

Chapter 2

Studies on avian PEO and epicardium derived cell differentiation potential in explant culture

A. Introduction

In vertebrate cardiogenesis, precardiac mesoderm differentiates into multiple cardiac cell types at early developmental stages (Schlueter & Brand, 2013). At first, two distinct cell types, which are contractile myocardium and inner endocardium, get formed. After that, an outer epicardial layer is found to appear, making an exterior protective covering of the embryonic heart (C. M. Braitsch, Kanisicak, van Berlo, Molkenin, & Yutzey, 2013; Yasuo Ishii, Garriock, Navetta, Coughlin, & Mikawa, 2010). Recently, numerous research articles have reported about the complex molecular interactions and signaling events of developing myocardium and epicardium during vertebrate heart development (Kennedy-Lydon & Rosenthal, 2015; Kruithof et al., 2006). Outer epicardium is a protective cardiac envelop, which is formed from the precursor extra-cardiac proepicardial organ or PEO (Asli, Xaymardan, & Harvey, 2014; Maya-Ramos, Cleland, Bressan, & Mikawa, 2013). PEO can be defined as a group of mesothelial cells, which is situated at the atrio-ventricular junction near venous pole of the vertebrate embryonic heart. In the next developmental stage, the PEO derived cells start to migrate over the whole myocardium and forms the outer epicardium, covering the entire embryonic heart (C. M. Braitsch et al., 2013; M. D. Combs et al., 2011). Several reports have shown that defects of epicardium development results in embryonic lethality and also a great number of cardiac abnormalities including deformed heart chambers, disorganized distribution of resident cell types and ventricular trabeculae after maturation (C. Braitsch & Yutzey, 2013; Y. Ishii, Langberg, Hurtado, Lee, & Mikawa, 2007; Jenkins et al., 2005). After epicardium development, a subset of embryonic epicardial (eEpi) cells invades through the myocardium and starts to delaminate from the epicardial area and undergoes epithelial to mesenchymal transformation (EMT) and differentiate into epicardium derived cells or EPDCs. These EPDCs are nothing but the different cardiac lineage specific cells which contribute smooth muscles, fibroblasts, endothelial cells and also

cardiomyocytes (CMs) during heart maturation (Austin, Compton, Love, Brown, & Barnett, 2008; Kruithof et al., 2006).

In response to adult cardiac injuries, cardiomyocytes (CMs) are very restricted to proliferate and regenerate in cardiac repair process. Therefore it affects a wide range of morbidity and mortality in mammals, including humans. There are several signaling pathways, including Wnt/ β -catenin and BMP2-pSmad /1/5/8 signaling, which are associated with molecular factors of CM differentiation and proliferation at the early heart developmental stages (Buermans et al., 2010; X. Cai et al., 2013; Santanu Chakraborty et al., 2013; Kruithof et al., 2006; Russell et al., 2011; Shelton & Yutzey, 2007). Duan et al. have reported that adult cardiac injury induction triggers the epicardium derived cells (EPDCs) to get reactivated, so that these can replenish new cardiac cell types (Duan et al., 2012). Moreover, other studies have revealed that EPDCs are multipotent progenitors which have significant contribution in CM differentiation via BMP/Fgf and Wnt/ β -catenin signaling (Kwon, Cordes, & Srivastava, 2008; Pennisi et al., 2003; Sasaki, Hwang, Nguyen, Kloner, & Kahn, 2013). In order to mention the major concerns of adult cardiac injuries these signaling mechanisms are insufficient to promote enough CM differentiation from existing cardiac progenitors to overcome the pathological and functional abnormalities. Although, recently, wide range of research focuses are directed towards the reactivation of many mechanisms of embryonic or developmental signaling pathways to regenerate and/or repair the extensive damage of muscle cells (myocardium), more detailed regulatory hierarchies remain poorly investigated. Among those signaling mechanisms, Wnt/ β -catenin signaling is involved in different cardiac developmental events including EPDC differentiation into resident cell types. Therefore, further studies on Wnt/ β -catenin signaling manipulation towards EPDC differentiation, have to be focused and inquired into the studies. Significant number of reports has unveiled the diverse role of Wnt signaling, which direct to carry on its detailed involvement in vertebrate cardiac development [discussed in chapter I]. On the other hand, several T-box transcription factors play major functions in regulation of embryonic heart development (Iio et al., 2001; Plageman & Yutzey, 2005; Fiona A. Stennard et al., 2003; Takeuchi, 2005). Among those T-box transcription factors, Tbx20 belongs to Tbx1 subfamily and it is known to express in multiple organs including heart (Greulich, Rudat, & Kispert, 2011). It has been reported after performing *Tbx20* loss-

of-function (LOF) and gain-of-function (GOF) assays, that *Tbx20* is associated with different cardiomyopathies (Kirk et al., 2007). Most important finding of *Tbx20* is that it is known to promote CM proliferation maintaining fetal characteristics in adult mouse hearts (Santanu Chakraborty et al., 2013). Also, it is added to the report that *Tbx20* elevates cell proliferation via Bmp2/pSmad/1/5/8 and PI3K/AKT/GSK3 β / β -catenin signaling pathways in both adult CMs *in vivo*, and neonatal CMs *in vitro*. So there might be an inter-relationship between *Tbx20* and Wnt/ β -catenin signaling regulating EPDC differentiation, which needs further researches. Also, *Tbx20* expression and its importance in both embryonic PEO and eEpi- derived cells remain undefined. Thus, overall *Tbx20* function in embryonic PEO and eEpi derived cell differentiation associated with Wnt/ β -catenin signaling requires further studies to enrich this area.

