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Temporal and contextual orchestration of cardiac fate by WNT-BMP synergy and threshold

Mahesh K. Verma, Nibedita Lenka*

National Centre for Cell Science, Ganeshkhind, Pune, Maharashtra, India

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Abstract

Cardiomyogenic development proceeds with a cascade of intricate signalling events that operate in a temporo-spatial fashion to specify cardiac cell fate during early embryogenesis. In fact, conflicting reports exist regarding the role of Wnt/β-catenin signalling during cardiomyogenesis. Here, we describe a dose-dependent and temporal effect of Wnt/β-catenin signalling on *in vitro* cardiomyogenesis using embryonic stem cells (ESCs) as a model system. We could demonstrate that canonical Wnt activation during early stage of differentiation either through ligand or by GSK3β inhibition helped in maintaining Oct4 and Nanog expressions, and in parallel, it promoted mesoderm and endoderm inductions. In contrast, it led to attenuation in cardiomyogenesis that was reversed by moderate concentration of DKK1, but not soluble Fz8. However, higher DKK1 could also block cardiomyogenesis, suggesting thereby governance of a particular signalling threshold underlying this developmental event. Interestingly, Wnt signalling activation at early stage modulated BMP4 expression in a stage-specific manner. Wnt activation, synchronized with BMP4 and brachyury up-regulation at early stage, correlated well with mesoderm induction. Conversely, Wnt activation led to BMP4 and Wnt5a down-regulation at late stage culminating in cardiomyogenic attenuation. Our findings suggested the existence of precise regulatory machinery with context-dependent role of Wnt for fine tuning mesoderm induction and its derivatives, through establishment of Wnt gradient during ESCs' differentiation. Moreover, contrary to mere activation/inhibition, a specific threshold of Wnt and BMP and their synergy seemed necessary for providing the guiding cues in orchestrating mesoderm induction and subsequent cardiomyogenesis.

Keywords: ES cells • Wnt signalling • BMP • cardiomyogenesis • mesoderm • cell fate

Introduction

Heart is the first functional organ during development comprising of heterogeneous cell types. Highly complex and tightly regulated cardiogenesis begins with commitment of cells to cardiac fate at early gastrulation. Needless to say, this involves a cascade of intricate signalling events that operate in a temporo-spatial fashion to specify cardiac cell fate during early embryogenesis. Hence, efforts are ongoing to better the understanding of cardiac development, explore the factors regulating it and to chalk out strategies for their enrichment *in vitro* with the ultimate implication in cell replacement therapy in mind. In fact, endoderm signals overlying mesoderm and the crosstalk among various factors specifies cardiac cell fate. Although a number of reports [1–5] indicate indispensable roles of BMP and FGF signalling during heart development, Wnt signalling remains contentious due to its both positive and negative influences [6]. While wingless protein, the mammalian equivalent of Wnt, is required for heart development in flies [7], Wnt proteins in vertebrates are known to inhibit cardiogenesis in heart field with its inhibition leading to cardiac induction [8–10]. Interestingly, Wnt signalling is also required for early mesoderm specification and development [11–15] that further yields mesoderm-derived tissues including heart. However, endogenous Wnt antagonists are known to be secreted from the organizer or anterior endoderm and are required for heart field specification [16, 17]. In fact, mutant phenotype of beta-catenin (β-catenin), the effecter molecule of canonical Wnt signalling cascade, was proven to be lethal at egg cylinder stage [18]. Thus, Wnt influence on cardiogenesis *in vivo* has remained quite paradoxical due to these conflicting reports.

*Correspondence to: Nibedita LENKA,
National Centre for Cell Science, Ganeshkhind,
Pune - 411007, Maharashtra, India.
Tel.: +91-20-2570-8112
Fax: +91-20-2569-2259
E-mail: nibedita@nccs.res.in, nibedital@yahoo.com

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Wnt is a secreted glycoprotein that follows both canonical and non-canonical pathways. During canonical signalling activation, Wnt binds to its receptor, Frizzled (Fz), thereby activating Dsh (Dishevelled) protein. The activated Dsh prevents GSK3β to phosphorylate β-catenin and hence leading to the accumulation of the latter in the cytoplasm, which then translocates to the nucleus and binds to the TCF/LEF transcription factors to transcribe the downstream target genes [19]. In the absence of Wnt signalling, β-catenin remains associated with a cytoplasmic complex containing CK1α, GSK3β, Axin and APC protein, following which β-catenin gets phosphorylated by GSK3β and undergoes degradation. Thus, nuclear localization of β-catenin designates the activated state of Wnt signalling. Wnt also posits a pivotal influence on early cardiogenesis *in vitro*. Embryonic stem cells (ESCs) with their unique potential of pluripotency are widely used to study early developmental proceedings *in vitro*. Maintenance and differentiation of these ESCs require specific intricate network of signalling events and ensuing levels designate these cells to opt between self-renewal and differentiation. In fact, a number of investigators have reported ESCs' differentiation into cardiomyocytes [20–23] and notable ambiguities do exist regarding Wnt influence during the same. The canonical Wnt pathway was seen having a positive influence during cardiac differentiation of pluripotent embryonal carcinoma (EC) cells [24]. While, Naito *et al*. [25] and Kwon *et al*. [26] demonstrated a temporal influence of Wnt signalling promoting cardiomyogenesis in ESCs, Liu *et al*. [27] described the opposite, showing inhibition of β-catenin by Sox17 leading to cardiomyogenic induction. Moreover, non-canonical Wnt signalling by Wnt11 was reported to induce cardiogenesis both *in vivo* and *in vitro* [28–30]. Nonetheless, it remained unclear whether Wnt signalling had a cell autonomous function in committing undifferentiated cells to adopt a cardiac fate, or it induced other cell types for non-cell autonomous signal transductions in the heart field, for cardiogenesis to occur. Hence, Wnt signalling during *in vitro* cardiomyogenesis has remained quite contentious too.

In this report, we could delineate Wnt influence on cardiomyogenic differentiation from ESCs, where activation of canonical Wnt signalling inhibited cardiac differentiation in a temporal and dose-dependent fashion. However, inhibition of Wnt signalling displayed a contrasting influence depending on the inhibitor type and concentration used. Interestingly, up-regulation in BMP4 and brachyury expressions at early stage of differentiation in response to canonical Wnt activation correlated well with mesoderm induction. However, it inhibited BMP4 and Wnt5a at late stage, corresponding with cardiomyogenic abrogation. Incidentally, non-canonical Wnt5a enhanced cardiomyogenesis at early time-point during differentiation. Hence, our investigation delineated an interesting paradigm of temporal Wnt-BMP4 crosstalk during differentiation. This multifaceted scenario also underscored the fact that Wnt activity at specific threshold might underlie cell fate decision machinery, directing the cells to opt between retention of undifferentiated state and acquiring specific cell fates in a context-dependent manner.

Materials and methods

Plasmid construction, transfection

Mouse beta-myosin heavy chain (βMHC) promoter (7.0 kb) was sub-cloned into pEYFP1 vector (Clontech, Mountain View, CA, USA) at SacI-SmaI sites following its excision from βMHC-PBS construct (a kind gift from Dr. Robbins) using SacI-HpaI restriction sites. About 10 μg of this DNA was linearized and the ESC line D3 (2×10^6 cells) was transfected using the same by nucleofection (Amaxa, Cologne, Germany). The G418-resistant stable clones designated as βMHC-EYFP were selected following the protocol [31].

ESC culture and differentiation into cardiomyocytes

The D3 line ESCs and βMHC-EYFP transgenic clones were maintained in culture using DMEM with leukaemia inhibitory factor (LIF- 1000 U/ml), L-glutamine, penicillin-streptomycin, non-essential amino acids and β-mercapto-ethanol as described [32] and were passaged every 48 hr. Differentiation of ESCs into cardiomyocytes was induced by generating embryoid bodies (EBs) in hanging drop (500 cells/20 μl drop) for 2 days (d0–2) [31] followed by suspension culture for 3 days (d2–5) using non-adhesive dishes. Medium used for differentiation was same as maintenance medium barring LIF. EBs were plated on gelatin-coated tissue culture dishes (24-well dish; 1EB/ well) on d5 and were monitored at various stages of development. Cardiomyogenic differentiation efficacy at different stages of development was discerned by monitoring EYFP expression in live culture using the inverted fluorescent microscope (Nikon, Japan), and counting (*i*) number of beating EBs, (*ii*) beating clusters/EB and (*iii*) area showing pulsation or EYFP expression during differentiation, staring from day 3 of plating (d8). $EYFP^+$ cardiomyocytes were also quantified by flowcytometry (FACS Calibur, Becton Dickinson, Singapore) using the standard protocol, as described [32]. Beating clusters/EB were represented either in fold difference to control keeping the count in control as 1 or in absolute numbers. Images were captured by DXM1200 camera and the $EYFP^+$ areas were determined using IPP software (MediaCybernetics, Bethesda, MD, USA). The area was calculated by marking individually all the $EYFP^+$ regions present in a single EB and pooling the total fluorescent positive areas/EB in a set and calculating the mean $EYFP^+$ area/set by considering all the EBs in a set.

