Enrichment differentiation of human induced pluripotent stem cells into sinoatrial node-like cells by combined modulation of BMP, FGF and RA signaling pathways

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Abstract
Background: Biological pacemakers derived from pluripotent stem cell (PSC) have been considered as a potential therapeutic surrogate for sick sinus syndrome. So it’s essential to develop high efficient strategies for enrichment of sinoatrial node-like cells (SANLC) as seed cells for biological pacemakers. It has been reported that BMP, FGF and RA signaling pathways are involved in specification of different cardiomyocyte subtypes, pacemaker, ventricular, and atrial cells.
Methods: During the differentiation process from human-induced pluripotent stem cell (hiPSC) to cardiomyocyte through small molecule based temporal modulation of the Wnt signaling pathway, signaling of BMP, FGF and RA was manipulated at cardiac mesoderm stage. qRT-PCR, immunofluorescence, flow cytometry and whole cell patch clamp were used to identify the SANLC. Results: qRT-PCR results showed that manipulating each one of bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and retinoid acid (RA) signaling was effective for the upregulation of SANLC markers. Moreover, combined modulation of these three pathways displayed the best efficiency for the expression of SANLC markers, which was further confirmed at protein level using immunofluorescence and flow cytometry. Finally, the electrophysiological characteristics of upregulated SANLC were verified by patch clamp method.
Conclusion: An efficient transgene-independent differentiation protocol for generating SANLC from hiPSC was developed, in which combined modulating BMP, FGF and RA signaling at cardiac mesoderm stage generates SANLC at high efficiency. This may serve as a potential approach for biological pacemaker construction.
Introduction
“Sick sinus syndrome” (SSS) is a group of heart arrhythmia caused by perturbed function of the sinus node that is composed of cardiac pacemaker cells. They include pathological and/or symptomatic sinus bradycardia, sinoatrial (SA) block and tachycardia-bradycardia syndrome. Although implantation of electronic pacemaker has been one of the most effective treatments at present it is associated with significant risks of infection, hemorrhage, and lead dislodging. Moreover, limited battery life and lack of autonomic neurohumoral responses further limit its usage [1, 2]. Therefore, the “biological
“pacemaker” derived from human pluripotent stem cells (hPSC) may provide a promising alternative treatment. Cardiomyocytes differentiation from hPSC is recognized as a powerful model to simulate the human embryonic heart development in vivo and is a promising source of cardiomyocytes for regenerative medicine. The embryonic heart development experiences several spatial-temporal stages from primitive streak to cardiac crescent and then to primitive heart tube, and the latter forms distinct anterior and posterior poles containing different mesodermal progenitors that give rise to different cardiomyocyte subtypes including ventricular, atrial and sinoatrial node (SAN)-like cardiomyocytes (SANLC) [3-5]. To date, most protocols were designed to generate heterogenous ventricular cardiomyocytes with a very small percentage of atrial and SANLC [6, 7]. Analyses of early developmental stages revealed that ventricular, atrial and SAN-like cardiomyocytes were derived from different mesoderm cell populations that could be distinguished based on their expression of CD235a [8, 9], RALDH2 [8] and TBX18 [10-12], respectively. In addition, it was found that different signaling pathways were involved different cardiomyocyte subtypes specification. For example, retinoic acid (RA) signaling at the mesoderm stage is required for atrial specification [8], while ventricular specification is highly dependent on the fibroblast growth factor (FGF) signaling [13-16]. A recent study showed that bone morphogenetic protein (BMP) signaling plays an important role in the specification of mesoderm progenitors into SANLC [17, 18]. These early discoveries suggest that hiPSC-induced cardiomyocytes can be directed to differentiate into SAN-like cardiomyocytes at cardiac mesoderm stage by activation of the BMP signaling pathways leading to SAN-like, and by simultaneous inhibition of signaling pathways leading to ventricular and atrial cardiomyocytes. Here, we have established a developmental-biology-based approach to enrich SANLC from hiPSC. We found in the present study that activation of BMP and simultaneous inhibition of RA and FGF signaling pathways during cardiac mesoderm stage of hiPSC differentiation can significantly enrich SAN-like cardiomyocytes. These findings will facilitate the study of human SAN development and may provide a rich source of cells for the development of biological pacemaker.

Materials And Methods
**Materials**

The information of purchased reagents and cell line used in the study: LHpb-YaabC3 hiPSC (HNF-P30-P11, OSINGLAY BIO, China), BJ human foreskin fibroblast cell line (CRL2522, ATCC, USA), HN4 human embryonic stem cell (hESC) line (HES-P20-P9, OSINGLAY BIO, China), Cell culture media RPMI/1640 (11875093, Thermo Fisher Scientific, USA), DMEM/F12 media (11320082, Thermo Fisher Scientific, USA), medium for hiPSC BioCISO (BC-PM0001, OSINGLAY BIO, China), GSK3 inhibitor CHIR99021 (S1263, Sigma, USA), FGF inhibitor PD (3044, Tocris Bioscience, UK), Wnt inhibitor IWP2 (3533, Tocris Bioscience, UK), ROCK inhibitor Y27632 (1254, Tocris Bioscience, UK), BMP activator BMP4 (120-05ET, PEPROTECH, USA), RA inhibitor BMS(SML1149, Sigma, USA), B-27 supplement with (17504-044, Thermo Fisher Scientific, USA) or without insulin, Matrigel (354277, Corning, UK), TRIzol Reagent (15596026, Thermo Fisher Scientific, USA), Real-time PCR reagents (208056, Qiagen, Germany). All primers/oligos were synthesized by Shenggong Biotech, China and listed in supplemental Table 1. All other reagents, unless specified otherwise, were products of Sigma.

**Cardiomyocytes differentiation from hiPSC**

Cardiomyocyte differentiation was performed in a growth factor and serum-free system by temporal modulation of the canonical Wnt signaling pathway with GSK3 inhibitor (Gi) and Wnt inhibitor (Wi), known as the GiWi protocol [19]. Briefly, 80-90% confluent hiPSC was harvested using 0.5mM EDTA, and resuspended with hiPSC maintaining medium at 0.5 x 10^5 cells per ml. Two ml of the cell suspension was seeded per well in a 12-well matrigel-coated plate with 2 ml of hiPSC at minus day4. At day 0 the medium was refreshed with RPMI/B-27 containing CHIR99021 (10 μM, GSK3 inhibitor) without insulin and continued to incubate for 24 h. The medium was replaced with RPMI/B-27 without insulin for another 48 h. On day 3 of the differentiation (72 h after addition of CHIR99021), medium was refreshed with RPMI/B-27 containing IWP2 (5 μM, Wnt inhibitor) without insulin for 48 h, followed by RPMI/B-27 without insulin from day 5 to 7. From day 7, the medium was refreshed with RPMI/B-27 containing insulin every 3 days. The beating cardiomyocytes can be seen as early as on day 8.