Here, mRNA expressions of various important cardiac lineage specific markers have been analysed using avian PEO and eEpi-derived cells *in vitro*. In embryonic avian hearts, PEO derived cells have shown elevated gene expression of selective CM specific markers (*Gata4*, *Nkx2.5*, *Mef2c*) including *Tbx20*, compared to eEpi derived cells. Moreover, lithium chloride (LiCl) mediated β -catenin stabilization on CM specification is being performed on both avian PEO and eEpi cells *in vitro*. Interestingly, β -catenin upregulation in eEpi cells results in increased *Tbx20* expression along with CM specific marker gene expression. Increased β -catenin also promotes greater number of sarcomeric myosin specific Mf20 positive cells with increased proliferative capacity in eEpi cells in culture. Together these data strongly suggest that Wnt signaling and associated increased *Tbx20* level might unlock and initiate a new insight into CM differentiation potential from eEpi cells *in vitro*. Overall, this report finds a novel source for cardiac progenitor cells and regenerative strategy for better cardiac protection and repair after injury.

2. Methodology:

a. Avian embryo collection

Fertilized incubated (38°C) chicken eggs were collected from local hatchery (State poultry farm, Tollygunge, Kolkata, West Bengal) at embryonic day 3, 4 and 5 (E: 3, 4 & 5) which are equivalent to Hamburger Hamilton (HH) stages 20, 24, 26 respectively (Hamburger & Hamilton, 1951). The specific avian breed used was *Gallus gallus domesticus* (Rhode Island Red and Black Australorp). All the fertilized eggs were procured in accordance with the University purchase guidelines.

b. RNA isolation, semi quantitative reverse transcriptase PCR

Total RNA was isolated from dissected and pooled twelve PEO tissue samples of E: 3 and E: 4 per experimental group using 200µl Trizol (Invitrogen, 15596026). Then 1µg of total RNA was reverse transcribed for cDNA synthesis by Bio-rad kit (iScript™ Reverse transcription supermix, 170-884) in 20µl total volume according to manufacturer's supplied protocol. Total RNA was also isolated from proepicardial (E: 4) and epicardial (E: 5) explants cell cultures using 200µl Trizol. Then cDNA was synthesized, taking 1µg of RNA by RT-supermix. Semi qRT-PCR was performed in 20 µl of total volume using DNA taq polymerase (Bioline, BIOTAQ DNA polymerase BIO-21040). These PCR were performed at 35 cycles using 20pmol of the following primer pairs (IDT) mentioned in the following annexure II.

c. Quantitative real time PCR (qRT-PCR) analyses

Quantitative real time PCR was performed for further validation of semi qRT-PCR in 10 µl of total volume using Bio-Rad Real Time PCR kit (SSO fast Eva green supermix, 172-52 03AP) according to previously standardized protocol (Alfieri et al., 2010b). Gene expression levels were determined by Cq value obtained from Bio-Rad CFX manager software normalized to Gapdh expression. qRT-PCR results for each gene represents the data generated from at least three biological and technical experiments (n=3). For both PEO tissues and explant cultures, gene expression was evaluated as increased or decreased fold change calculated by experimental Cq values generating ΔCq and $\Delta\Delta Cq$. Statistical significance was calculated for observed differences by student's t-test and considered significant for p values (≤ 0.05).

d. Avian PEO and eEpi culture

After collecting fertilized incubated chicken eggs from the hatchery, embryos were isolated in Phosphate buffer saline (PBS) solution. Proepicardial organ or PEOs were dissected out from atrio-ventricular (AV) junction in whole chick embryonic heart at E: 4. Dissected PEOs from 24 embryos were seeded onto a 0.01% collagen (CollagenI Rat tail, 3mg/ml, A10483-01) coated two-well chamber slides (SPL, 30122 or Eppendorf, 0030742052) with 1ml complete media containing M199 (Invitrogen, 11150-059) and supplemented with 10% fetal bovine serum (Invitrogen, 10270), 1% penicillin/streptomycin (Pen-Strep) (Invitrogen, 15140122). After 16 hrs in culture the prematurely beating explants, indicative of myocardial cell contamination were removed to establish a pure PEO explant culture. After 24hrs, the PEO explant cultures were used to perform all the experiments.

Embryonic epicardial (eEpi) explant cultures were established by isolating whole hearts from chick embryos at E: 5. Collected whole hearts were cultured in 0.01% collagen coated plates containing M199 complete media and allowed to attach at the bottom for overnight. Then the hearts were removed leaving the formed eEpi monolayers. These eEpi monolayer cultures were consequently used for further experimentations.

e. Embryonic Rat myoblast culture (H9C2):

Embryonic rat myoblast cell line, H9C2 was maintained in cell plates containing Dulbecco's Modified Eagle Medium (DMEM) complete media, following passage numbers. Cells were seeded according to experimentations from the stock plates and further procedures were performed.

f. Activator assays in eEpi cells in explant culture

For Wnt signaling activation, lithium chloride (LiCl) or recombinant Wnt3a protein is used as described previously by others (Alfieri et al., 2010b; Sinha, 2004). After successful establishment of eEpi culture, cells were treated with 15mM Lithium Chloride (LiCl) (Sigma, 203637) or 150ng/ml recombinant Wnt3a protein (Abcam, ab81484) supplemented with M199+Pen-strep media as described before. After 48 hrs

of culture control cells were kept in M199+Pen-strep and experimental plates were kept in LiCl or Wnt3a supplemented M199+Pen-strep medium for 24 hrs. After indicated hrs, control and treated plates were carried forward for detailed gene expression analyses and immunostaining.

g. Inhibitor assay in eEpi cells in explant culture

eEpi cultures were established as stated before and treated with Xav939 (Abcam, ab120897) for Wnt signaling inhibition assay. After 48hrs of eEpi culture, 5 μ M of Xav939 were supplemented with M199+Pen-Strep and treated to the eEpi cells for 7hrs as described previously (Santanu Chakraborty et al., 2013). After indicated hrs, control and treated plates were carried forward for detailed gene expression analyses.

h. Immunostaining

PEO and eEpi explant cultures were fixed in 1% paraformaldehyde followed by 4% paraformaldehyde (Affymetrix, 19943) and washed with 1x phosphate buffer saline (PBS) and PBST solution (PBS+0.01% tween 20). Then immunostaining was performed according to manufacturer's supplied protocol. The following antibodies were used for immunofluorescence (IF): Mf20 (1:200 dilution, DSHB, ID-AB_2147781), Ki67 (1:400 dilution, ab15580). A mouse polyclonal secondary antibody (Goat anti-Mouse IgG H&L, Alexafluor 488, ab 150113) directed against Mf20 was used at 1:1000 dilutions in blocking solution. Likewise a rabbit polyclonal secondary antibody (Goat anti-Rabbit IgG H&L, Alexafluor 488, ab 150077) was applied against Ki67 primary antibody at 1:1000 dilutions in blocking solution. All primary antibody incubations were done for overnight at 4°C.