Influence of Wnt signalling on cardiomyogenesis

Conditioned media were prepared from control (L cells; CCM), L-Wnt3a (W3CM) and L-Wnt5a (W5CM) fibroblast cells as per supplier's instructions (ATCC, Manassas, VA, USA). To study the effect of Wnt signalling on cardiac differentiation, CCM/W3CM was added to culture medium at a final concentration of 10% v/v at different time-points. BIO (6-Bromoindirubine-3'-oxime; Calbiochem, La Jolla, CA, USA) [33], recombinant Wnts (rWnt3a, rWnt5a), DKK1 and Fz8 (R&D, Minneapolis, MN, USA) were added as indicated. DMSO served as the vehicle control for BIO with its concentration ranging 0.1% and 1% depending on the BIO concentration (1 and 10 μM, respectively) used. To study early differentiation in monolayer culture, 500 cells/well were plated on 24 multi-well dishes and maintained without LIF and with either DMSO or BIO till 6 days. To investigate the effect of BMP signalling on cardiomyogenesis whether in concert with or independent of

Wnt, either exogenous BMP2 or BMP4 were added to the medium or the endogenous BMP was inhibited using either Chordin/Noggin or anti-BMP4 blocking antibody both in the presence and absence of Wnt modulators.

Immunocytochemistry

ESCs or EBs grown either in suspension or on gelatin-coated glass coverslips were used for immunocytochemistry following the standard protocol [32]. Antibodies used were β-catenin, Oct4, Bry, Troponin-T, Nkx2–5 (all from SantaCruz, Santa Cruz, CA, USA), α-actinin (Sigma, St Louis, MO, USA) and the corresponding secondary antibodies coupled with Cy2/Cy3/Cy5 (Chemicon, Temecula, CA, USA). Samples were processed for negative control by substituting primary antibodies with FBS. Cells were observed under the confocal microscope (LSM 510, Zeiss, Germany) to verify immunostained pattern.

RNA isolation and RT-PCR

Total RNA was isolated from ESCs and EBs at different stages using TRIzol reagent (Invitrogen), following the manufacturers protocol. The residual genomic DNA was removed by DNAse treatment (RQ1-DNAse, Promega, Madison, WI, USA), followed by acid-phenol: chloroform (Ambion, Austin, TX, USA) extraction. About 3 μg of total RNA was used for first strand cDNA synthesis using oligo dT primers and SuperScript II reverse transcriptase (Invitrogen, New York, NY, USA). While 2 μl of reaction (1:10 diluted) was used for PCR, 5 μl of the same was used for real time quantitation (qPCR) using SYBR-Green super mix (Bio-Rad, NSW, Australia). The data were normalized to β-actin and represented as fold of control that was set as 1. The primer sequences used are listed in Table S1.

TCF-dependent luciferase activity

ESCs were transfected with Super8X-TOP or -FOP flash vectors (kind gift from Dr. Moon) (2 μg) and various treatments were given as indicated. Cells were harvested 72 hrs post-transfection and luciferase activity was measured by LucLite luciferase assay (Perkin Elmer, Boston, MA, USA), by analysing the ratio between TOP and FOP-flash activities.

Statistical analysis

All data were presented as mean ± S.E.M. with each set of experiment being repeated 3–9 times. Statistical significance was calculated using t-test (Sigma plot) and represented as ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$.

Results

Generation of stable ESC clones and cardiac differentiation

A number of stable ESC clones (βMHC-EYFP) were established harbouring EYFP reporter gene under the regulatory control of the cardiac-specific βMHC gene promoter [34] (Fig. 1A). This, in fact, facilitated demarcating 'live', the cardiomyocytes among the heterogeneous cell types generated during ESCs' differentiation. These clones were successfully differentiated into cardiomyocytes as monitored by spontaneous pulsating activity, the indicator of initiation of cardiomyogenic differentiation. EYFP expression was first detected in 7–8 days old EBs soon before pulsation initiation (Fig. 1B), and was not seen in undifferentiated ESCs. In fact, the fluorescence increased as differentiation proceeded and remained confined only to beating areas (Fig. 1B). The cardiomyocyte-specific EYFP expression was authenticated by immuno-cytochemical characterizations (Fig. 1C). Further, the cardiac differentiation efficiency was quantitated by counting number of beating clusters/EB and percentage of EBs that were beating (Fig. 1D and E). While almost all EBs showed pulsation by d10 of differentiation, beating clusters/EB increased with days analysed (1.3 ± 0.2, 4.1 ± 0.3, 5.4 ± 0.3 at d8, d10 and d13, respectively).

Influence of canonical Wnt signalling activation on cardiac differentiation

Initially we intended to discern endogenous canonical Wnt signalling status by monitoring the β-catenin expression pattern during differentiation of ESCs to cardiomyocytes. Since, a conclusive picture on nuclear localization of β-catenin could not be discerned by immunostaining (Fig. S1A), we resorted to either activating or inhibiting Wnt signalling during various stages of differentiation and investigated its influence on cardiac differentiation from ESCs *in vitro*.

Inhibition of GSK3β by BIO increased β-catenin levels in cytoplasm/nucleus (Fig. 2A) and hence, signalled the activated state of canonical Wnt signalling. In fact, BIO treatment during differentiation initiation stage (d0–2) resulted in a dose-dependent attenuation in cardiac differentiation (Fig. 2B and C). While with 100 nM BIO only number of beating clusters/EB was decreased (0.76 ± 0.04 fold relative to DMSO control), with 1 μM BIO, both percentage EBs beating (46.6 ± 0.9% of DMSO control) and number of beating clusters/EB (0.28 ± 0.08 fold) were decreased at d10 of differentiation. Interestingly, a marginal enhancement in cardiac differentiation was seen in 10 μM BIO treated EBs (0.45 ± 0.08 fold in beating clusters; 63.9 ± 7.3% beating EBs) instead of further attenuation compared to the 1 μM treatment, indicating the attainment of saturation. Nevertheless, overall cardiac differentiation was inhibited at all concentrations of BIO used compared to DMSO control (Fig. 2B and C). BIO treatment also decreased $EYFP^+$ areas, when used at both 1 and 10 μM concentrations (Fig. 2D). We further examined its temporal effect by adding BIO at various time-points during differentiation. BIO decreased cardiac differentiation at all the time-points studied and its effect was most detrimental at d2–5 differentiation regimen and also with continuous (d0–10) exposure, where not a single EB showed pulsation when used at both 1 (Fig. 2E and F) and 10 μM (data not shown) concentrations. To rule out the possibility of BIO inhibiting only rhythmic contractions without affecting cardiac differentiation, EYFP expression in these EBs was monitored at different