**Enriched differentiation of SAN-like cardiomyocytes from hiPSC by BMP4 (B), PD (P) and BMS (M) treatment**
Based on the GiWi protocol that it caused high efficient pan-cardiomyocytes as shown above, different concentrations of BMP4 (0, 1.25, 2.5, 5 ng/ml), PD (0.480, 720, 960 nM) and BMS (0, 1 μM) were added alone between day 5 and 7 after differentiation for 2 days. The qRT-PCR analysis was performed to evaluate the mRNA levels of SAN markers at day 16 to determine the optimal concentration of each compound. To investigate the synergistical effect, the cells were treated with combinations of BMP4, PD and BMS with the optimal concentration from day 5 to 7 during the differentiation process. The markers of pacemaker cells were evaluated by analysis of qRT-PCR, immunofluorescence and flow cytometry at day 21. Electrophysiological characteristics were analyzed using Action potential (AP) recording at day 60. The schematical protocol was shown in Figure 4A.

**RNA isolation and qPCR**

Total RNA was isolated using Trizol method (15596026, Invitrogen, USA). One μg of total RNA was reversely transcribed in a total volume of 10μl with ReverTra Ace qPCR RT Master Mix kit (FSQ-201, TOYOBO, Japan) following manufacturer’s instructions. The cDNA was diluted 3 times, and 1 μl was used for real-time PCR in a 20 μl reaction using SYBR Green Real Time PCR Mix (204143, Qiagen, Germany). The PCR conditions were 95°C for 2min, followed by 40 cycles of 95°C for 20” and 60°C for 15”. All primers were listed in supplement Table 1. The expression of target gene was normalized to that of GAPDH and calculated using 2^(-ΔΔCt) method.

**Immunofluorescence**

Single hiPSC cells and induced cardiomyocytes were seeded in a µ-Slide 8 well (80827, ibidi) coated with Matrigel at the density of 2 × 10^4 per well for 48 h. Cells were fixed with 4% (w/v) Paraformaldehyde (PFA) for 15 min at room temperature, permeabilized, and incubated with the following primary antibodies: anti-OCT4 antibody (#2750, Cell Signaling Technology, USA), anti-NANOG antibody (#3580, Cell Signaling Technology, USA), anti-SSEA4 antibody (#4755, Cell Signaling Technology, USA), anti-TRA-1-60 antibody (#4746, Cell Signaling Technology, USA), anti-Ki67 antibody (ab15580, abcam, USA), anti-NKX2.5 antibody (ab91196, abcam, USA), anti-cTNT antibody from mouse (MS-295-P1, Thermo Fisher Scientific, USA), anti-cTNT antibody from rabbit (15513-1-AP,
Proteintech, China) anti-α-actinin antibody (A7811, Sigma, USA), anti-SHOX2 antibody (ab55740, abcam, USA), anti-TBX18 antibody (ab115262, abcam, USA), anti-COUPTFⅡ antibody (PP-H7147-00, R&D, USA), anti-MLC2V antibody (MABT180, Sigma, USA) and anti-TBX3 antibody (ab154828, abcam, USA), followed by the following species-specific fluoresce-conjugated secondary antibodies: alexa fluor 488 labeled goat anti-rabbit IgG (A-11008, Invitrogen, USA), alexa fluor 488 labeled goat anti-mouse IgG (A-11001, Invitrogen, USA), alexa fluor 594 labeled goat anti-rabbit IgG (R37177, Invitrogen, USA) and alexa fluor 594 labeled goat anti-mouse IgG (A-11005, Invitrogen, USA). The cells were then counterstained using 0.5 µg/ml of DAPI (4083, Cell Signaling Technology, USA) for 15 min at room temperature. After rinsing with PBS, the chambers were mounted and visualized under fluorescence microscopy (IX83, Olympus, Japan). Corresponding antibody isotype control, mouse IgG (ab205719, abcam, USA), rabbit IgG (ab205718, abcam, USA), mouse IgG (ab190369, abcam, USA), mouse IgG1 (#5415, Cell Signaling Technology, USA), mouse IgG3 (#37988, CST, USA) were used.

**Flow cytometry**

The induced cardiomyocytes in 12-well plate were digested with 0.25% trypsin with 0.5mM EDTA into single cell suspension and washed with PBS. Cells were fixed with 4% formaldehyde for 10 min at room temperature and chilled on ice for 1 min. Permeablization was performed by adding one tenth of ice-cold 100% methanol slowly to the pre-chilled cells and continue to incubate on ice for 30 min. Cells were then blocked with blocking buffer (0.5% BSA in PBS) for 10 min, incubated with the following primary antibodies, anti-OCT4 antibody (#2750, Cell Signaling Technology, USA), anti-NANOG antibody (#3580, Cell Signaling Technology, USA), anti-SSEA4 antibody (#4755, Cell Signaling Technology, USA), anti-TRA-1-60 antibody (#4746, Cell Signaling Technology, USA), anti-Ki67 antibody (ab15580, abcam, USA), anti-cTNT antibody (MS-295-P1, Thermo Fisher Scientific, USA), anti-NKX2-5 antibody (ab91196, abcam, USA), and anti-SHOX2 antibody (ab55740, abcam, USA) for 1 h at room temperature, then washed with PBS, and followed by incubation with the corresponding species-specific fluorescence-conjugated secondary antibodies, alexa fluor 488 labeled goat anti-mouse IgG (A-11029, Invitrogen, USA) alexa fluor 488 labeled goat anti-rabbit IgG (A-11034, Invitrogen, USA), alexa fluor 647 labeled goat anti-mouse IgG (A-21235, Invitrogen, USA), and alexa fluor 647
labeled goat anti-rabbit IgG (A-32733, Invitrogen, USA) for 30 min at room temperature. Cells were analyzed using flow cytometry machine (651155, BD FACS Verse, BD Bioscience, USA) according to the manufacturer’s protocol.