On the other hand Epicardial explant cultures were fixed in 1% Paraformaldehyde and followed by 4% Paraformaldehyde and washed with 1xNP40 (IGEPAL® CA-630, Cat. No.198596, Sigma-Aldrich, NP40 supplement) and PBS. Then Phalloidin staining was done using Alexa488 tagged Phalloidin (Life Technologies, Ref.No.A12379). For both experiments nuclei were stained with DAPI (abcam, ab104139) in fluoroshield mounting medium.

i. Bright-field and fluorescence microscopy

Chick embryos were dissected out from fertilized eggs at E: 3, E: 4 and E: 5 and kept in ice cold 1xPBS. Then these embryos were oriented sagittally and light microscopic images were taken using inverted microscope with camera attachment (Nikon SMZ800). PEO and eEpi explant cultures were also being imaged at bright-field using inverted microscope (Zeiss). For immunostained slides, fluorescence was detected using upright and inverted fluorescent microscopes (Zeiss lab, flu microscope and Leica fluorescence microscope) and images were captured using zeiss software: ISC capture and Leica software: LAS X.

j. Coomassie brilliant blue staining

For morphological study, the isolated proepicardial (PEO) and embryonic epicardial (eEpi) cells were stained with coomassie brilliant blue after 48 hrs of culture. Cultured cells were washed once with 1x PBS and given coomassie brilliant blue solution for 30 mins. After that coomassie blue stained slides were air-dried for 1-2 hrs and bright field images were captured using inverted microscope (Zeiss).

k. Flow Cytometry

First, eEpi cultures were established as mentioned earlier. After 48hrs of culture, sub confluent (95-98%) eEpi cells were trypsinized, counted and harvested after a brief centrifugation at 3000 rpm for 7mins. Approximately 3×10^6 cells were taken for each experiment. Subsequently, cells were washed once in ice cold binding buffer (1xPBS with 2% fetal bovine serum and 0.02% sodium azide) and re-suspended in the same buffer. The cells were then aliquoted (100 μ l) and incubated with primary antibody Wt1 [abcam, cat # 89901] or c-Kit [cell signaling technology, cat # D13A2]) with 1:50 dilution at 4°C for 40mins. After that cells were washed again with cold binding buffer and then incubated with FITC-conjugated secondary antibody (Alexafluor 488, abcam) at 4°C for 40 mins. Again cells were washed with ice cold binding buffer and final sample was prepared by adding 1% paraformaldehyde. Next, samples were analyzed by BD Accuri™, Flow cytometer, BD Biosciences. Only secondary antibody was applied to make negative control (Ruiz-Villalba, Ziogas, Ehrbar, & Pérez-Pomares, 2013).

1. Statistical Analyses

Student's t-test, specifically one sided two-tailed was used to detect the statistical significance ($p \leq 0.05$) and to derive all the fold changes in gene expression assays. All experiments were carried out at least in both biological and technical triplicates. For cell size measurement, minimum of 2500 cells was measured in each set of experiments under 20X objective. In addition, the entire auto calculated values were taken and after generating the mean values in each set of experiments, control value was set at 1. Then fold changes of respective experiments, were calculated with respect to the control value by unitary method. Also for Ki67 and Mf20 experiments, a minimum of 500 cells was counted in each set of experiments ($n=3$). All the cell count values were taken and after generating the percentages in each set of experiment, control value was set at 1. Then fold changes of respective experimental sets, were calculated with respect to the control as mentioned previously.

Overall, all the statistical analyses have been reported with standard error of mean (SEM) subjected to student's t-test ($p \leq 0.05$).