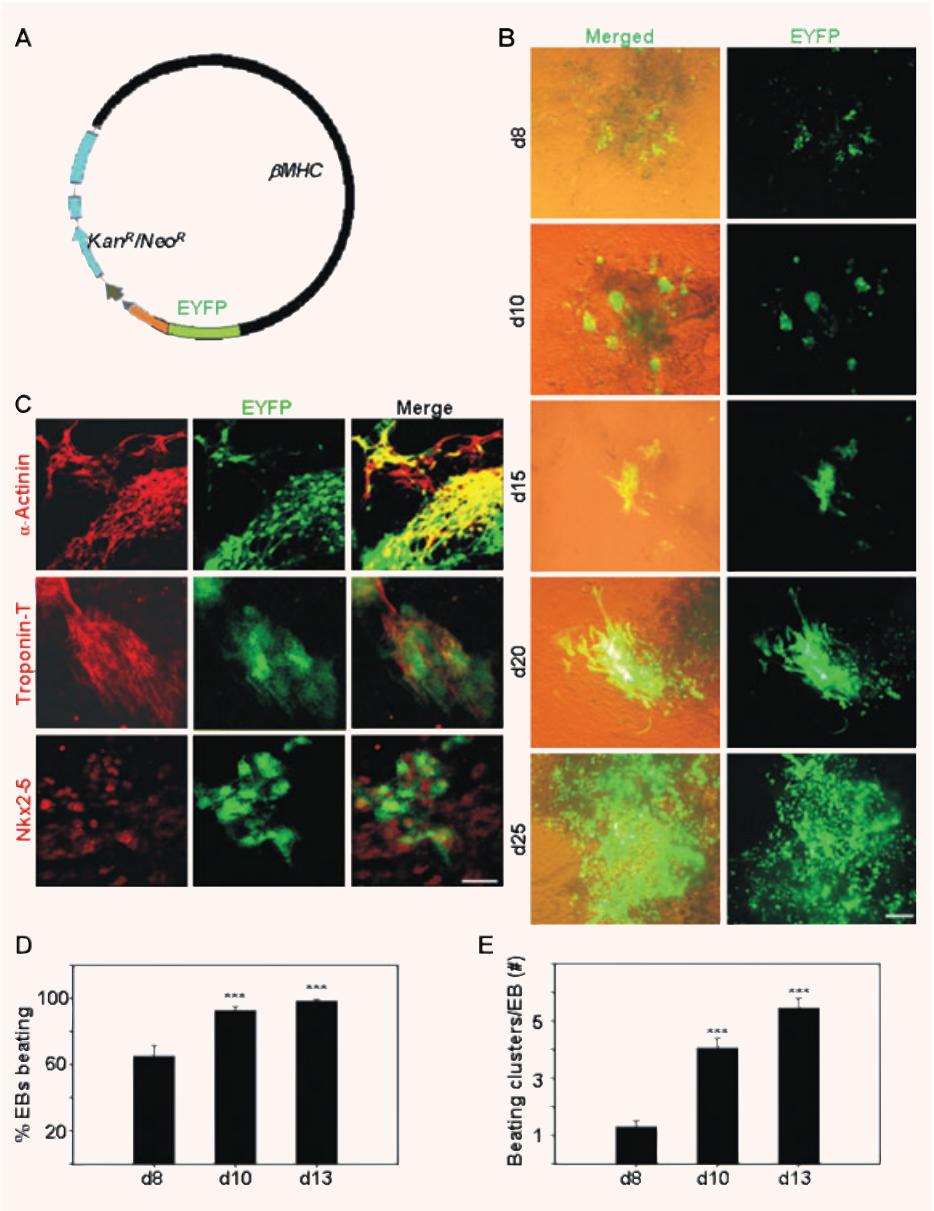

Fig. 1 Generation and characterizations of βMHC-EYFP clone. (**A**) Vector construct used to generate stable ESCs clones to demarcate cardiomyocytes expressing βMHC promoter driven EYFP during differentiation. (**B**) EYFP expression profile during differentiation revealed EYFP expression commencement from d8 onwards in differentiating EBs. (**C**) Immuno-cytochemical analysis during differentiation and authentication of cardiac-specific EYFP expression. EYFP positive cells showed co-localization with endogenous cardiac markers; α-actinin, Troponin-T and Nkx2–5, thereby specifying those as cardiomyocytes. (**D, E**) Quantitation of cardiac differentiation from ESCs by counting percentage of EBs beating (**D**) and beating clusters/EB (**E**) at the indicated time-points. $n = 5–8$: mean ± S.E.M. Scale: 100 μm (B), 50 μm (C).

time-points (Fig. S1B). In line with inhibition in pulsating activity, either less (d0–2 and d5–8) or no (d2–5 and continuous) $EYFP^+$ cells were detected in EBs treated with BIO. A radical decrease in cardiac differentiation markers and transcription factors was also seen in d0–2 BIO-treated EBs compared to DMSO control, when analysed at both cardiac commitment stage (d5; Fig. 2G) and later (d10; Fig. 2H). However, ESC-specific markers, Oct4 and Nanog, were seen up-regulated in those (Fig. S1C and D). This interesting fate modulation by BIO suggested Wnt signalling operating in a temporal fashion, with early time-point (d0–5) remaining more susceptible to BIO-induced abrogation of cardiac differentiation.

Since, GSK3β has a pleiotropic role [35] and BIO blocks GSK3β activity, further confirmation of our findings was attempted by activation of the Wnt signalling by Wnt ligand itself. Accordingly, conditioned medium, W3CM, was opted to activate canonical Wnt and CCM was used in parallel as control. Cells with W3CM but not CCM exhibited increased levels of nuclear β-catenin (Fig. 2A), thereby authenticating its usage in the activation of Wnt/β-catenin signalling. Accordingly, W3CM was added at different time-points during cardiac differentiation to assess its temporal effect. A rare occurrence in pulsating activity, associated with decreased EYFP expression in EBs, marked the cardiomyogenic

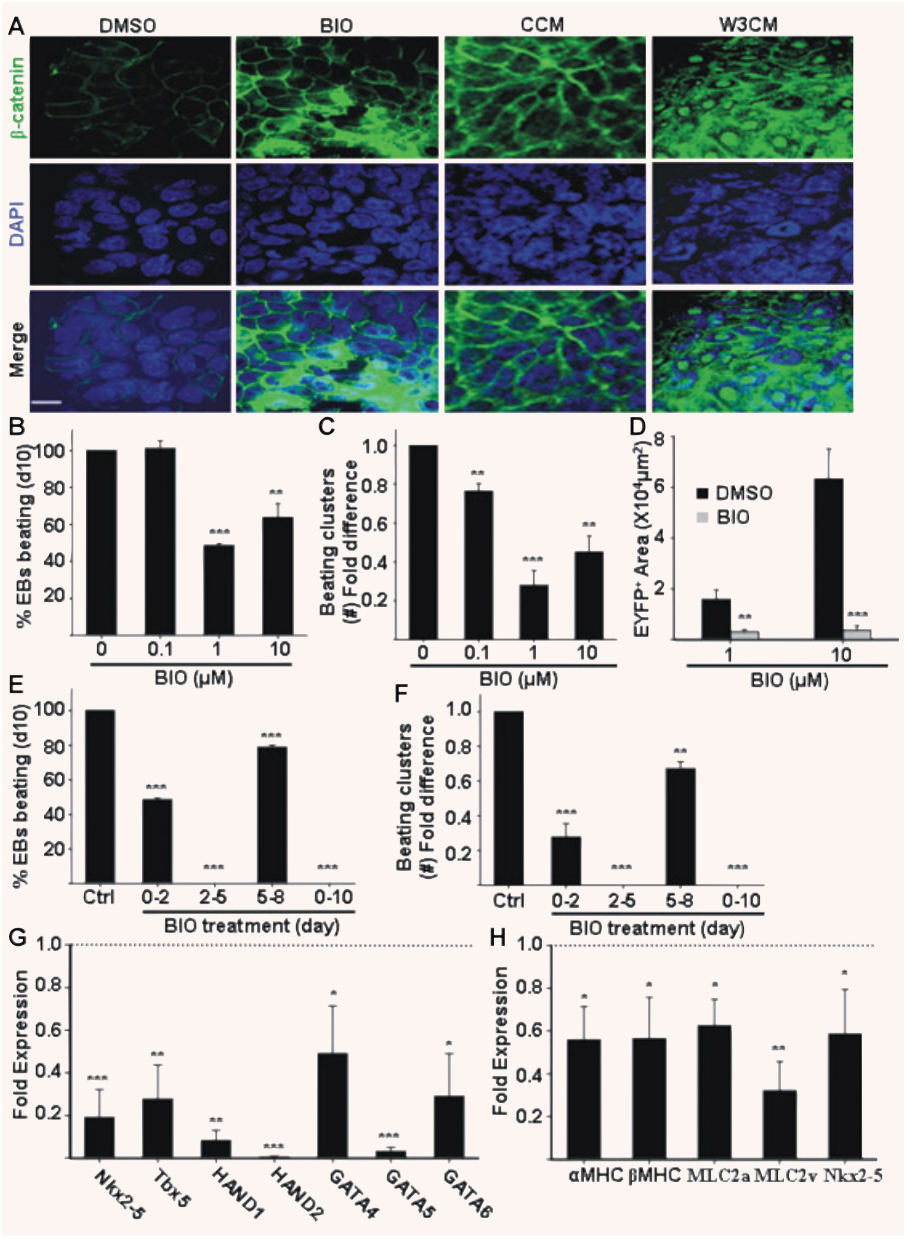

Fig. 2 Authentication of canonical Wnt activation and inhibition of cardiac differentiation upon BIO exposure. (**A**) Increase in the immunopositivity of β-catenin upon BIO and W3CM treatment (d0–2) in d2 EBs indicated the activation of canonical Wnt/β-catenin signalling. DAPI (blue) was used for nuclear staining (Scale: 20μM). (**B, C**) Dose-dependent effect of BIO during cardiac differentiation was monitored at d10 by counting percentage EBs beating (**B**) and beating clusters/EB (**C**). BIO exposure at 1 uM resulted in attenuation in cardiac differentiation. (**D**) EBs showed reduced $EYFP^+$ area with BIO exposure during d0–2 time period compared to vehicle control. (**E, F**) BIO also exerted its effect in a temporal fashion by decreasing the percentage EBs beating (**E**), and the beating clusters/EB (**F**) at the indicated time-points compared to control (Ctrl). (**G, H**) qPCR analysis of cardiac marker expression in EBs at d5 (G; BIO: 10 μM) and d10 (H; BIO: 1 μM) of differentiation, dotted line indicating the control value set as 1. $n = 3$–4: mean ± S.E.M.