**Action potential (AP) recording**

AP recording was performed following El-Battrawy I’s protocol with some modifications [20]. Briefly, on 60 days after cardiomyocytes differentiation, induced cardiomyocytes were dissociated into single cell suspension by 30 min’s typeⅠcollagenase (2 mg/ml) followed by 3 min’s Trypsin (0.25%) without EDTA. $1 \times 10^4$ cells were seeded into a 3.5 cm dish containing a lysine-treated glass coverslip and incubated for 3 days. AP was recorded using the whole cell patch clamp electrophysiology method. Briefly, the adherent cells on the coverslip were placed in the recording chamber and perfused with bath solution containing 140 mM NaCl, 1 mM MgCl₂, 5 mM KCl, 1.8 mM CaCl₂, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 mM glucose, (The pH was adjusted to 7.40, and the osmolality to 301 ± 3 mOsm, respectively). The patch pipettes were pulled from borosilicate glass capillaries (7-000-0650-LHC, Drummond, USA) by a horizontal puller (PC100, NARISHIGE, Japan) and had resistances of 1.5–3 MΩ. Pipette solution consisted of 110 mM K-glucuronate, 20 mM KCl, 1mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 5 mM ethylene glycol tetraacetic acidpotassium chloride (EGTA-KOH), 5 mM ATP-Mg²⁺, 5 mM Na-phosphocreatine. The pH was adjusted to 7.2 by KOH and the osmolality to 290 ± 3 mOsm. A Multiclamp 700B amplifier was used to record APs, and data were analyzed using a custom software.

**Statistical analysis**

Experimental data are presented as ‘Mean ± SD’ with at least three repeats. Comparisons between multiple groups were performed using one-way analysis of variance (one-way ANOVA), with $p < 0.05$ was considered statistically significant.

**Results**

**Characterization of hiPSC**

hiPSC and human embryonic stem cell (hESC) was purchased from OSINGLAY BIO company. To confirm the authenticity of the hiPSC, the molecular signature of hiPSC was validated by both qRT-PCR
and Immunofluorescence (IF). The qRT-PCR results showed that the hiPSC expressed stem cell-specific markers, OCT3/4, SOX2, and NANOG at similar levels as hESC which serves as a positive control. In contrast, these markers were non-detectable in the terminal differentiated human terminal differentiated fibroblast cells (Bj) as a negative control, (Figure 1A). The IF results showed that the hiPSC was proliferative (Ki67 positive) (Figure 1B) and heavily stained for NANOG, OCT4, SSEA4 and TRA-1-60, which were localized in nuclei (for NANOG and OCT4) and plasma membrane (for SSEA4 and TRA-1-60), respectively (Figure 1C-F). This was further confirmed using flow cytometry analysis (Figure 1G, 1H). These results demonstrate that the hiPSC possesses their pluripotency and self-renewal capability.

Identification of hiPSC induced cardiomyocytes

Cardiomyocytes were induced by the widely used GiWi protocol originally proposed by Lian, et al [19], where the induction process was temporally initiated using GSK3 inhibitor (Gi) and followed by temporal inhibition of the Wnt signaling pathway using Wnt inhibitor (Wi). To optimize the induction protocol, different concentrations of the Gi, CHIR were added on day 0 of the differentiation and continued to incubate for 24 h, and the markers of mesoendoderm (BRACHYURY) and cardiac mesoderm (MESP1) were evaluated on day 1 and day 3 using qRT-PCR. CHIR dose-dependently increased the expression of both BRACHYURY (Figure 2A) and MESP1 (Figure 2B) on day 1, with CHIR at 10 μM reached the highest level. Similar concentration-response curves were observed on day 3 for both markers (Figure 2A and 2B). However, the overall levels of BRACHYURY (Figure 2A) were significantly decreased, while the levels of MESP1 (Figure 2B) were significantly increased, at different concentrations of CHIR compared to those levels on day 1. These observations suggest fate conversion from mesoendoderm into cardiac mesoderm. The successful induction of cardiomyocytes at day 21 from differentiation was confirmed by both IF, showing positive staining for cardiomyocytes marker, cTNT and α-actinin (Figure 2C), and by flow cytometry, showing that around 85% of the induced cardiomyocytes expressed the cardiac sarcomere proteins, cTNT (Figure 2D). Quite high percent of beating cardiomyocytes was observed at day 21 as showed in SUPPL-Video 1. This further confirmed our optimized differentiation procedure.
**BMP, RA and FGF signaling pathways contribute differently to the differentiation of cardiomyocytes toward SAN-like cells**

Although it is generally agreed that ventricular, atrial and SAN-like cardiomyocytes are derived from different progenitor cells emerging as early as mesoderm stage, and different signaling pathways contribute differently to the directional differentiation toward ventricular, atrial and SAN-like cardiomyocytes, the timing of manipulation of the signaling pathways remains controversial. To determine the timing of manipulation, we evaluated the temporal expression of cardiac mesoderm marker (MESP1), cardiac progenitor marker (NKCX2-5) and pan-cardiomyocyte marker (TNNT2) by qRT-PCR. As shown in Figure 3, with the progression of cardiomyocyte differentiation the expression of MESP1 reached the highest level on day 5 and sharply dropped on day 7 and 10, suggesting that day 5 is equivalent to the developmental cardiac mesoderm stage. Whereas NKX2-5 expression dramatically increased and reached the highest level on day 7 equivalent to the cardiac progenitor stage, and TNNT2 were up-regulated in a time-dependent manner (Figure 3A). These results suggest that it is from day 5 to day 7 during which cells experienced fate conversion from the cardiac mesoderm to the progenitor. Given that the SANLC development originates as early as cardiac mesoderm, the optimal time window of 48 h between day 5 and day 7 was determined to manipulate the SANLC-enriched differentiation process using different signaling pathway modulators. The schematical illustration of SANLC-enriched differentiation protocol based on GiWi method was shown in Figure 4A.