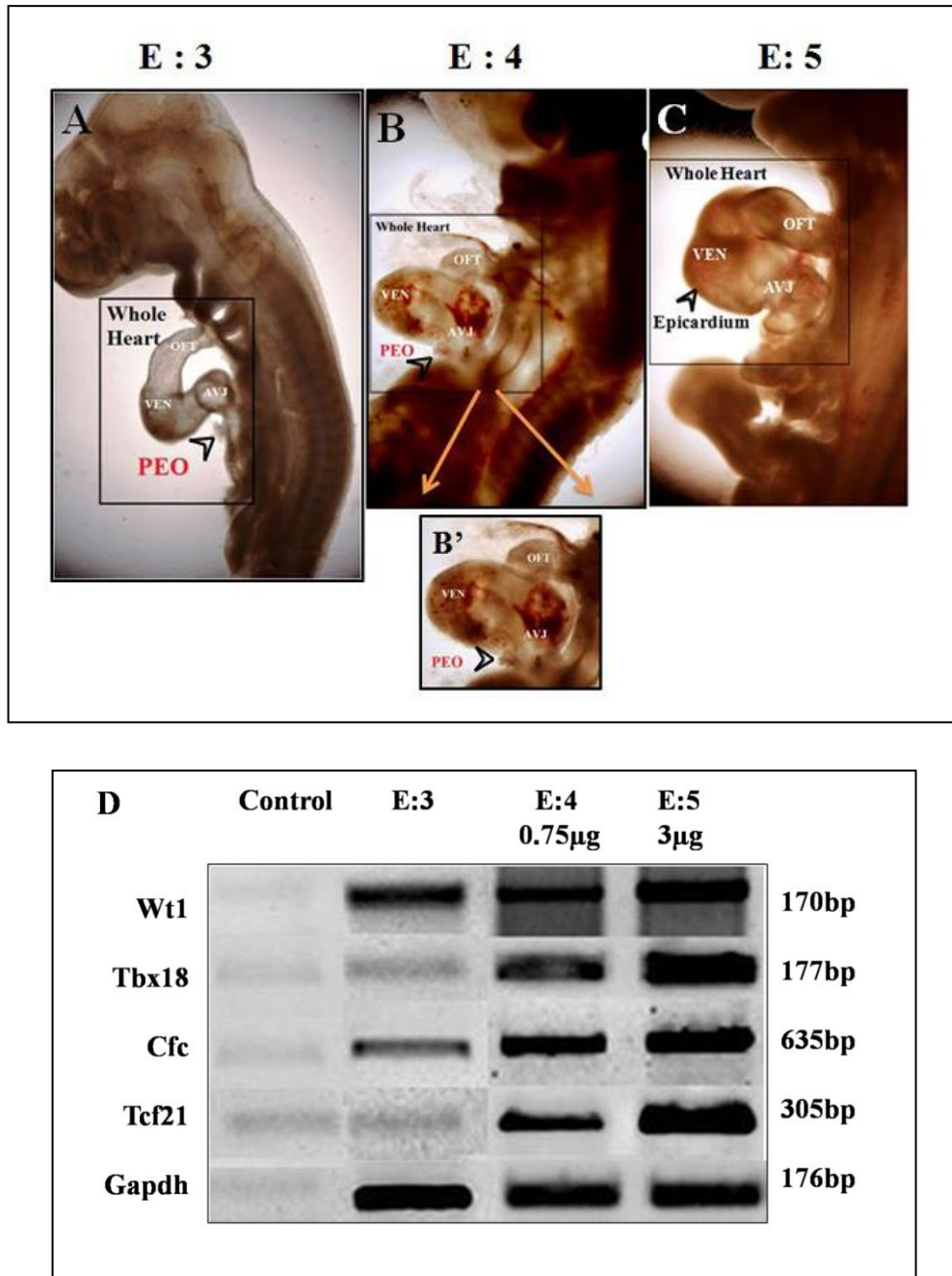
C. Results

a. Localization of epicardial precursor organ, proepicardial organ (PEO) in developing avian embryos

According to earlier reports, it has been suggested that PEO localizes at Hamburger and Hamilton (HH) stage 16-17 or embryonic day (E: 4.5), however, no other report have shown the detailed localization and subsequent development of PEO to embryonic epicardium (eEpi) in successive embryonic days previously. To show the localization of the fully formed PEO and also for the subsequent eEpi formation in developing embryos, we have taken the images of whole avian embryos at E: 3, E: 4 and E: 5 stages which are equivalent to the HH stages 20, 24 and 26 respectively (Hamburger & Hamilton, 1951). The whole embryos are dissected out from fertilized avian eggs and villous protrusions like group of cell clusters, which is symbolic of the developed PEO, has been found at atrio-ventricular (AV) junction of whole heart under bright-field microscope. The developing PEO was sighted at E: 3 embryos and when the PEO gets completely developed, then it has been localized at E: 4 embryos (figure 1A and figure 1B respectively). In contrast, we have shown the localization of developed eEpi at E: 5 in whole avian embryonic heart. Overall, at E: 3, developing PEO has been marked by arrowheads in the whole heart region of the embryo (figure 1A). As expected, distinct PEO has also been marked at the AV junction near sinus venosus of the whole heart in the embryo at E: 4 (figure 1B). Magnified view of PEO localization has been shown in figure 1B'. Then at E: 5, eEpi is marked over the outer layer of the whole embryonic heart, shown in figure 1C. Thus, fully formed PEO localizes at E: 4 whole chick embryos.

Selective PEO Specific marker gene expressions have been analysed to validate PEO localization. For that, we have performed gene expression analyses of PEO specific markers (*Wt1*, *Tbx18*, *Cfc*, *Tcf21*) by semi quantitative reverse transcriptase PCR (semi q RT PCR) on dissected PEO tissues from E: 3 and E: 4 avian hearts. In E: 4, two concentrations of RNA (0.75 μ g and 3 μ g) have been used as a template to synthesize PEO specific cDNAs for semi QRT-PCR analyses. Here, all these PEO markers (*Wt1*, *Tbx18*, *Cfc*, *Tcf21*) have shown elevated expression in E: 4 developed PEO compared to E: 3 developing PEO (figure 1D). In addition, PEO specific markers have been expressed in a concentration dependent manner (0.75 μ g versus 3 μ g of RNA template). Samples without DNA taq polymerase, are used as controls and *Gapdh* is used as loading control. Hence, *in vivo* localization and gene expression data are strongly suggestive of fully formed PEO in developing chick embryo at embryonic day 4 (E: 4).

Figure 1



[Figure 1: Localization and isolation of Proepicardial Organ (PEO) in developing chick embryos. (A-C) Light stereo microscopic images of left sagittally oriented chick embryo. A: Image of whole chick embryo at embryonic day 3 (E: 3) indicating looped heart (boxed region) and developing Proepicardial organ or PEO (arrowhead) at the atrio-ventricular junction (AVJ). B: Image of chick embryo at embryonic day 4 (E: 4) with looped heart and developed PEO (arrowhead) at the AVJ. B': Zoomed view of boxed looped heart region of same image B shows outflow tract (OFT), ventricle