blockade upon W3CM treatment during the initiation of differentiation (d0–2), the stage corresponding to the generation of EBs in hanging drops (Fig. 3A–C). However, no appreciable difference in cardiac differentiation was noted in EBs treated during d2–5 period (Fig. 3A and B) unlike that with LiCl (data not shown) or BIO. While sustained activation of canonical Wnt signalling by continuous exposure to W3CM completely abrogated cardiomyogenesis without any beating EBs or EYFP expression, cardiac differentiation remained unaltered with CCM (Fig. 3A–C). The observation was further authenticated by using rWnt3a protein (Fig. 4A–G). Consistent to the findings with W3CM, rWnt3a treatment at d0–2 time period blocked cardiac differentiation in a dose-dependent manner and 50 ng/ml showed maximum abrogation among treatments (beating clusters 0.45 ± 0.07 fold; beating EBs 73.0 ± 4.5%) (Fig. 4A and B). While 1 ng/ml recombinant protein had no effect on cardiac differentiation compared to the control, 10 ng/ml attenuated it (0.68 ± 0.04 fold) without changing the number of EBs beating (95.8 ± 2.9%). Interestingly, rWnt3a could also hinder cardiac differentiation (beating clusters 0.49 ± 0.04 fold; beating EBs 82 ± 3.17%) at d2–5 time-point similar to BIO

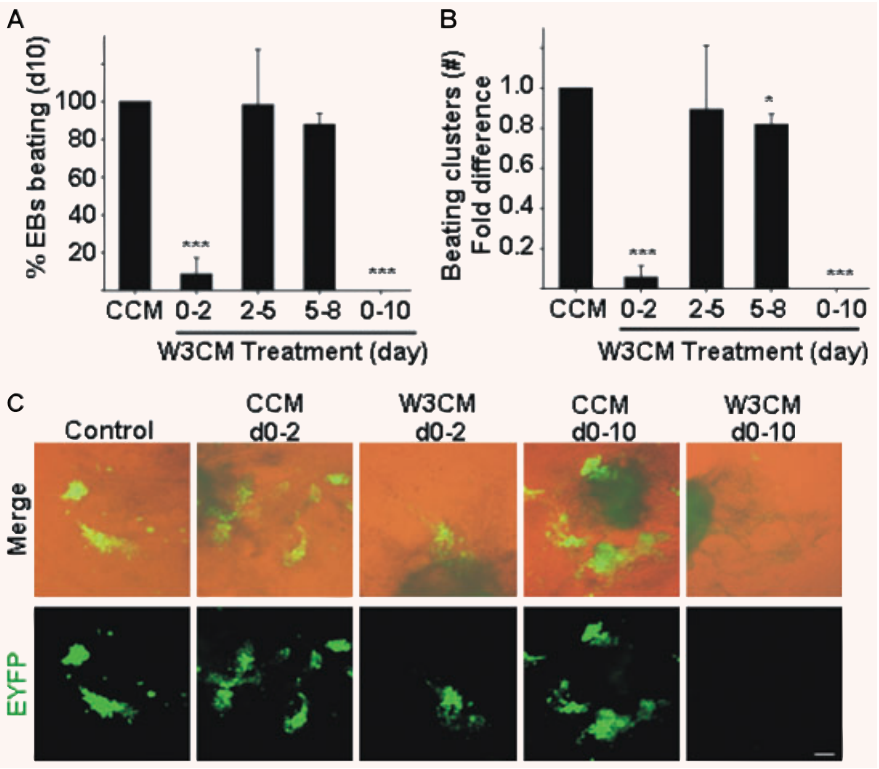

Fig. 3 Canonical Wnt signalling and inhibition of cardiac differentiation (d10). (**A, B**) W3CM inhibited cardiomyogenesis in a temporal fashion as quantified by counting percentage EBs beating (**A**) and beating clusters/EB (**B**). (**C**) In W3CM-treated EBs during d0–2, EYFP expression was less compared to CCM and it was further reduced in EBs with continuous treatment. $n = 3$: mean ± S.E.M. Scale: 100 μm (**C**).

(Fig. 4C and D). However, it did not alter the same during d5–8 treatment. There was also pronounced decrease in $EYFP^+$ population in treated EBs (Fig. 4E–G). Together, these data clearly suggested that canonical Wnt signalling that acted through β-catenin could inhibit ESCs' differentiation into cardiomyocytes and the critical time window of action resided early during differentiation.

Non-canonical Wnt signalling and cardiac differentiation

Since canonical Wnt signalling was inhibitory, it became imperative for us to investigate whether or not non-canonical Wnt has similar/opposite influence during cardiomyogenesis. Accordingly, Wnt5a that acts generally as a non-canonical Wnt ligand was used to study its role during cardiac differentiation. Contrary to Wnt3a, rWnt5a (200 ng/ml) did not have any appreciable influence at any of the time-points studied except d0–2 (Fig. 5A–E). The morphology of EBs also remained comparable in both control and treated groups at d5 (data not shown). Although no difference was seen in percentage EBs beating, the increase in beating clusters/EB (1.45 ± 0.09 fold), the $EYFP^+$ area as well as the percentage of $EYFP^+$ cells (Fig. 5B–E) suggested a positive influence of Wnt5a on cardiac differentiation during d0–2 time regimen. Thus, non-canonical Wnt5a might be effective only during early stage of cardiomyogenesis from ESCs *in vitro*.

Specific activation of the TCF-dependent luciferase activity

To validate the authenticity of Wnt activation and its repercussion on cardiomyogenesis, the reporter activity was assessed using Super8X-TOP-flash system [36]. The activated β-catenin binds to TCF in the nucleus for the transcriptional activation of its target genes. While Super8X-TOP-flash contains eight TCF binding sites upstream of luciferase coding sequence, Super8X-FOP-flash contains the mutated sites in the consensus sequence. The W3CM but not W5CM could enhance the luciferase activity when measured after 72 hrs post-transfection (Fig. 5F). Among all, BIO was found to be the most potent one imparting maximum luciferase activity. These results unequivocally deciphered the specificity of canonical Wnt signalling activation, conferred through its ligands or through GSK3β inhibitor used during the investigation.

Inhibition of the Wnt signalling and cardiomyogenesis

To further validate our observation, we inhibited Wnt signalling by DKK1 [37] and Fz8 (soluble Fz receptor) and observed, whether the effect described with Wnt activation would be reversed. While DKK1 is believed to inhibit only canonical Wnt signalling, FZ8

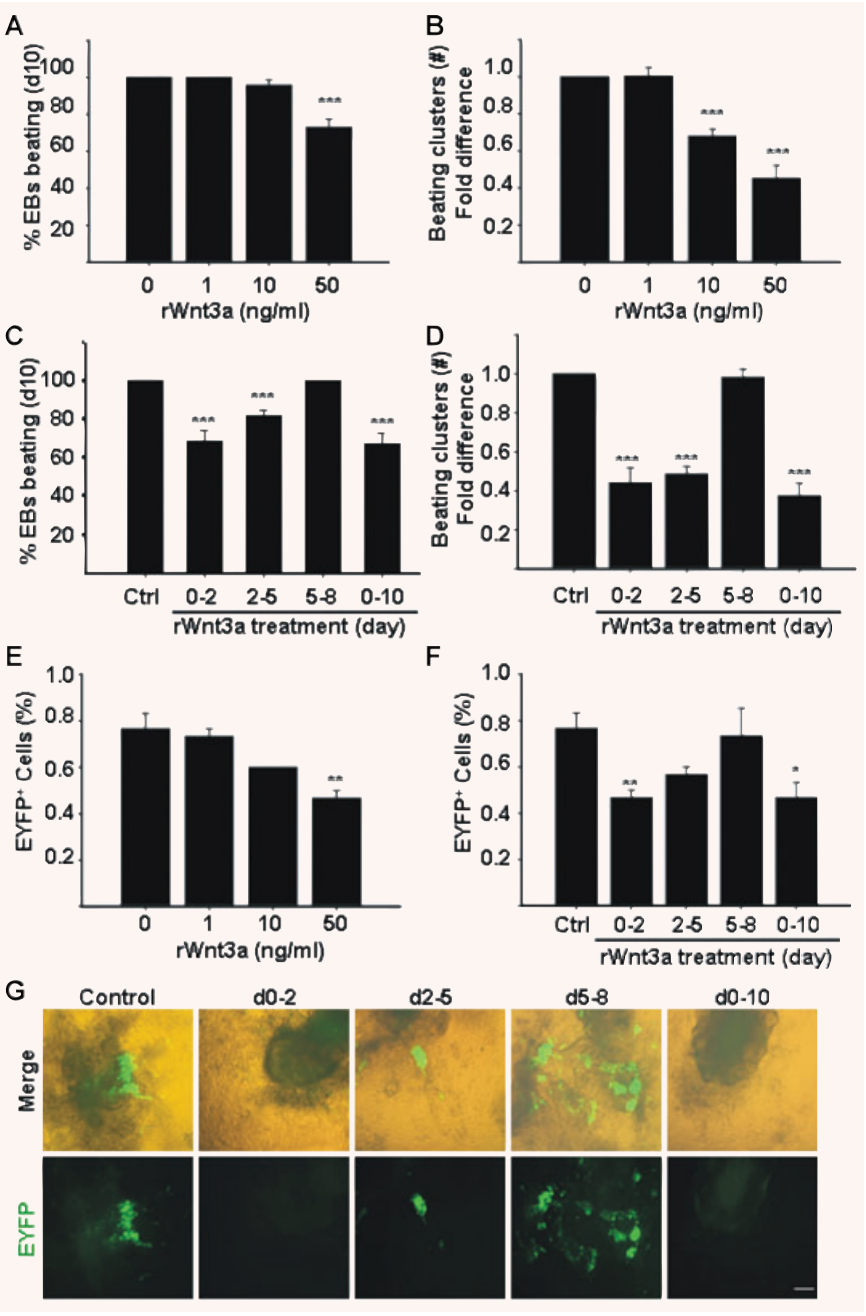

Fig. 4 Canonical Wnt signalling inhibits cardiac differentiation (d10) in a dose-dependent and temporal fashion. (**A, B**) rWnt3a (d0–2) blocked cardiac differentiation from ESCs in a dose-dependent manner with 50 ng/ml showing maximum reduction among treatments. (**C, D**) EBs at early stages and with continuous exposure were more sensitive to rWnt3a (50 ng/ml) block compared to control (Ctrl). (**E, F**) Flowcytometry quantification revealed reduction in EYFP$^+$ cardiomyocytes following rWnt3a treatment in a concentration-dependent manner (**E**, d0–2) as well as in a temporal fashion (**F**), when treated (50 ng/ml) at various time regimens. (**G**) Live microscopic monitoring of EYFP expressing cardiomyocytes with or without rWnt3a exposure at various time-points during differentiation. Scale: 100 μm. $n = 3$–6: mean ± S.E.M.