BMP4 has been shown to increase the proportion of SANLC from hiPSC via activating the bone morphogenetic protein (BMP) signaling pathway. To optimize the concentration of BMP4 on the differentiation of hiPSC to the SANLPC, hiPSC was stimulated with different concentrations of BMP4 for 2 days and the expression of SAN markers, SHOX2, TBX18, TBX3 and HCN4 were analyzed at day 16. Consistent with a previous report that lower concentration of BMP4 could increase the proportion of SANLPC from hiPSC, BMP4 at 1.25 ng/ml maximumly increased the expression of the SAN markers, SHOX2, TBX18, TBX3 and HCN4, but not the pan-cardiomyocyte marker, TNNT2 that showed no response at lower concentrations and reached statistical significance only at 5 ng/ml (Figure 3B).
Interestingly, further increase the concentrations of BMP4 tended to concentration-dependently weaken the induction effect of BMP4 on the expression of TBX18, SHOX2 and TBX3 (Figure 3B). 5 ng/ml was therefore chosen as the optimal concentration for BPM4.

NKX2-5-mediated fibroblast growth factor (FGF) signaling has been proposed to contribute to the differentiation of hiPSC to ventricular cardiomyocytes. To optimize the inhibition of FGF signaling pathway on the SANLPC generation, FGF inhibitor, PD was added in the hiPSC and the expression of SHOX2, TBX18, TBX3, HCN4 and TNNT2 were analyzed at day 16. The result showed that PD concentration-dependently up-regulated all the SANLPC markers. As to TNNT2, PD at 450nM significantly up-regulated its expression, which was concentration-dependently inhibited by higher concentrations of PD (Figure 3C). Taken together, our observations indicate that PD at 960 nM is optimal.

Finally, the effect of retinoid acid (RA) signaling on SANLPC differentiation was also investigated at day 16. Treatment of hiPSC with 1μM RA signaling inhibitor, BMS significantly increased the expression of the markers of pacemaker cells, HOX2, TBX18, TBX3, HCN4 and of pan-cardiomyocyte marker, TNNT2 (Figure 3D).

**Combined modulation of the three signaling pathways synergistically promoted the differentiation of hiPSC to SANLC**

Having optimized the conditions of manipulating bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and retinoid acid (RA) signaling pathways individually on the differentiation of hiPSC to SANLC, we tested whether combined usage of the three modulators BMP4, PD, and BMS (They were abbreviated as B, P and M correspondingly in Figure 4B) has any synergistical effect. The scheme of SANLC-enriched differentiation protocol based on GiWi method was shown in Figure 4A, where the detailed information regarding time points for treatment and testing analysis was included. As shown in Figure 4B, BMP4 + PD, BMP4 + BMS, PD + BMS, and BMP4 + PD + BMS (They were abbreviated as BP, BM, PM, BPM correspondingly in Figure 4B)combinations all significantly increased the expression of the markers of pacemaker cells, SHOX2, TBX18, TBX3, and HCN4 and of pan-cardiomyocyte marker TNNT2 compared to those of the GiWi protocol, with the three-combination having the best induction...
efficiency (Figure 4).

**The BMP4 + PD + BMS (BPM) induction protocol leads to biased differentiation of hiPS to SANLC.**

Since the BMP4 + PD + BMS (BPM) induction protocol significantly increased the gene expression of the markers of pacemaker cells, we next evaluated the expression of the aforementioned markers at protein level using IF and flow cytometry on day 21. IF results showed that most nuclei were positive for transcription factors, SHOX2, TBX3 and TBX18 (Figure 5A-C). In agreement, flow cytometry data showed that BPM induction protocol generated a higher percentage of SANLC (defined by cTNT\(^+\)/NKX2.5\(^-\)) (55.1 ± 5%) compared to the traditional GiWi induction protocol (34.1 ± 2%) (Figure 5D). When SANLC was defined by cTNT\(^+\)/SHOX2\(^+\), BPM protocol again generated a higher percentage of SANLC (44.5 ± 2% in the BPM versus 22.4 ± 5% in the GiWi) (Figure 5E). The results of Figure 5D and 5E were further confirmed by IF analysis of double staining for cTNT/NKX2.5 and cTNT/SHOX2 as showed in SUPPL-Figure 1. It seemed that the SAN cells identified by cTNT\(^+\)/NKX2.5\(^-\) and cTNT\(^+\)/SHOX2\(^+\) were more likely to display the similar morphological characteristics indicated by elongated or spindle-like shapes consistent to the previous report.

It may be inferred that enriched SANLC population by BPM resulted in the reduced ratio of other two cardiomyocytes subtype, ventricular and atrial cells. To further evaluate the effect of BPM, the expression of ventricular and atrial related markers was tested using qPCR analysis at day 16. Consistent with our prediction, the levels of both atrial (MYL7 and COUPTFII) (SUPPL-Figure 2A) and ventricular (MYL2 and NKX2.5) (SUPPL-Figure 2B) markers were significantly decreased in BPM group. These results were further supported by IF analysis of COUPTFII and MLC2V whose coding gene is MYL2 (SUPPL-Figure 2C). The markers of another automatic cells subtype, sinoatrial node (SAN) cell was investigated using qPCR analysis at day 16. The results showed that the expression of SAN markers, MSX2 and TBX2 was reduced by BPM (SUPPL-Figure 3).

**SANLC generated by the BPM protocol displays the typical electrophysiological characteristics of pacemaker cells**
To evaluate the automaticity of the SANLC, cells were observed under microscopy and the beating rate was recorded. The representative movies of beating cardiomyocytes as shown in Supp-Vdeo2 (GiWi group) and Supp-Vdeo3 (BPM group) displayed that the beating rate of SANLC was significantly higher in the BPM protocol (n = 6) than the GiWi protocol (n = 6) (Figure 6A). Since hyperpolarization-activated cyclic nucleotide-gated channel (HCN4) contributes the most to the automaticity in SAN, inhibition of the HCN4 would decrease the automaticity of the SANLC. Consistent with our prediction, inhibition of the HCN4 using Zatebradine hydrochloride significantly decreased the beating rate of SANLC from the BPM protocol (n = 7) than the GiWi protocol (n = 6), suggesting a higher percentage of SANLC in the BPM protocol than the GiWi protocol (Figure 6B). We then analyzed the AP using whole cell patch clamp technique 60 days after the differentiation. Based on the morphology of AP, ratio of AP duration at 90% repolarization (APD90) to APD50, upstroke velocity and maximum diastolic potential, SANLC from both groups contained ventricle-like, atrial-like, and pacemaker-like cells (Figure 6C). However, the percentage of pacemaker-like cells was significantly higher in the BPM protocol than the GiWi protocol (Figure 6C).