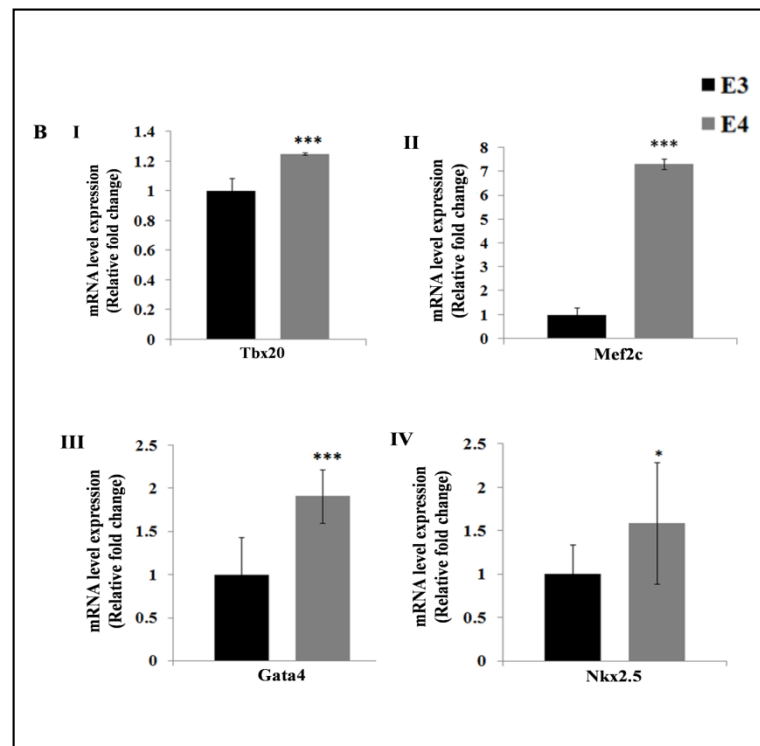
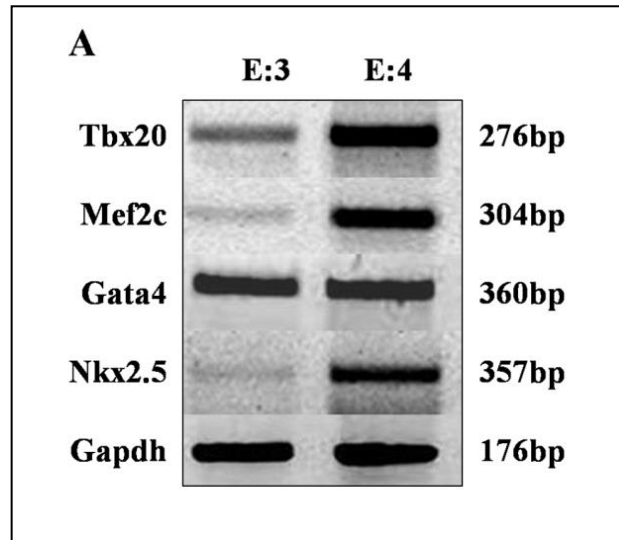
(VEN), AVJ and PEO. **C:** Image of chick embryo at embryonic day 5(E:5) with well developed looped heart (boxed region) associated with outflow tract (OFT), ventricle (VEN), AV junction (AVJ) and the outer Epicardial layer. **D:** Gene expression analyses using qRT-PCR were performed in concentration dependent manner on cDNA synthesized from total RNA isolated from chick PEO at embryonic day 3 and 4 (E:3 and E:4). At E: 3, PEO specific gene markers (*Wt1*, *Tbx18*, *Cfc*, *Tcf21*) and T-box transcription factor, *Tbx20* mRNA were detected. Expression of *Tbx18* and *Tcf21* mRNA were relatively low at E: 3 compared to their expression at E: 4. In contrast, *Wt1* mRNA expression pattern was similar in both developmental stages of cardiogenesis. Semi-quantitatively (0.75 µg and 3 µg of RNA template used) all these genes are intensely expressed at E: 4 PEO. *Gapdh* was used as a loading control. (n=3)]

b. Gene expression analyses of *Tbx20* and cardiomyocyte (CM) lineage specific markers in isolated PEO tissue during avian cardiogenesis

Tbx20, a T-box family transcription factor, is known to express in different cardiac cell lineage specific cells, during early developmental stages of cardiogenesis. Therefore, to find out the differential expression of *Tbx20* during early as well as later stages of PEO development, a comparable gene expression pattern has been analysed on isolated RNA pools from E: 3 & E: 4 PEO tissues of avian embryos. Here, the baseline expression of *Tbx20* and CM specific marker genes has been performed by semi qRT-PCR using 0.75 µg concentration of RNA template for PEO specific cDNA synthesis. Further, these data are validated by quantitative real time PCR (qRT-PCR). On isolated PEO tissue at E: 3, *Tbx20* and CM specific *Mef2c* and *Nkx2.5* have shown decreased expression compared to *Gata4* with increased expression. Altogether, CM specific markers (*Mef2c*, *Gata4*, and *Nkx2.5*) and *Tbx20* have shown increased expression at E: 4 PEO compared to E: 3 PEO tissues (figure 2A). Here also, *Gapdh* is used as loading control. Again, this data is confirmed by real time PCR, which determines that *Tbx20* and CM specific markers (*Mef2c*, *Gata4*, *Nkx2.5*) have been significantly increased at E: 4 by 1.25 fold, 7.31 fold, 1.91 fold and 1.59 fold respectively (figure 2B, I-IV) as compared to E: 3 PEO tissues. Thus, these data

strongly indicate that *Tbx20* and CM specific markers have markedly expressed in E: 4 PEO in developing avian embryos.