binds to all Wnts with different affinity due to its conserved cysteine-rich domain inhibiting both canonical and non-canonical signalling. Strikingly though, we could monitor a decrease in cardiac differentiation (76.3 ± 3.8% beating EBs and 0.47 ± 0.09 fold beating clusters) after Fz8 (200 ng/ml) treatment during d0–2 time-point (Fig. 6A–C and H), and with no difference at lower Fz8 concentrations used (data not shown). A decrease in cardiac differentiation was also evident after continuous treatment (75.7 ± 4.9% beating EBs and 0.72±0.14 fold beating clusters). However, ESCs differentiated in presence of DKK1 showed a dose-dependent contrasting effect (Fig. 6D–H). Since control showed 100% of the EBs beating (Fig. 6D), DKK1 influence on differentiation was evident by counting beating clusters/EB (Fig. 6E), assessing the area encompassed demarcated by EYFP expression (Fig. 6F and G) and quantitating the EYFP$^+$ cells (Fig. 6H). DKK1, when used at moderate (50ng/ml) concentration during d0–2 time-point, led to

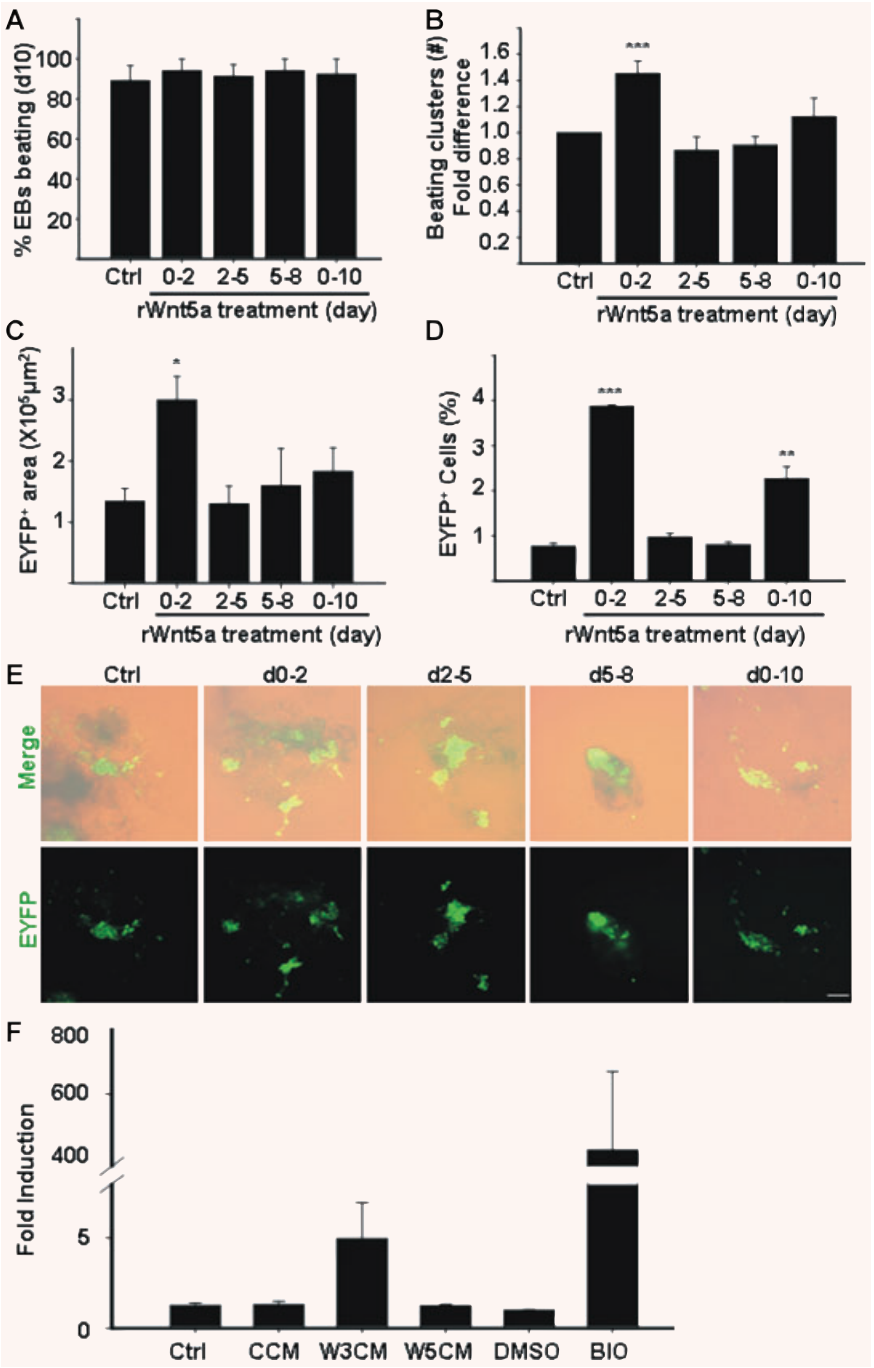

Fig. 5 Non-canonical Wnt signalling and cardiac differentiation (d10). (**A–E**) rWnt5a showed temporal influence on cardiac differentiation. While no difference was noted in percentage EBs beating with rWnt5a exposure during various time-points of differentiation compared to control (**A**), increase in beating clusters/EB was evident only with d0–2 exposure (**B**). (**C**) EYFP expression in rWnt5a-treated EBs remained unaltered irrespective of the treatment regimen except at d0–2, where it showed an increase in $EYFP^+$ area. (**D**) Flowcytometry analysis also revealed significant increase in $EYFP^+$ cells following rWnt5a treatment during d0–2 as well as with continuous exposure. (**E**) Live monitoring of EYFP expressing cardiomyocytes with or without rWnt5a exposure at various time-points during differentiation. (**F**) W3CM but not CCM could increase the luciferase activity, whereas with non-canonical Wnt5a conditioned medium (W5CM), it was comparable to control (ctrl). Treatment with BIO (1 μM) could also drastically enhance the luciferase activity compared to DMSO control. $n = 3–6$ mean ± S.E.M. (Scale: 100 μm in E).

a significant increase in cardiac differentiation (1.46 ± 0.09 fold beating clusters/EB) accompanied by an increase in $EYFP^+$ cells and areas. However, it had an opposite influence at higher concentration (500 ng/ml; 0.56 ± 0.06 fold beating clusters/EB) compared to control, thus suggesting a threshold effect. Supporting evidences were also obtained by quantifying the transcripts of cardiac-specific cytoskeletal proteins (α/βMHC, MLC2a, MLC2v, etc.) that showed enhanced expression with DKK1 at 50 ng/ml and reduction at 500 ng/ml (Fig. S1E). Continuous exposure of DKK1 (50 ng/ml) could also enhance cardiomyogenesis (data not shown). Thus, blocking of canonical Wnt signalling to a specific level might promote cardiomyogenesis from ESCs. Additionally,