Different expression profile of the ion channels could account for the different electrophysiological property among SAN, ventricular and atrial cells subtype. To further confirm the SANLC-biased effect of BPM, a series of ion channel genes were checked by qPCR at day 16. In addition to the SANLC markers (SHOX2, TBX18, TBX3, and HCN4) detected (Figure 4 and Figure 5), two other SANLC markers, ISL1 and HCN1, displayed significant increasing by BPM treatment (SUPPL-Figure 4A). The qPCR analysis of potassium ion channels showed that BPM increased the SANLC specific channel genes (KCND2, KCNK2 and KCNN4) but reduced the levels of KCNQ1 specifically for ventricle and KCNJ5 for atria (SUPPL-Figure 4B). Regarding the calcium ion channels, the expression of SANLC channel genes (CACNA1A and CACNB1) were upregulated by BPM contrasting with obvious reduction of CACNA1C, as a ventricular ion channel marker (SUPPL-Figure 4C). Similar result of sodium ion channel was obtained, as evidenced by drastic increased SCN3B that is specific for pacemaker cells and significantly downregulated SCN5A, a ventricular specific ion channel gene (SUPPL-Figure 4D). In addition, the result of testing the gap junction channel related genes showed that BPM treatment
caused remarkable induction of SANLC specific CX30.2. However, expression levels for both CX43 of ventricle and CX40 of atria were dropped (SUPPL-Figure 4D).

Discussion
In this study, using the system of cardiomyocytes differentiation from hPSC by temporal manipulating the canonical Wnt signaling pathway as a model to simulate cardiac development, we discovered that SANLCs can be significantly enriched by simultaneous manipulation of BMP, FGF and RA signaling pathways. These biasedly enriched SANLCs express SAN-specific markers, are sensitive to HCN4 channel blocker, and possess the electrophysiological property of native SAN cardiomyocytes. Biased differentiation of cardiomyocytes to SANLCs could be achieved by either manipulating the expression of SAN-specific transcription factors or using pathway-specific activators/inhibitors. For example, TBX18 is restrictedly expressed in SAN where it promotes the development of pacemaker cardiomyocytes and at the same time prevents the activation of genes leading to chamber cardiomyocyte development. Accordingly, forced expression of TBX18 in hiPSC resulted in increased differentiation to SAN-like cardiomyocytes [21]. In addition, over-expression of TBX18 could convert the human working cardiomyocytes, adult rat bone mesenchymal stem cell and adipose derived stem cells into functional SAN-like cardiomyocytes [22, 23]. However, these methods of genetic manipulation for SAN regeneration are not desirable in future clinical applications. Previous investigations have demonstrated that SAN like cells could be induced from different cell types by gene modification-dependent strategy. In our study, we established a gene-free and chemical-induced method for highly efficient differentiation of SAN like cells from hiPSC, which is more amendable in future clinical use. In a recent study, Protze SI, et al introduced a gene-free method for SAN cells induction from hPSC [24]. Based on the system of embryonic body-based cardiomyocytes differentiation, they showed that modulating the BMP and RA signaling pathway enables highly efficient NKX25-/cTNT+ SAN cells induction (55±5%) indicated by flow cytometry results which is similar to our results (55.1±5%). It suggested that transgene-independent method may serve as a faster, simpler and higher efficient strategy for SAN cells generation. BMP signaling has been reported to participate in the induction of cardiac mesoderm and formation of
the first heart field [17, 25]. Low concentration of recombinant BMP4 could induce cardiac mesoderm specification from hPSC and more importantly, increased the proportion of SAN-like progenitor cells in a time window and in a concentration sensitive manner [8]. In line with these observations, our investigation showed that treatment of hiPSC with low concentration of BMP4 during the cardiac mesodermal stage increased the yields of SAN-like cardiomyocytes. However, increasing the dosage of BMP4 weakened the induced expression of SAN-specific markers.

FGF signaling is indispensable for promoting and maintaining the characteristics of ventricle during the early stage of heart development. Activation of FGF signaling sustains ventricular development in the early stage by maintaining the number of and the electrical characteristics of ventricular cardiomyocytes, while inhibition of FGF signaling results in a gradual accumulation of atrial cardiomyocytes, and a concomitant decreasing number of ventricular cardiomyocytes [26]. Further studies showed that NKX2-5 is the downstream regulator of the FGF signaling [13, 14]. Indeed, our study found that inhibition of FGF signaling downregulated the expression of ventricle specific markers including NKX25, while enhanced the expression of SAN makers.

RA signaling is not only essential for normal heart development but also involved in the differentiation and specification of atrial cardiomyocytes. Previous studies have shown that activation of RA signaling in hPSC differentiation is sufficient to generate cardiomyocytes displaying both electrophysiological characteristics and gene expression profile of atrial cardiomyocytes. However, study by Protze SI et al. showed that activation of RA signaling increases the expression of some markers of SAN, and does not affect the induction efficiency in hPSC differentiation [24]. Our study showed that antagonizing RA signaling significantly increased the expression of SAN-specific markers SHOX2, TBX3 and HCN4 that promoted SAN differentiation. Considering the discovery by Protze SI et al. that timely activation of, and the optimal activation of RA signaling promotes the maturation of SAN, we tend to believe that the biological effect of RA signaling is not just involved in atrial development but also in SAN differentiation, which warrants future investigations. It is worth mentioning that the effect of RA in the SAN development may be in the spatiotemporal dependent manner. In fact, Protze SI et al. showed that the effective time window of RA treatment enhancing pacemaker characteristics is 3-7 days in
the hPSC differentiation [24].