Figure 2



[Figure 2: Marker gene expression analyses in chick embryonic proepicardial organ (PEO). A: qRT-PCR was performed on synthesized cDNA from RNA isolated from E: 3 & E: 4 PEO tissues. This result indicates markedly increased expression of

*Tbx20, Mef2c and Nkx2.5 in E: 4 PEO than E: 3 PEO. Although similar expression was detected for Gata4 in both E: 3 and E: 4 PEO tissues. B: Real Time PCR was performed to quantify the fold change in Tbx20, Mef2c, Gata4 and Nkx2.5 expression in E: 3 versus E: 4 PEO tissues. In this data, Tbx20, Mef2c, Gata4 and Nkx2.5, all the markers are upregulated by 1.25 fold ($p=0.05$), 7.31 fold ($p=0.04$), 1.59 fold ($p=0.05$) and 1.91 fold ($p=0.007$), respectively (I-IV). Statistical significance was determined by Student's t-test, where * denotes $p\leq 0.05$. ($n = 3$)*

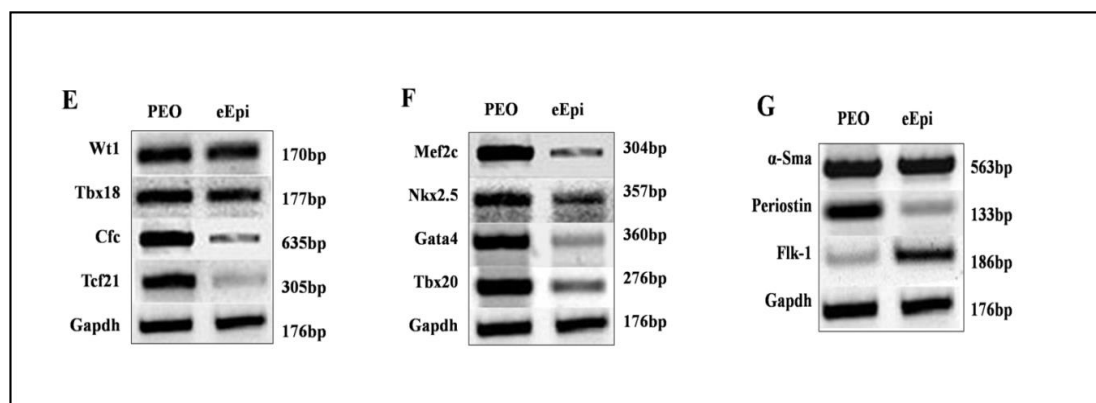
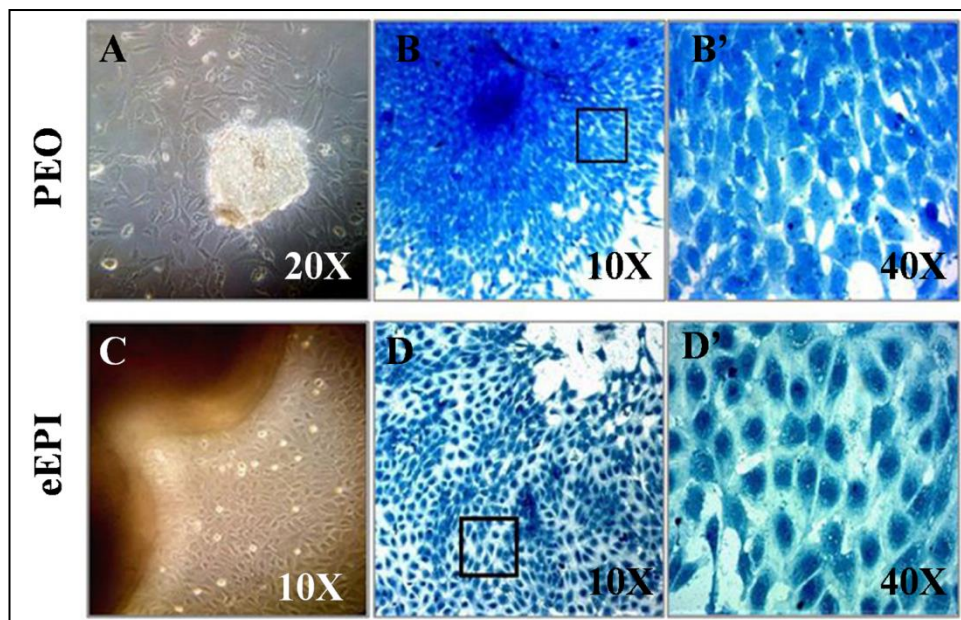
c. Increased level expressions of CM lineage specific marker genes have been detected in PEO derived cells compared to eEpi cells

For *in vitro* studies, both primary avian PEO and eEpi explant cultures have been established from E: 4 and E: 5 embryos respectively. The bright-field image is viewed in figure 3A which shows the PEO monolayer cells, migrated and grown from explanted PEO tissue. Next, to determine the overall cellular morphology, coomassie brilliant blue stain was used to stain the cells in cultures. Bright-field images are also shown in figure 3B, B' which depict the diverse and multiple cardiac cell types with distinct morphologies. In contrast, figures 3C, D and D' have shown a typical cobblestone like embryonic epicardial cell morphology, which is detected by both the bright field images of unstained and coomassie brilliant blue Stained images. Overall, it is conclusive that both PEO and eEpi explant cultures have been successfully established.

Differential gene expression patterns of various cardiac lineage specific cell markers have been analysed by semi qRT- PCR with 1 μ g of RNA template used to synthesize cDNA, after successfully establishing PEO and eEpi explant cultures. At first, as expected, it is detected that PEO specific *Cfc* and *Tcf21* have increased expressions in PEO explant cultures, whereas both PEO and eEpi specific *Wt1* and *Tbx18* have shown comparable expression in both PEO versus eEpi (figure 3E). In addition, CM specific markers (*Mef2c*, *Nkx2.5*, *Gata4*) have resulted in increased band intensity in PEO cells compared to eEpi cells. Similarly, increased *Tbx20* specific band intensity has been detected in PEO cells (figure 3F). Additionally, the gene expression pattern of a small number of non-CM markers like smooth muscle specific α -*Sma* (Alpha smooth muscle actin), fibroblast specific *Periostin* and endothelial lineage specific

Flk-1, have also been analysed. Here, *α-Sma* indicates very much intense expression in both PEO and eEpi whereas *Periostin* indicates the increased expression in PEO compared to eEpi cells. Moreover, *Flk-1* has demonstrated increased expression in eEpi compared to PEO (figure 3G). *Gapdh* is used as loading control. Hence, above data suggest that several cardiac lineage specific markers, markedly CM markers have shown upregulated expression in PEO cells compared to eEpi cells and other non-CM lineage specific markers have been divergently expressed in both PEO cells and eEpi cells in culture. Overall, these data might demonstrate a new insight about the differentiation potential of both PEO and eEpi progenitor cell pools towards several cardiac lineage specialization at least in explant culture.

