRNA levels of representative NOTCH signal pathway genes after E2A knockout. Data were normalized to GAPDH level and presented as mean ± SEM. * P value < 0.05, ** P value < 0.01, *** P value < 0.001 (Two-tailed Student’s t-test). (b) Expression of NOTCH target genes HES1 and HEY1 in day30 CMs of WT and E2A−/− after treated with NOTCH inhibitor. Data were normalized to GAPDH level and presented as mean ± SEM. * P value < 0.05, ** P value < 0.01, *** P value < 0.001 (ANOVA). (c) Cell areas of E2A−/− CMs increased after inhibition of NOTCH signal pathway. (d) Statistic of cell size of E2A−/− CMs after interfering NOTCH signaling. (e) Increased expression of cTnT and MLC-2v in E2A knockout CMs after treated with NOTCH inhibitor (RO4929097). All data were presented as mean ± SEM. * P value < 0.05, ** P value < 0.01, *** P value < 0.001 (Two-tailed Student’s t-test). (f) mRNA levels of structures genes were partially rescued in E2A−/− CMs after inhibition of NOTCH signal pathway. Data were normalized to GAPDH level and presented as mean ± SEM. * P value < 0.05, ** P value < 0.01, *** P value < 0.001 (ANOVA). (g) mRNA expression of L-type calcium channels genes was increased, while which of T-type calcium channels and several other ion channels were decreased in E2A−/− CMs after treated with NOTCH inhibitor. All data were presented as mean ± SEM. * P value < 0.05, ** P value < 0.01, *** P value < 0.001, # P value < 0.0001 (ANOVA).
commonly accepted approach [19,23,24], showed in our study, a marked increase in the percentage of nodal-like cells in E2A knockout hESCs-CMs. Second, E2A knockout hESCs-CMs monolayers displayed random electric activity and increased pacing points in MEA assays, which is consistent with the property of SAN cardiomyocytes. Third, a substantial increasing number of E2A knockout cardiomyocytes displayed morphological and functional properties substantiating the nodal-like cell phenotype. Pacemaker cells derived from hESCs have been characterized by a special appearance as the round shaped and relatively smaller mononuclear cell (10-30 μm in size) [25], which is distinct from multinuclear working type cardiomyocytes. A substantial increasing number of E2A knockout hESCs-CMs displayed the morphology matching the special appearance of pacemaker cells derived from hESCs. Fourth, in our study, bulk RNA sequencing analysis demonstrated that the expression patterns of representative genes, such as ion channel encoding genes and contractile structure genes, were more close to the nodal-like cells. Moreover, expression of the key SAN development transcription factors SHOX2, TBX3 and TBX18 were all up-regulated in E2A knockout cardiomyocytes. By analyzing the public ATAC-seq and DNase-seq data from hESC, cardiac progenitors (CP), cardiomyocytes, and fetal heart tissues, we traced the conserved E-box binding motifs near the promoters of these transcription factors, which indicated the possible regulation of E2A on their expression. Further, we proved that E2A directly inhibited TBX3 expression by luciferase reporter assays. TBX3 is known to play a vital role in specification and development of the conduction system during vertebrate embryogenesis [26,27]. TBX3 overexpression also efficiently programed mESCs or hiPSCs into sionoaidal bodies exhibiting highly beating rates and robust capability of pacing myocardium in vitro [28-30]. Vincent van Ef and et al., concluded a gene program including TBX3, SHOX2, ISL1, HOX family members, BMP and Notch signaling components are conserved between human and mouse [31]. Single cell sequencing analysis done by Liang and et al. determined TBX3 and SHOX2 are core in the gene regulatory network of pacemaker activity [32]. And functional genes encoding HCN channels, K+ channels, Ca2+ channels are all expressed in SAN across species; while the expression of voltage-gated potassium channel gene was low in SAN cells. These gene expression pattern was similar in our hESCs-derived cardiomyocyte samples from day15 after differentiation. Of note, two new SAN specific markers, Smoc2 identified by Vincent van Ef and et al., and Vsnl1 characterized by Liang in above studies, were also significantly up-regulated in our E2A knockout cardiomyocytes. All these data support our findings that E2A ablation promoted pacemaker cells formation from hESCs specific differentiation.