The effect of signal pathway modulators has been generally considered to be sensitive to the concentrations. For example, study by Protze SI, et al showed that BMP4 treatment at the low concentration range (1.25–5 ng/ml) could enhance the differentiation of SANLC from hiPSC [24]. In contrast, higher concentrations significantly inhibited the whole cardiomyocytes differentiation including SANLC [24]. Similarly, our study showed that the induction effect of BMP4 on the expression of SANLC markers gradually weakened when increasing the concentrations (1.25, 2.5, 5 ng/ml) (Figure 3B). One of our interesting observations is that each two combinations (BMP4 + PD, BMP4 + BMS, PD + BMS) seemed not to show the obviously additional effect on the expression of SANLC markers compared to the individual treatment (BMP4, PD, BMS), which is in sharp contrast with three combinations (BMP4 + PD + BMS). It suggests that the cardiomyocytes differentiation from hiPSC simulating the fetal heart development is a very complex process in a highly coordinated spatio-temporal manner, in which the terminal effect of combing different signaling pathways modulators was determined by a number of factors, such as concentration and time window.

**Study Limitation**

The present study has several limitations that is worth mentioning. A new efficient transgene-independent differentiation protocol for generating SANLC from hiPSC via combined modulating BMP, FGF and RA signaling was not completely verified at the protein levels when compared to a broader coverage of the mRNA levels. However, several SANLC specific markers (SHOX2, TBX18 and TBX3) in BPM-caused cells were confirmed by immunocytochemistry (Figure 5A, 5B and 5C). The coded transcription factors by these three marker genes has generally been thought to play the most important role in SAN cells specification, patterning and maturation [12, 24]. The mostly well-recognized double staining for cTNT/NKX2.5 was used to further verify the protein levels of SANLC population by BPM treatment by flow cytometry and immunocytochemistry (Figure 5D, SUPPL-Figure 1) [24, 27], and it was further consolidated by another identification standard of SHOX2/cTNT (Figure 5E, SUPPL-Figure 1). Our in vitro studies demonstrated that BPM-enriched cells display the basic characteristics of SAN cells including higher beating rate, typical action potential and sensitivity to
SAN cell specific HCN4 channel inhibitor. However, the abilities of BPM-enriched cells to engraft within the heart and to drive the working cardiomyocytes at physiological rates in vivo animal model remain to be addressed.

Conclusion
In summary, we have used a developmental-biology-guided approach to establish a transgene-independent highly efficient differentiation protocol for generating SAN-like cardiomyocytes from hPSC. We find that activation of BMP signaling and simultaneous inhibitions of both RA and FGF pathways during cardiac mesoderm stage of hiPSC differentiation lead to a SAN-specific gene expression landscape favoring pacemaker cell specification (Figure 7). This provides a rich source of SAN cardiomyocytes to further study its biology and the potential applications in the treatment of arrhythmia-related disease.

Abbreviations
pluripotent stem cells, PSC; human induced pluripotent stem cell, hiPSC; sinoatrial, SA; sinoatrial node-like cells, SANLC; sick sinus syndrome, SSS; atrioventricular node AVN; bone morphogenetic protein, BMP; fibroblast growth factor, FGF; retinoic acid, RA; immunofluorescence, IF; action potential, AP; GSK3 inhibitor and Wnt inhibitor, GiWi.

Declarations

Acknowledgements
N/A

Author contributions
Guarantor of integrity of entire study: Rui Zhou and Bin Liao. Study concepts: Feng Liu, Rui Zhou, and Bin Liao. Study design: Linli Wang, Rui Zhou, and Bin Liao. Literature research: Feng Liu, Xiaojie Hou, and Yibing Fang. Experimental studies: Feng Liu, Yibing Fang, Xiaojie Hou, Ying Yan, Haiying Xiao, Dongchuan Zuo and Jing Wen. Data acquisition: Feng Liu and Yibing Fang. Data analysis/interpretation: Xiaojie Hou and Ying Yan. Statistical analysis: Ying Yan and Yibing Fang. Manuscript preparation: Feng Liu, Xiaojie Hou, and Rui Zhou. Manuscript editing: Xitong Dang, Zhichao Zhou and Linli Wang. Manuscript revision/review: Xitong Dang, Zhichao Zhou and Rui Zhou. Manuscript final version approval: Rui Zhou and Bin Liao.
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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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Supplementary Figure Legends

SUPPL-Figure 1. Validation of the enriched differentiation of SANLC by the BMP4/PD/BMS (BPM). Representative IF analysis at day 21 showed that both CTNT+/NKX2.5- and CTNT+/SHOX2+ populations representing SANLC accounted for around 50% and 40% of the total cells indicated by DAPI respectively, which is consistent with the corresponding flow cytometry analysis. Scale bars, 100 μm.

SUPPL-Figure 2. Evaluation of the ventricular and atrial working cardiomyocytes in the BMP4/PD/BMS (BPM)-induced SANLC population. (A, B) Compared to the GiWi control, the expressions of specific atrial markers (MYL7 and COUPTFII) (A) and ventricle markers (MYL2 and NKX2.5) (B) were remarkably downregulated in BPM enriched SANLC at day 16 indicated by qPCR.
analysis (t test, * p < 0.05 versus GiWi control, n = 3). (C) Representative IF analysis further showed that quite low fractions of COUPTFIΙ+ and MLC2V+ (corresponding MYL2 gene) population was observed in total cells indicated by DAPI. Scale bars, 100 μm. Expression values of all PCR analyses were normalized to the housekeeping gene GAPDH. Data are presented as ‘Mean ± SD’.

SUPPL-Figure 3. Evaluation of the atrioventricular node (AVN) cells in the BMP4/PD/BMS (BPM)-induced SANLC population. Compared to the GiWi control, the expressions of specific AVN cells specific markers (MSX2 and TBX2) were significantly decreased in BPM group at day 16 as shown by qPCR analysis (t test, * p < 0.05 versus GiWi control, n = 3). Expression values of the PCR analysis was normalized to the housekeeping gene GAPDH. Data are presented as ‘Mean ± SD’.

SUPPL-Figure 4. Evaluation of the expression of ion channel coding genes in the BMP4/PD/BMS (BPM)-induced SANLC population. A series of more other SANLC markers were checked using qPCR analysis 16 days after cell culture. (A) ISL1 (specific SANLC transcription factor) and HCN4 (SANLC specific pacemaker channel) were dramatically increased. (B-D) The expression of potassium, calcium and sodium ion channels distinguishing three types of cardiomyocytes (SANLC, ventricle and atria) were determined. (B) BMP treatment resulted in significant upregulation of SANLC potassium channel genes (KCND2, KCNK2 and KCNN4) in contrast with obvious decrease of ventricle (KCNQ1) and atria channels (KCNJ5). (C) Calcium ion channels related genes (CACNAIA and CACNB1) were increased by BPM while CACNA1C for ventricle was reduced. (D) BPM treatment remarkably increased the expression of SCN3B, SANLC specific sodium channel and decreased the level of SCN5, ventricle sodium channel. (E) Gap junction channel genes specific for SANLC (CX30.2), ventricle (CX43) and atria (CX40) were also tested. (t test, * p < 0.05 and ** p < 0.01 versus GiWi control, n = 3). Expression values of all PCR analysis was normalized to the housekeeping gene GAPDH. Data are presented as ‘Mean ± SD’.