Figure 3



[Figure 3: Several cardiac lineage specific marker gene expression analyses on embryonic chick primary Proepicardial organ (PEO) and embryonic epicardial (eEpi) explant cultures. (A-B') Establishment of Chick primary PEO explant culture. A: Bright field image (20X) of primary chick PEO explant culture. Based on aggregated and grape like cell morphology, PEO had been dissected out from atrio-ventricular (AV) groove from chick embryo at embryonic day 4 (E: 4) and cultured for 24hrs. B: For identification of PEO cellular morphology, bright-field images (10X) are taken from cultured primary chick PEO derived cells after staining with Coomassie brilliant blue. B': Magnified view (40X) of boxed region of image B for better visualization of PEO cell morphology in explant culture. (n=3) (C-D') Establishment of Chick primary eEpi culture. C: Bright-field image (10X) of primary chick embryonic epicardial cells. At E: 5, chick whole hearts were isolated from embryo and harvested for 24hrs. Cells were migrated from outer epicardial layer. D: For further identification of cobblestone like cell morphology of epicardial cells, Coomassie brilliant blue staining was performed and bright field images (10X) were taken. D': Magnified view (40X) of boxed region of image B for better visualization of embryonic epicardial cell morphology in explant culture. (n=3) E: PEO specific *Wt1*, *Tbx18*, *Cfc* and *Tcf21* marker gene expression analyses by qRT-PCR in both PEO and eEpi derived cells. *Wt1* and *Tbx18* express similarly in both the explants where as developmental PEO specific *Cfc* and *Tcf21* were increasingly expressed in PEO than eEpi derived cells. F: Cardiomyocyte (CM) specific marker genes including *Tbx20* expression had been detected also by qRT-PCR. *Mef2c*, *Gata4*, *Nkx2.5* and *Tbx20* mRNA were all increased in PEO compared to eEpi derived cells. (n=3) G: Gene expression analyses of other cardiac non CM lineage markers including smooth muscle specific, α -*Sma*; fibroblast specific, *Periostin*; and endothelial specific, *Flk1* have also been demonstrated. α -*Sma* strongly expressed in both PEO and eEpi derived cells where as *Periostin* shows increased expression in PEO compared to eEpi derived cells and *Flk1* shows increased expression in eEpi compared to PEO derived cells.(n=3)]

d. Wnt signaling manipulation in eEpi cells indicate increased CM lineage specific gene expression in vitro

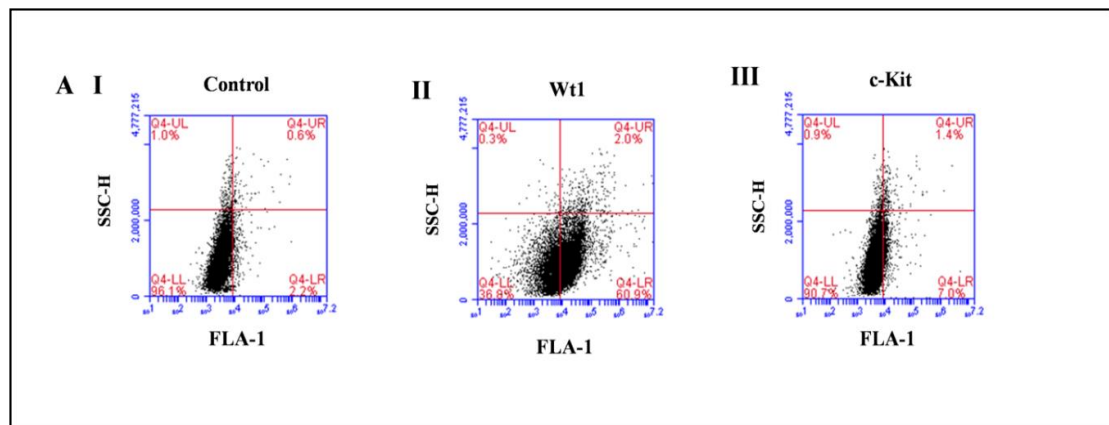
After successful establishment of eEpi cells in culture, flow cytometry has been done using epicardium specific marker Wt1, in order to find out the enrichment of epicardial specific cells in culture. Additionally, we have also applied c-Kit (CD117) as it is known as one of the early cardiac progenitor marker as a positive control, to compare with Wt1 marked cells. However, the percentages of Wt1⁺ cells and c-Kit⁺ cells are determined in eEpi cells in culture. Results from flow cytometric quadrants are portrayed in Figure 4 A I-III and here the respective numbers of Wt1⁺ cells and c-Kit⁺ cells have been recorded. Figure 4A I depicts the negative population of both c-Kit⁺ cells and Wt1⁺ cells which is 2.2% of negative control, whereas figure 4A II & III show the 60.9% of Wt1⁺ cells and 7% of c-Kit⁺ cells in that cultured population respectively. This data distinctly suggest the salient enrichment of Wt1⁺ cells in an isolated eEpi cell population *in vitro*. Furthermore, as expected, only 7% of c-Kit⁺ cells clearly demonstrate that eEpi cell populations get mostly differentiated beyond early cardiac progenitor markers like c-Kit *in vitro*.