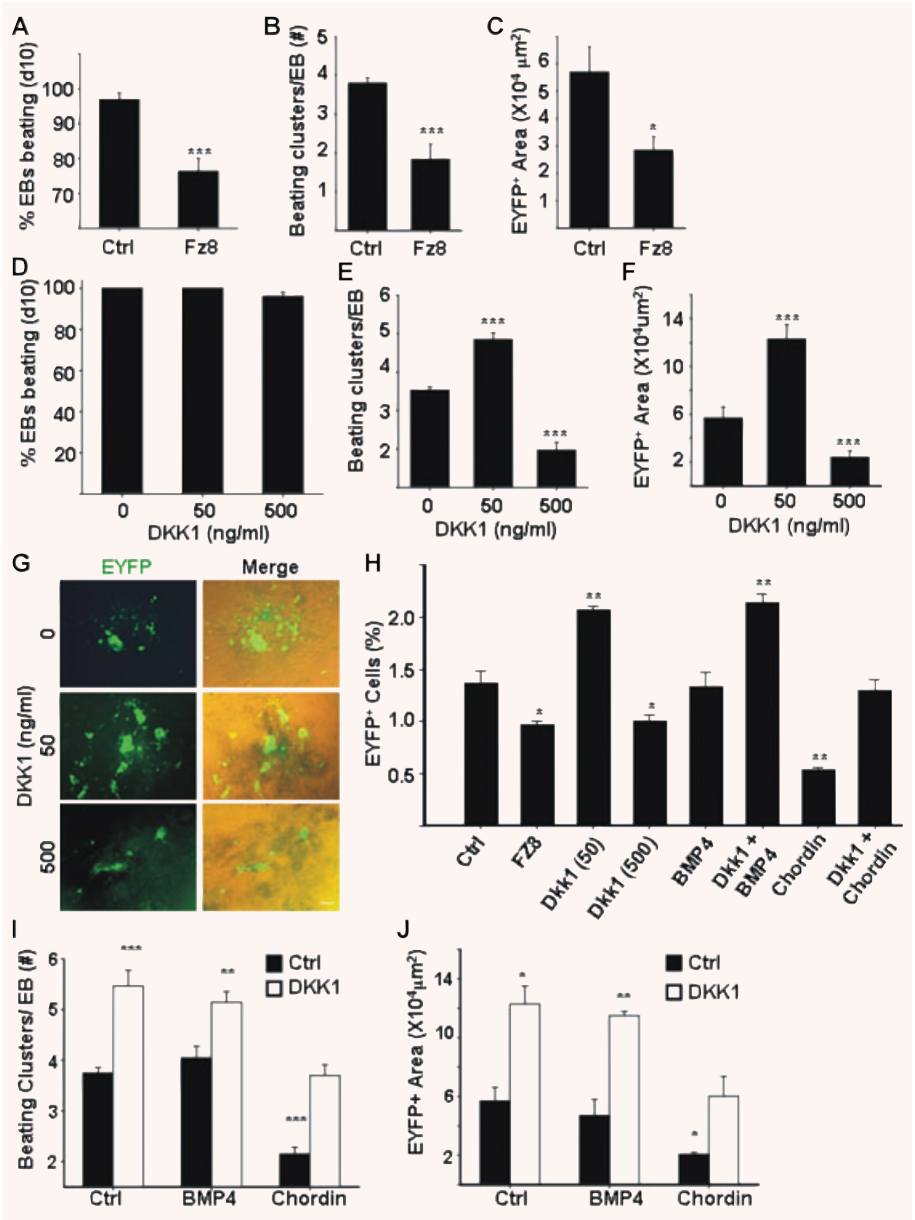

Fig. 6 Inhibition of Wnt signalling and its influence on cardiomyogenesis (d10) and Wnt-BMP synergy. A–C: Fz8 (200 ng/ml) exposure during d0–2 inhibited cardiac differentiation as determined by counting percentage EBs beating (**A**), beating clusters/EB (**B**) and EYFP$^+$ area (**C**). (**D**–**G**) DKK1 exposure (d0–2) influenced the cardiac differentiation in a dose-dependent manner. While, percentage EBs beating remained unchanged (**D**), variation was noted in the number of beating clusters/EB (**E**) and in the EYFP$^+$ area (**F, G**) upon DKK1 treatment. While lower DKK1 (50 ng/ml) treatment could promote cardiomyogenesis, the same at higher concentration (500 ng/ml) abrogated it. (**H**) Flowcytometry analysis revealed decrease in EYFP$^+$ cardiomyocytes following Fz8 exposure. While DKK1 at 50 ng/ml promoted EYFP$^+$ cardiomyocytes generation, it inhibited the same when used at 500 ng/ml. Exogenous supplementation of BMP4 did not show any appreciable influence on cardiomyogenesis as determined by no difference with respect to EYFP$^+$ cardiomyocytes generation (**H**), beating clusters/EB (**I**) and EYFP$^+$ area (**J**) compared to control. However, blocking endogenous BMP4 with Chordin inhibited cardiomyogenic differentiation from ESCs, the effect of which was nullified by blocking Wnt signalling with DKK1 (50 ng/ml) treatment indicating a possible synergy between Wnt and BMP underlying cardiomyogenic developmental programme. n = 3–6: mean ± S.E.M. (Scale: 100 µm in G).

opposing effect of Fz8 and DKK1 supported the involvement of non-canonical Wnt signalling during early cardiomyogenesis.

Wnt-BMP crosstalk influencing cardiomyogenesis

To ascertain possible crosstalk of Wnt with other guiding cues and their influence on cardiomyogenesis, EBs were treated with BMPs at d0–2 time-point. Interestingly, no apparent difference in cardiac differentiation was observed with BMP exposure compared to control. Comprehending that this might be due to serum presence in the medium that already contains BMPs, we blocked endogenous BMP signalling using Chordin (200 ng/ml). This resulted in a decrease in cardiac differentiation, as monitored by counting beating clusters/EB, the EYFP$^+$ cells and the area encompassed (Fig. 6H–J). However, this inhibition was nullified by DKK1 (50 ng/ml) (Fig. 6H–J) indicating the existence of a possible interaction between Wnt and BMP4. Supporting evidences were obtained by quantifying BMP4 transcripts that showed an increase at d2 (during mesoderm induction) and a decrease at d5 (during cardiac specification/induction) of differentiation with BIO exposure (0–2d treatment) compared to control (Fig. 7A). Interestingly,