However we also noticed that E2A displayed a positive regulation of SHOX2 and TBX5 expression which is conflict with the alternation in the expression level of these genes after E2A ablation. Given that bHLH family proteins usually function as homodimer or heterodimer, the different regulation of E2A on TBX3 and SHOX2 expression may resulted from lacking of other bHLH family partners in our in vitro luciferase reporter assay system. It is possible that, in vivo, E2A binds to different bHLH family partners to form different heterodimers and perform precise regulation of cardiomyocyte specification in cardiac differentiation of hESCs. An insight into the dimerization brought by the shared bHLH domain may help identify the key mechanism of E2A in future study. More importantly, we assumed there may exist some indirect function of E2A to finalize its regulation on the downstream targets. Interestingly, we got a hint from the KEGG and GSEA analysis of our RNA-seq data at successive stages during the differentiation process. The NOTCH pathway was dramatically activated in E2A knockout early cardiomyocytes around day7 post differentiation (Supplementary Fig. 6a). Furthermore, the receptors, ligands, and downstream targets of NOTCH signaling were all significantly up-regulated in E2A knockout late cardiomyocytes around day30 post differentiation (Fig. 6a). With respect to the NOTCH signaling participants, including HEY2, NOTCH1 and JAG1, our data also support the previous data showing that in vitro or in vivo activation of myocardial NOTCH signaling by overexpressing the Notch intracellular domain (NICD) reprogrammed CMs to a CCS phenotype [22]. And overexpression of NOTCH extracellular domain (NICD) was proven to stimulate the expression level of TBX5 and other regulators [22,33]. By further confirming that NOTCH signaling inhibitor partially reverses the altered differentiation phenotypes of E2A knockout hESCs (Fig. 6). Considering the accordant changes of these regulators’ expression level with the activity of NOTCH signaling, we think this synergistic effect of E2A on above molecular determinants in SAN development is one of the biggest factors in high yield of nodal-like cells in E2A knockout hESCs-derived cardiomyocytes. One limitation of our current study is not able to isolate the funny current which serves as a distinct feature of mature nodal like cells and contributes to generate the diastolic depolarization [34]. It is worth noting that the abundance of typical sinoatrial HCN4 did not change in our E2A knockout cardiomyocytes, which is responsible for the If current generation and serves as a functional marker of pacemaker cells [35-37]. One of the possible reasons is that our current small molecule-based cardiac specific differentiation protocol was performed to yield a majority of chamber cardiac myocytes from embryonic stem cells. Sartiani et al. [38] indicated that hESCs-derived cardiomyocytes at early stage express a robust If current which decreased in the late stage. Meanwhile the decreased mRNA level of HCN4 was also observed with time. But Bosman et al pointed out that although the mRNA abundance of HCN4 decreased during matura- tion of hESC-CMs, the HCN4 protein level actually increased [39]. And considering E2A knockout cardiomyocytes in our differentiation system may yield the immature nodal like cells, these typical sinoatrial gene expression pattern will be more inclined to nodal-like pacemaker cells after adjusting differentiation condition. Thus, it is important to isolate If current from E2A knockout CM in our future study. And If blocker and caffeine can be applied to minimize the membrane clock or debilitate the Ca2+-clock which will provide more detailed information about Ca2+ handling in E2A knockout cardiomyocytes.

Interestingly, the electric activity recorded by MEA indicated the exceptional conductive ability of E2A knockout cardiomyocytes. Moreover, patch clamp recording revealed that E2A knockout markedly increased the proportion of nodal-like cardiomyocytes derived from hESCs, while at the same time suppressed the number of ventricular-like cardiomyocytes. This notion leads to a critical question as to in which stage E2A plays a role in the specification or differentiation of nodal-like cells. Previous studies provided evidences that overexpression of vital transcription factors reprogrammed ventricular cardiomyocytes into pacemaker-like cells in vitro [40-42]. In our study, we also noticed that there was a significant down-regulation of genes (e.g. MESP1, NKX2.5, ISL1) in early mesoderm development and cardiac progenitors derived from E2A knockout hESCs (Fig. 2b). Importantly, among these genes, NKX2.5 is a major regulator for cardiomyocyte specification and differentiation. Previous studies also showed that pacemaker cells are recruited from NKX2.5-negative progenitors [5]. Thus, the down-regulation of NKX2.5 in E2A knockout cardiomyocytes progenitors may be key to lineage specification for nodal-like cells. Moreover, we also observed the increased expression of SHOX2, which is well-known to antagonize the expression of NKX2.5 [43,44], in E2A knockout cells. These results suggested that the changed transcriptional regulation of these genes in E2A knockout hESCs during cardiac specification may result in the altered differentiation outcome. To further investigate the mechanisms of changes in cardiac-specific differentiation regulated by E2A deficiency, we also focused our analysis on the genes encoding signaling molecules regulating early mesoderm and cardiogenic specification. Signalling molecules of BMP, Retinoic Acid, WNT and other family served as the cardiac-inducing factors in a spatiotemporal-dependent manner to activate expression of the NKX2.5, GATA4 and T box.
factors [18,45-47]. Our current small molecule-based cardiac specific differentiation protocol was performed by adding small molecules regulating Wnt signaling to yield cardiac myocytes from embryonic stem cells [18]. As mentioned, upstream regulator analysis from IPA predicted WNT3A as one of the top 10 upstream molecules leading to the observed changes in gene expression after E2A knockout. We noticed that the activity of WNT signaling pathway were dramatically changed compared with that in the WT hESC differentiation (Supplementary Fig. 7). The canonical WNT 11 is known to activate NKX2.5 and GATA4, while WNT3a and WNT8 were shown to suppress cardiogenesis in differentiating ES cells in vitro [48,49]. It is possible that E2A interrupted the activity of WNT signaling and thus affect the specification pattern of cardiac progenitors. To manipulate the activity of these signalling pathways could be an effective way to improve the yield of nodal-like cells for human cell based biopacemakers in the future. Yechikov S and et al. recently reported they improved the yield of pacemaker-like cells up to 2.4 fold by interfering the activity of NODAL and WNT pathway in cardiac mesoderm stage [42]. Here our data indicated that beyond its direct regulation of SAN specific genes, E2A may also play multiple actions on regulating the activity of NOTCH and other pathways to augment the proportion of nodal like cells in the heterogenous cardiomyocyte population. To address the above question, single cell sequencing could be a better solution to isolate subpopulations from the heterogenous cardiomyocytes and traces developmental fates of cardiomyocyte lineages during directed differentiation in the future.