Table
Table1. Primer sets for qRT-PCR analysis.
| Gene     | Direction | Sequence (5'-3')                      | Product size (bp) |
|----------|-----------|---------------------------------------|------------------|
| NANOG    | Forward   | TTTGTGGGCCTGAAGAAAACCT                | 91               |
|          | Reverse   | AGGGCTGCTCCTGAATATAACGA               |                  |
| SOX2     | Forward   | GCCAGAGTGGAAAACCTTTTTGTGA             | 145              |
|          | Reverse   | GGCACCGTGTATCTATATTCTCTCTGTCAA        |                  |
| OCT4     | Forward   | CTTGGTTGATCCTCGAGGACCT               | 121              |
|          | Reverse   | CCAATCGGAGTTGCTCTCCAC                |                  |
| BRACHYURY| Forward   | CAGTGGCAGTCTCAGGTTAAGAGGA            | 122              |
|          | Reverse   | CGCTACTGCAAGGTTGGAAC               |                  |
| MESP1    | Forward   | AGCCCAATGAGCAAGGGCAACT               | 82               |
|          | Reverse   | AAGGAACACTTCCGAAAGGTTCA              |                  |
| NXX2-5   | Forward   | CAGGTGTGCCGCTGCTCTTT                 | 111              |
|          | Reverse   | CAGCTCTTCTCTTTTTCTAGGA               |                  |
| TNNT2    | Forward   | CAGCTTTCTTCTTTCTCGCTCTA             | 157              |
|          | Reverse   | TTATTAATAT GGTTAGGGTTGAGCTGAAG       |                  |
| SHOX2    | Forward   | CAAAGAGGAGATCCGAGAAGGAT              | 122              |
|          | Reverse   | AGTTGGTGCTGTCTCAAAAGGCC             |                  |
| TBX18    | Forward   | GACGATCTTCTCTCTCTCAGA                | 124              |
|          | Reverse   | CTATCTCTAGGCGAGTAAATCTCTCA           |                  |
| TBX3     | Forward   | CCGGCTCTCCACATTTGTAAGA               | 104              |
|          | Reverse   | GTATGACTGCAAGCCTGATGA                |                  |
| HCN4     | Forward   | TGGACACCGCCTTACAAATGTG               | 157              |
|          | Reverse   | CGCGGCAATCTCCCTTAAAGGA               |                  |
| MYL7     | Forward   | AACATCTGCTCTGACCTCAAGG               | 127              |
|          | Reverse   | AACATGCTCTGACCTCAAGG                 |                  |
| MYL2     | Forward   | TGAGAGACACCTTTTTCTGGG                | 139              |
|          | Reverse   | GCCGACCGCCTCCTAACTCTGCG             |                  |
| MSX2     | Forward   | CTTGGTAAAGGCTTCTGGAC                | 133              |
|          | Reverse   | ATATGCTCCTCTACTCTCTGACGCC            |                  |
| TBX2     | Forward   | TACGCTCTGTACGAGGAGAC                | 157              |
|          | Reverse   | TACGACTCTCTCTCAGCTCAAG              |                  |
| ISL1     | Forward   | TCCTATGTGTGGTTGCTGCC                | 194              |
| Cx30.2   | Forward   | CCGAGAACCTTGGCTTGGTA                | 98               |
|          | Reverse   | GACGAAAACCCGCTGACTCAAGG             |                  |
| Cx43     | Forward   | TACAAAAACAGCGAGAGGTT                | 139              |
|          | Reverse   | TGGGGACACACTCTCTTTGGTC              |                  |
| Cx40     | Forward   | AGAGTGTTGAGAAGGACCGGGA              | 70               |
|          | Reverse   | AACAGATGCGAAAACACTCTTGCT             |                  |
| HCN1     | Forward   | GCCATCTGAGCAAGTTGAG                 | 178              |
|          | Reverse   | TCAGCAGGCAATATCTCTCCCA              |                  |
| KCND2    | Forward   | GGTTCTCTGGCTAGAAGTAAGT              | 91               |
|          | Reverse   | GCACCATGTCACATACACCTCA               |                  |
| KCNK2    | Forward   | TGGAACACAGACTCCTCTTGCG              | 150              |
|          | Reverse   | CTGCAGCAACACATCTCTGTGGCA             |                  |
| KCNN4    | Forward   | CCGAGACGGAGGCTGCTGTTAAT             | 81               |
|          | Reverse   | AGCCGATGTCAGGAATGTGG               |                  |
| KCNJ5    | Forward   | CCCACAACAGGGAGAGGTTG               | 170              |
|          | Reverse   | AGCCGATGCTGGGATGTGTT                |                  |
| KCNQ1    | Forward   | GGCGGGAGCTTCAACACCCT               | 169              |
|          | Reverse   | CAGGAGGCTTCCACATCTCCAGAA            |                  |
| CACNA1A  | Forward   | GTCTGGGAAAGAAGTGTCCG               | 151              |
|          | Reverse   | GCTCCTCTATGGCAATCTTTG               |                  |
| CACNB1   | Forward   | CCAGTGCCCAACAGGAGACCG              | 181              |
|          | Reverse   | CGAGTGATGAGAGATCTCGCC               |                  |
| CACNA1C  | Forward   | GACGTGCTGTACTGGGCCA                 | 125              |
|          | Reverse   | AACTCTCGCATTACAGCCACCC             |                  |
| SCN5A    | Forward   | GAGAGGAGTCCAGGGAAC                | 194              |
|          | Reverse   | AACTCTGCTCTTGAGGCCGAC               |                  |
| SCN3B    | Forward   | AGCCATTTCTGTAGGCGCAAGC             | 156              |
|          | Reverse   | CTTCCAGGCCTCTGAAGCT                |                  |