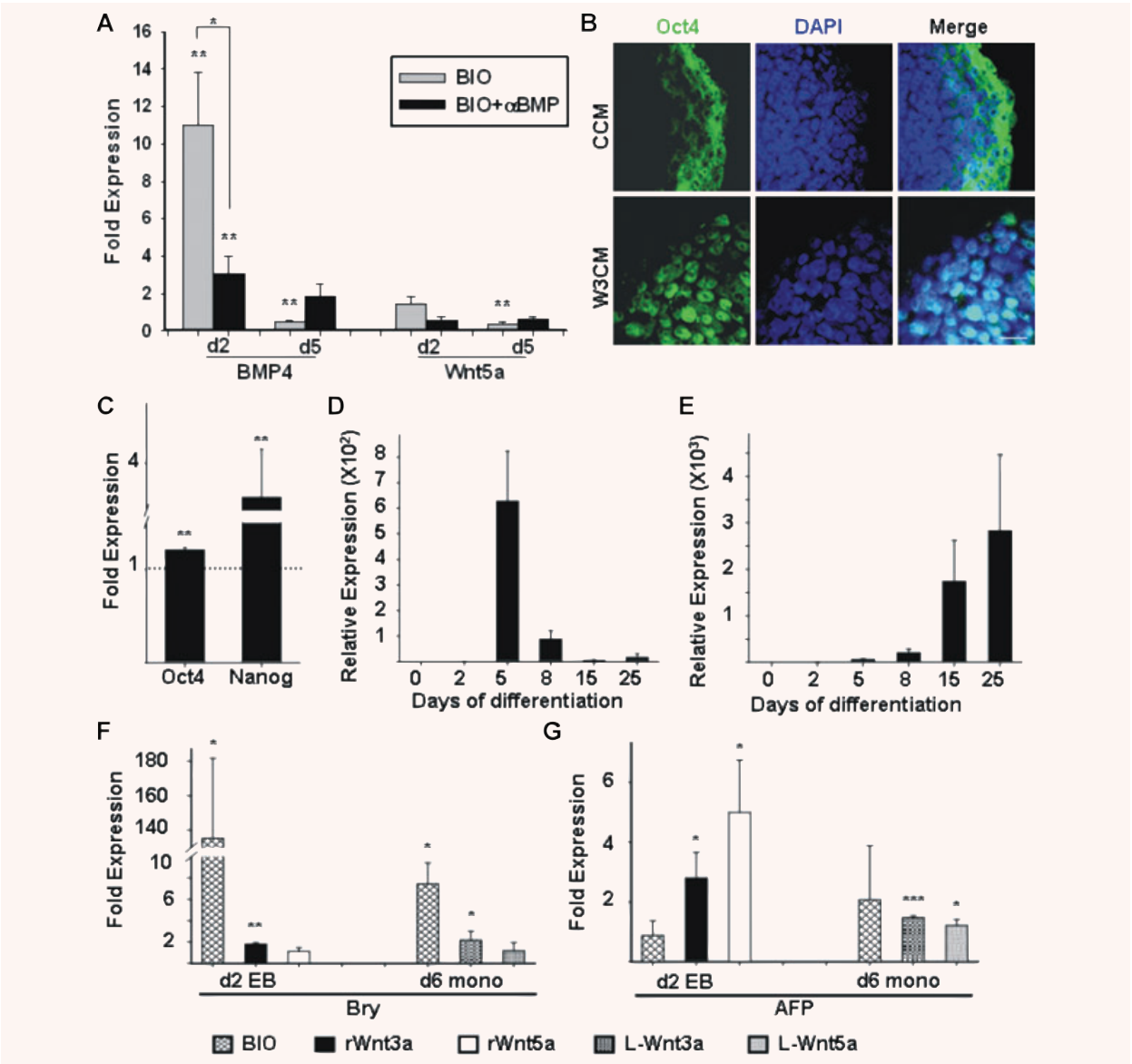

Fig. 7 Wnt-BMP crosstalk and modulation of ESCs' differentiation onset. (**A**) BIO exposure at d0–2 had a contrasting regulatory influence on BMP4 and Wnt5a expression in d2 and d5 EBs, respectively. Blocking of BMP signalling (d0–2) by using either BMP4 blocking antibody or Noggin in presence of BIO influenced the BMP4 transcript level at d2 indicating a probable interaction between the two. The values have been plotted as fold difference to untreated control (set as 1) at the said time-points. (**B**) An apparent increase in Oct4 levels and its nuclear localization was seen in W3CM treated EBs (d2). DAPI (blue) was used to mark the nucleus. Scale: 50 μm. (**C**) BIO treatment (d0–2) increased both Oct4 and Nanog transcripts in d2 EBs. (**D, E**) Expression profile of Bry (**D**) and AFP (**E**) relative to βactin during ESCs differentiation, as analysed by qPCR. (**F**) Bry expression was up-regulated in d2 EBs upon exposure to either BIO or rWnt3a but not rWnt5a (d0–2). ESCs grown for 6 days (d6) with either BIO or on L-Wnt3a feeders in monolayer culture (mono) could also show increase in Bry expression. (**G**) BIO did not alter AFP expression in EBs (d2) and also in cells in monolayer culture (d6), whereas Wnt3a and Wnt5a could up-regulate it. $n = 3–9$: mean ± S.E.M.

Wnt5a expression was also decreased in d5 EBs upon BIO exposure with no difference at d2 when compared with the control. To further substantiate these findings, BMP signalling was blocked during the same period (0–2d) using either Noggin or blocking antibody against BMP4. No significant difference in BMP4 transcripts was obtained between control and BMP antagonists group (data not shown). Intriguingly, BMP blockade in presence of BIO showed increased BMP4 at d2 that resided in between BIO and

control groups and with no difference at d5 when compared with the control (Fig. 7A). Expression of Wnt5a, however, remained unaltered with BMP blockade. Together our findings brought forth an interesting paradigm of temporal BMP modulation by Wnt, and its resultant effect on cardiomyogenesis.

Canonical Wnt signalling and meso- and endoderm formation

Since activation of canonical Wnt signalling at different levels was found to be inhibitory for cardiac differentiation, it became plausible to dissect out the mechanism by which Wnt inhibited cardiomyogenesis. To discern whether activation of Wnt/β-catenin signalling during differentiation caused an overall suppression in differentiation due to presence of undifferentiated ESCs, cells were differentiated either in presence or absence of W3CM, and morphology of EBs was observed. EBs exposed to W3CM were more dense with an increased Oct4 expression in both d2 (Fig. 7B) and d5 EBs (data not shown) than those treated with CCM. BIO could also increase the expression of Oct4 and Nanog (Fig. 7C and Fig. S1C and D). This led us to hypothesize that Wnt activation at early stage might have promoted undifferentiated ESC phenotype in differentiating EBs, hence affecting their proper differentiation into cardiomyocytes. However, it did not rule out the possibility of overall differentiation impairment of ESCs to all the germ layers following Wnt activation. Hence, we further sought to investigate whether Wnt exerted its effect in a temporal fashion on mesoderm/endoderm commitment and specification, and whether or not the same early time window was effective during this process.

Expression patterns of mesoderm lineage marker, Bry and endoderm marker, α-feto-protein (AFP), were studied to discern the role of Wnt signalling during germ layer specification. Under normal condition, Bry transcripts in control were found to be up-regulated by d2 of differentiation that attained a peak at d5 (Fig. 7D) and down-regulated subsequently. However, AFP was up-regulated by d5 and increased as differentiation proceeded (Fig. 7E). Thus, the early time-point (d2) was chosen to study the effect of Wnt during germ layer specification. A sharp increase in Bry expression was observed in EBs following BIO-mediated Wnt activation (d0–2) indicating mesoderm induction, as ascertained by qPCR (Fig. 7F) and immunoassaying (Fig. S1F). In fact, increased Bry expression with rWnt3a but not rWnt5a could further authenticate the specificity of canonical Wnt signalling underlying this activation (Fig. 7F). However, both rWnt3a and rWnt5a but not BIO could up-regulate AFP expression in d2 EBs (Fig. 7G). This indicated that probably difference in mechanism of activation or extent of Wnt signalling by Wnt/BIO triggered this differential effect. Together, our data suggested canonical Wnt signalling having a context-dependent biphasic role that could promote undifferentiated ESCs and in parallel it could specify mesoderm and endoderm inductions.

Further to assess the same during spontaneous differentiation in a monolayer culture, ESCs were grown at low density for 6 days, either in presence or absence of BIO and Wnt inhibitors. BIO influenced drastic up-regulation in Bry expression without affecting that of AFP (Fig. 7F and G), thereby suggesting that BIO might have expedited the mesodermal differentiation process in mono-culture too. However, no appreciable effect of DKK1 or Fz8 on the differentiation process was observed (data not shown). To validate these findings further, ESCs were cultured on either of L/L-Wnt3a/L-Wnt5a fibroblasts as feeders till 6 days. An up-regulation in Bry expression was seen only in cells grown on L-Wnt3a, but not on L/L-Wnt5a feeders (Fig. 7F). Similarly, a significant up-regulation in AFP expression was seen in cultures grown on both L-Wnt3a and L-Wnt5a feeders, consistent with our findings on the EB-mediated differentiation (Fig. 7G). Together, our data revealed a temporal influence of canonical Wnt signalling on expediting the initiation of both meso- and endoderm inductions while inhibiting cardiac differentiation. Interestingly, these events remained restricted to early stages only, thus, suggesting that the cardiac inhibitory effect of Wnt might be operational subsequent to mesoderm commitment/specification. Additionally, Wnt activation promoting undifferentiated ES cells and inducing mesoderm in parallel brought forth to an intriguing aspect of Wnt threshold acting in synergy with other modulators such as BMP in designating the cell fate and influencing multifaceted events during ESCs differentiation.

Discussion

Cardiac development is one of the complex developmental events during embryogenesis integrating heterogeneous cell types including cardiomyocytes, the functional entities of heart. Our study delineated the inhibition of canonical Wnt signalling augmenting ESCs' differentiation into cardiomyocytes in a temporal manner. This was in consistence with earlier *in vivo* findings in *Xenopus* and avian embryos (8–10) including the recent report on mouse [38, 39]. In fact, the early time window of differentiation was more sensitive to Wnt treatments than the later ones and Wnt, while promoting mesoderm induction could suppress cardiomyogenesis, the anterio-lateral plate mesoderm derivative, depending on its threshold.

Gastrulation during embryonic development marks the onset of three germ layers specification and EBs during ESCs' differentiation resemble the gastrulating embryos. The d0–2 and d2–5 windows in EBs were found to be the most effective time regimens for Wnt treatment and its consequent regulation of cardiomyogenic developmental events. This augmented various possibilities such as: (*i*) Wnt acted by blocking ESCs differentiation *per se*, (*ii*) it inhibited mesoderm formation or (*iii*) it might have directed the nascent mesoderm to other mesodermal derivatives rather than cardiac. Our data, in fact, demonstrated that Wnt activation maintained both Oct4 and Nanog expressions during differentiation, and in parallel, it promoted both mesoderm and endoderm formation. This dual fated scenario underscored the fact that a specific threshold of Wnt activity might underlie the cell fate decision machinery directing that to opt between the retention of undifferentiated state or acquiring specific cell fates. Following the same

rationale, it might as well be comprehended that low Wnt activity might tilt the mesodermal-committed population towards cardiac ones rather than the non-cardiac lineage subsequent to their specification. The dose response of DKK1 on cardiomyogenesis showing the opposite influence indeed supported this. Hence, precise regulatory machinery might exist to fine tune the developmental proceedings by establishing Wnt gradients.

Though nascent mesoderm adopts cardiac, skeletal, bone or haematopoietic cell fates, it still remains unclear how and when this decides to commit to a particular cell derivative. Our study has clearly demonstrated canonical Wnt signalling inducing mesoderm formation in EBs both *in vivo* and *in vitro* similar to earlier reports [11–15, 40]. In the vertebrate system, presumptive cardiac fate specification commences from anterio-lateral plate mesoderm where cardiac crescent cells, the precursors of heart, get signals from adjacent endoderm and become specified for cardiac fate [41]. However, posterior lateral mesoderm also adopts cardiac fate when co-cultured with anterior endoderm [8]. Incidentally, canonical Wnt being active in posterior mesoderm was reported to induce blood cells formation, whereas the anterior one promoted cardiac mesoderm following signals from anterior endoderm in form of Wnt antagonists [8]. Our results indeed indicated that mesoderm formed following activation of canonical Wnt did not adopt cardiac fate. In chick embryo too, Wnt inhibited heart formation where neural tube blocked cardiogenesis in adjacent anterior paraxial mesoderm [10], and the early stages of development were more responsive to the treatment [42]. Similarly, Wnt antagonists could induce ectopic heart formation in *Xenopus* embryos [9]. Thus, *in vivo* data suggested that inhibition of Wnt signalling in vertebrates was required for cardiac fate specification. Conversely, the segment polarity gene wingless, a homologue of vertebrate Wnt, was required for tubular heart formation in *Drosophila* [7]. Our data corroborated well with *in vivo* findings that were seen in chick and *Xenopus*, demonstrating canonical Wnt inhibition at an early time window could promote cardiac fate during differentiation and a sustained activation proving most detrimental for the same.