Based on these criteria, we reasoned that E2A deficiency interfered both the specification and differentiation of nodal-like cells in our cardiac-specific differentiation system. It has drawn much attention for E2A-mediated transcriptional regulation contributed in differentiation of many cell lineages, including lymphoid, muscle, and neuronal cells [50-53]. However its role in cardiac development remains unrevealed. Of note, Cunningham and others pointed out that E2A acts as the antagonist of cardiogenetic mesoderm formation from mESCs and the inhibitory activity were regulated by another bHLH protein member, Id proteins [10]. In their work, E2A knockdown by siRNA efficiently promoted the emergence of KDR positive cardiogenic mesoderm progenitors. Their conclusion about the regulatory effect of E2A is inconsistent with our study. This may be caused by the very dissimilarity between mouse and human ESCs and in their in vitro culture/differentiation conditions. Interestingly, we also noticed that the expression level of the other two E proteins (HEB and E2-2) were up-regulated in E2A knockout hESCs and cardiomyocytes (data not shown). The complementarity or redundancy among these E proteins have been fairly confirmed in embryonic differentiation and development [54]. Whether combined or total manipulation of E protein expression benefit the differentiation efficiency of nodal-like cardiomyocytes worth further exploration. Moreover, E47 and E12, two variants of E2A generated by alternative splicing, have been identified to display different roles in stem cell pluripotency maintenance and differentiation [55-57]. Since they have biased preferences for heterodimer partners and E box motifs, it will be worth to explore the potential role of these two isoforms and their partners during cardiomyocytes differentiation in the future.

In summary, our study revealed a new role of the transcription factor E2A during directed differentiation of hESCs into cardiomyocytes that E2A ablation substantially increased the proportion of nodal-like cells. Our study may provide an important clue to develop the approach to generate distinct cardiomyocyte subtypes, and further provide SAN-like cardiomyocytes for biological cardiac pacemaker therapy.

Contributors

Xiuya Li, Fei Gao, Xiaochen Wang and Qianqian Liang: Considered equal contribution; Xiuya Li and Fei Gao carried out the majority of the experiments, performed analyses, and generated data figures and supplemental information, and Xiuya Li wrote the first draft of our manuscript; Xiaochen Wang carried out Whole cell patch clamp recordings generated data figures, and participated in writing the manuscript; Qianqian Liang and Aobing Bai contributed significant roles in RNA-seq and subsequent Bioinformation analysis; Zhuo Liu, Xinyun Chen and Ermin Li: Contributed to technical support for experiments described in the manuscript. Sifeng Chen, Chao Lu and Ruizhe Qian participated in data interpretation; Ning Sun, Ping Liang and Chen Xu: conceived the study, provided funding acquisition and finalized the manuscript.

Xiuya Li, Fei Gao and Chen Xu verified the underlying data.

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Data sharing statement

Please contact Dr. Xu and Dr. Sun for requesting all the data and reagents described in this article.

Declaration of Competing Interest

The authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103575.

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