Figures
Characterization of the hiPSC. (A) hiPSC expressed high levels of the pluripotency marker genes, OCT3/4, SOX2 and NANOG, which were comparable to hESC, a human embryonic stem cell (t test, NS not significant, ND not detectable; n = 3). (B) Ki67, Pluripotency markers of hiPSC was identified using IF method. (C-E) Pluripotency markers of hiPSC (NANOG, OCT4, SSEA4 and TRA-1-60) were confirmed by immunoflorescence (IF) assay. (G, H) Representative flow cytometry analysis further confirmed the pluripotency of the hiPSC. Scale bars, 100 μm. Expression values of all PCR analyses were normalized to the housekeeping gene GAPDH. Data are presented as ‘Mean ± SD’.
Figure 2

Characterization of the hiPSC-derived cardiomyocytes. (A, B) CHIR concentration-dependently increased the expression of BRACHYURY (A) and MESP1 (B) in differentiated cells at day 1 and day 3 (t test, *p < 0.05, **p < 0.01 and *** p < 0.001 versus 0 μM or day 1; n = 3). (C) The hiPSC-induced cardiomyocytes expressed cTNT and α-actinin indicated by IF assay in induced cardiomyocytes from day 21 differentiation. (D) Representative plots of flow cytometry displaying high yields of cardiomyocytes derived from hiPSC at day 21. Scale bars, 100 μm. Expression values of all PCR analyses were normalized to the housekeeping gene GAPDH. Data are presented as ‘Mean ± SD’. 
Optimization of the timing and concentration of small molecule chemicals targeting FGF, RA
and BMP signaling pathways for enriched SANLC differentiation. (A) The expression of cardiac mesoderm marker (MESP1), cardiac progenitor marker (NKK2-5) and cardiomyocyte marker (TNNT2) at day 3, 4, 5, 7, and 10, respectively by qRT-PCR (t test, *p < 0.05, **p < 0.01 and *** p < 0.001 versus day 3; n = 3). (B) The expression of SHOX2, TBX18, TBX3, HCN4 and TNNT2 was evaluated by qRT-PCR at day 16 after the differentiation of hiPSC-induced cardiomyocytes was treated with BMP4 at the indicated concentrations at day 5-7 (t test, *p < 0.05, **p < 0.01 and NS not significant versus 0 μM; n = 3). (C) The expression of SHOX2, TBX18, TBX3, HCN4 and TNNT2 was analyzed by qRT-PCR at day 16 of the differentiation after the induced cardiomyocytes were treated with PD at the indicated concentrations at day 5-7 (t test, *p < 0.05, **p < 0.01, *** p < 0.001 and NS not significant versus 0 μM; n = 3). (D) The expression of SHOX2, TBX18, TBX3, HCN4, and TNNT2 was analyzed by qRT-PCR at day 16 of the differentiation after the induced cardiomyocytes were treated with BMS at the indicated concentrations at day 5-7 (t test, *p < 0.05 and **p < 0.01 versus 0 μM; n = 3). Expression values of all PCR analyses were normalized to the housekeeping gene GAPDH. Data are presented as ‘Mean ± SD’. 
The BMP4/PD/BMS (BPM) method promotes the differentiation of hiPSC toward SANLC. (A) Scheme of the GiWi-based protocol used for enriched differentiation of SANLC from hPSC via treatment of BMP4, PD and BMS. Gi, GSK inhibitor (CHIR); Wi, Wnt inhibitor (IWP2); iCM, induced cardiomyocytes. (B) Compared to GiWi control, the levels of the expression of SHOX2, TBX18, TBX3, HCN4, and TNNT2 (from left to right) generally displayed the gradual increasing tendency from the individual treatment of BMP4 (B), PD (P) and BMS (M) to the two combination of BMP4 + PD (BP), BMP4 + BMS (BM) and PD + BMS (PM), and BMP4 + PD + BMS (BPM) caused the highest expression of the markers above as shown by qPCR analysis at day 16 (t test, *p < 0.05, **p < 0.01, *** p < 0.001 and NS not significant versus GiWi control; n = 3). Expression values of all PCR analyses were normalized to the housekeeping gene GAPDH. Data are presented as ‘Mean ± SD’.
Figure 5

Validation of the enriched differentiation of SANLC by the BMP4/PD/BMS (BPM). (A-C) Representative IF analysis showed that SANLCs expressed SAN-specific transcription factor, SHOX2, TBX3 and TBX18. Scale bars, 100 μm. (D) Representative flow cytometry analysis showed that BPM significantly increased the percentage of CTNT+/NKX2.5- cells as compared to the GiWi group (t test, *** p <0.001 versus GiWi control; n = 5). (E) Representative flow cytometry analysis showed that BPM significantly increased the percentage of CTNT+/SHOX2+ cells as compared to the GiWi group (t test, *** p <0.001 versus GiWi control; n = 5). Data are presented as ‘Mean ± SD’.
SANLCs induced by the BMP4/PD/BMS (BPM) possess typical electrophysiological characteristics of SAN. (A) Spontaneous beating frequency of SANLCs was significantly higher in the BPM (n = 6) than the GiWi groups (n = 6) (t test, *** p <0.001 versus GiWi control followed by Bonferroni’s post hoc test). (B) HCN4 channel inhibitor treatment caused significantly decreased beating rate in the BPM (n =7) compared to the GiWi groups (n = 7) (t test, ** p <0.01 versus GiWi control followed by Bonferroni’s post hoc test). (C) Representative ventricule-like, atria-like, and sinus node-like action potential (AP) curves were recorded by whole cell patch clamp (left panel). The percentage of cells with pacemaker-like AP was remarkably increased in the BPM compared to the GiWi group (6/18 vs 2/15, BPM vs GiWi) (t test, *** p <0.001 versus GiWi control). Data are presented as ‘Mean ± SD’.
Graphic abstract. Working model of enriched differentiation of SANLC from hiPSC by the BMP4 + PD + BMS (BPM) treatment versus the GiWi alone method. Simultaneous activation of BMP and inhibition of RA and FGF signaling pathways during cardiac mesoderm stage of the GiWi-induced hiPSC differentiation strongly favors a SAN-specific gene transcriptional landscape leading to enhanced pacemaker cell specification.

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