Contrary to *in vivo* data, notable ambiguity existed regarding Wnt's influence on cardiac differentiation *in vitro*. Our findings on mesodermal induction from ESCs by Wnt, while negated the report of Naito *et al.* [25], it supported that of Gadue *et al.* [14] and Lindsley *et al.* [13]. Keller's group [14, 15] had demonstrated that Wnt could promote the Bry^+ mesodermal cells from ESCs. While early $Bry^+/Flk1^+$ cells had haemangioblast fate, late $Bry^+/Flk1^-$ ones took cardiac fate, though, these $Flk1^-$ cells eventually became Flk^+ [43, 44]. In line with these findings, the early Wnt signalling by BIO/Wnt3A that expedited the process of mesoderm formation might have helped adopt haematopoietic fate rather than the cardiac one. Though Nakamura *et al.* [24] showed blocking of Wnt pathway by Fz8 attenuating DMSO-induced cardiac differentiation in EC cells, the activation of the pathway alone (without DMSO) could never generate beating cardiomyocytes in those. Blocking of Wnt pathway was further shown to be either inhibiting or promoting cardiac differentiation in a temporal fashion [25]. We could also demonstrate that Fz8 could attenuate cardiac differentiation possibly by inhibiting both canonical and non-canonical pathways. Conversely, DKK1 that inhibited only canonical Wnt could induce cardiomyogenesis in a temporal and a concentration-dependent manner. In fact, an early inhibition by moderate concentration of DKK1 promoted the same with little or no effect at late stages, thus, indicating that Wnt might have exerted its influence in a temporal fashion [26, 38, 45].

Wnt signalling was also shown to inhibit heart formation in a non-cell-autonomous manner through β-catenin mediated inhibition of homeodomain transcription factor Hex in endodermal cells, residing in close proximity to the cardiogenic mesoderm [46]. Sox17, an endodermal transcription factor, could in turn inhibit β-catenin, thereby activating Hex in endodermal cells and promoting cardiogenesis in the adjacent mesoderm [27, 47]. Similarly DKK1 was also found to induce cardiogenesis in a non-cell-autonomous manner involving Hex [48]. Kwon *et al.* [26] have reported the positive involvement of Wnt signalling during heart development using loss- and gain-of-function β-catenin mutants. Even in ESCS, they have shown cardiac promoting effect of Wnt3a (150 ng/ml) and have demonstrated the proliferation of cardiac progenitors due to cyclin D2 up-regulation in response to Wnt activation. However, the conditions used by them did not yield optimum EB differentiation in contrast to ours where 100% of the EBs showed pulsation by d10 of cardiac differentiation. Moreover, they have targeted a stage subsequent to mesoderm induction, while our investigation has highlighted the criticality of early stages. We have shown at d0–2 (corresponding to the germ layer specification stage) and d2–5 (the cardiac progenitor specification stage) of differentiation especially the former one where consistency in response was observed with respect to various Wnt activators and inhibitors used, Wnt exerted its crucial influence modulating various developmental events including cardiomyogenesis. Hence, it is quite intriguing to have variable findings while working on a similar system and it may be due to the time and strength of treatments used by various groups. The probability of cells remaining at the progenitor stage [26] without undergoing further differentiation was also ruled out because of the striking influence obtained in response to DKK1. Additionally, there was noted decrease in the expression of various cardiac-specific markers both early and late expressing ones (NKx2–5, GATA4, Tbx5, HAND1, HAND2, α- and βMHCs, MLC2a, MLC2v, etc.) in response to Wnt activation and the opposite noted with moderate DKK1. As seen with DKK1, while this at a moderate concentration used by us could induce cardiomyogenesis supporting the findings of Gadue *et al.* [14], the same at higher concentration totally abrogated it similar to the report by Naito *et al.* [25] and Kwon *et al.* [26]. This unequivocally strengthened our supposition of a specific Wnt threshold modulating cardiomyogenesis in a temporal fashion instead of mere activation or inhibition. Further, it encouraged us to discern the crosstalk of Wnt with other factors influencing early cardiomyogenesis from ESCs *in vitro*.

Role of BMPs in mesoderm specification is well studied [11, 40, 49] and that in concert with FGF helps in cardiac cell fate

specification [2, 50, 51]. To discern whether Wnt and BMP acted synergistically or independent of each other we intended to block BMP in presence of both Wnt activators and inhibitors. Since Wnt activation had cardiomyogenic inhibitory influence, it was more convincing for us to have BMP inhibition in presence of Wnt inhibitors. Accordingly, when both DKK1 and Chordin were used in combination, the cardiomyogenic efficiency followed neither of the patterns seen with independent treatments. In fact, the response was intermediate and that led us to presume as though DKK1's promotory effect could nullify the inhibitory effect of Chordin. This indeed highlighted the positive influence of BMPs on cardiomyogenesis. A recent report by Klaus *et al.* [38] has also demonstrated the influence of BMP and canonical Wnt signalling during early cardiogenesis *in vivo,* highlighting a temporal and a threshold-dependent effect. However, formation of cardiac crescent cells and expression of various cardiac markers, despite the looping defect in the β-catenin conditional knockouts used in their study, might have been due to the presence of an intact non-canonical Wnt signalling. Our data have revealed non-canonical Wnt signalling having a positive influence on cardiomyogenesis from ESCs *in vitro* when treated at the early stage only. Since EBs resemble the developing embryos, it is conceivable that EB during hanging drop (d0–2) would have initiation of three germ layers specification, while the subsequent stage in suspension will have committed progenitors. With the same rationale d2 EB would have mesoderm committed cells and d5 the cardiac progenitors [26]. Hence, decrease in BMPs and Wnt5a, the non-canonical Wnt at d5, in response to early activation of canonical Wnt was presumed to have caused decrease in subsequent cardiac differentiation.

The positive influence of BMP did pose the possibility that Wnt and BMP either acted independently or synergistically. Indeed, our study has brought forth an interesting paradigm based on BMP4 expression profile in response to Wnt activation, where Wnt exerted its effect by a stage-specific modulation of BMP4 [38]. Intriguingly, the increased expression of BMP4 at d2 of differentiation in response to Wnt activation that showed decrease upon BMP blockade, though the level remained higher than control, in fact strengthened our interpretation regarding the second possibility. Moreover, BIO induced BMP4 expression at d2 also correlated well with Bry increase, implicating existence of probable synergy between Wnt and BMP in promoting mesoderm induction. The delineation of a direct and an inverse relation between canonical Wnt signalling and BMP4 during mesoderm induction and cardiomyogenesis respectively, has unequivocally emphasized the multifaceted role of Wnt during early cell fate determination. Further work would be needed to substantiate this and to delineate the underlying mechanistic basis for the same.

Overall, our investigation has evidently demonstrated for the first time that instead of mere activation or inhibition, it might be specific signalling threshold of Wnts/BMPs and the synergy among these guiding cues that operate in a temporal fashion could underlie the cell fate decision machinery, directing it towards various lineages in parallel. The knowledge gained would further help in enriching the *in vitro* generated cardiomyocytes in view of potential implications in regenerative medicine.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Canonical Wnt signalling and regulation of ESCs differentiation. (**A**) Sub-cellular localization of β-catenin at various stages of cardiac differentiation. (**B**) BIO exerted its effect in a temporal fashion by decreasing the EYFP expression at the indicated time-points, compared to control (Ctrl). (**C**) BIO treatment (1 μM) in early differentiating EBs (d2) increased the ESC marker Oct4 expression and its nuclear localization compared to DMSO control. (**D**) Oct4 and Nanog were up-regulated in d5 EBs upon BIO (10 μM) treatment (d0–2) compared to DMSO control (set as 1) as analysed by qPCR. (**E**) A significant increase was seen in the expression of αMHC and βMHC, the cardiac-specific cytoskeletal markers, with DKK1 treatment (d0–2) at moderate concentration (50 ng/ml), while the same was decreased at higher (500 ng/ml) DKK1 as analysed by qPCR at d10. (**F**) Early mesoderm marker Bry showed increased expression upon BIO (1 μM) treatment in d2 EBs. DAPI (blue) was used to visualize the nucleus. $n = 4$: Mean ± S.E.M. Scale: 20 μm in A, C and F; 100 μm in B.

Table S1. List of primers and their sequences used for RT-PCR.

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