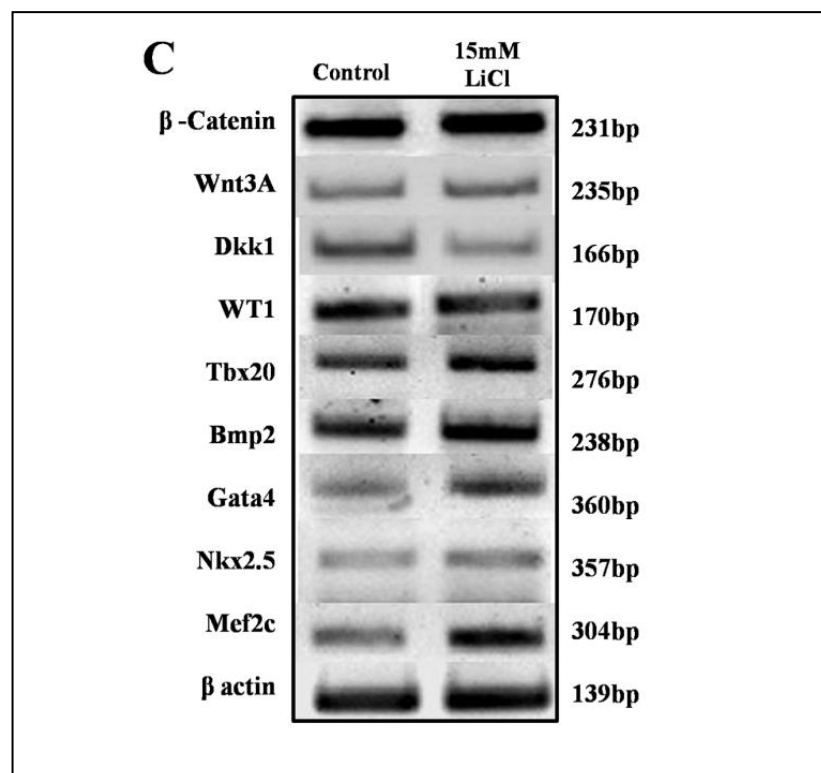
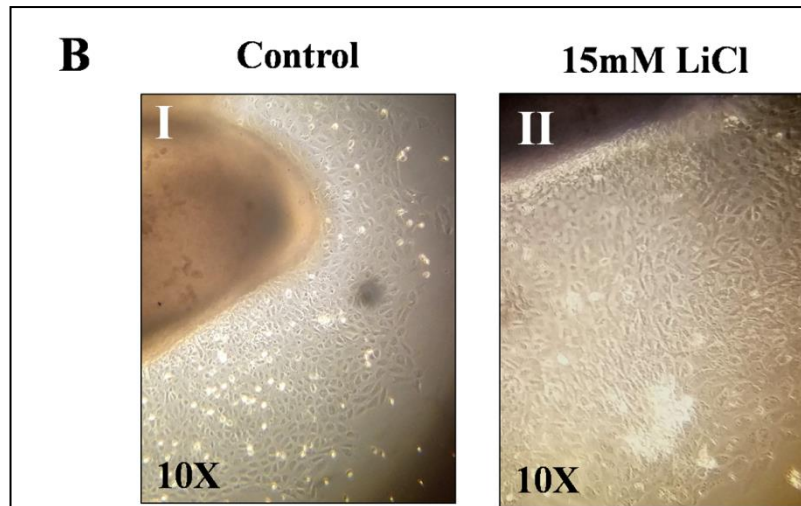
After validation of Wt1 enriched population of eEpi cells, Wnt/ β -catenin signaling has been manipulated on avian primary eEpi explant cultures by treating with 15mM of LiCl. At first, in figure 4B I & II, bright-field images of both LiCl treated and untreated control eEpi explant cultures have been shown. After that, semi qRT-PCR and real time PCR data show that the Wnt signaling specific agonist gene markers, β -catenin, *Wnt3A*, expressions have been significantly increased, whereas antagonist of Wnt signaling, *Dkk1* expression is decreased upon LiCl treatment on eEpi cells. Moreover, expression of *Tbx20*, and its downstream target gene *Bmp2* and CM specific markers (*Mef2c*, *Gata4*, *Nkx2.5*) are also significantly increased in LiCl treated eEpi culture by semi qRT-PCR (figure 4C). Likewise, in consistency with semi qRT-PCR data, qRT-PCR data have validated that β -catenin, *Wnt3A*, *Tbx20*, *Bmp2*, *Mef2c*, *Gata4*, *Nkx2.5* mRNA have been increasingly expressed (2.37, 2.96, 1.29, 7.31, 13.64, 1.07, 4.53 folds respectively) and *Dkk1* mRNA has been decreasingly expressed (0.84 fold) in LiCl treated eEpi cells compared to untreated control eEpi cells (figure 4D I-VIII). In contrast, we have also analysed the mRNA expressions of several non-CM marker genes (*α -SMA*, *Periostin*, *Flk-1*), which have shown unchanged expressions upon LiCl treatment on eEpi cells compared to untreated controls (Figure 4 E and F I-III). Although, *Periostin* have indicated an increased expression trend in LiCl treated eEpi cells, but it is insignificant. Hence all these data firmly suggest that Wnt signaling

activation via LiCl treatment promotes increased expressions of CM lineage specific marker genes in eEpi cells in cultures.

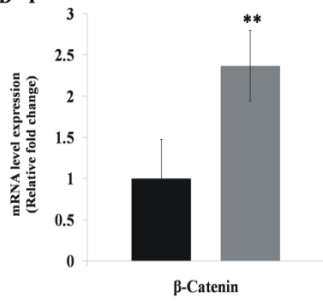
Altogether, these data strongly demonstrate that there might be a relatively unknown regulatory mechanism of Wnt/ β -catenin signaling mediated CM lineage specification in eEpi cells *in vitro*.

Figure 4

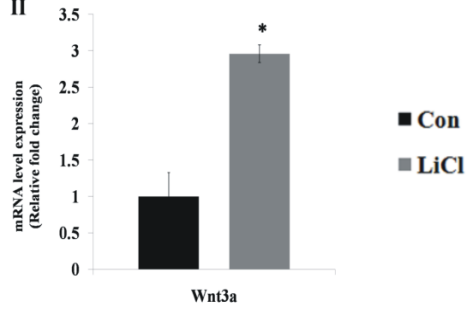




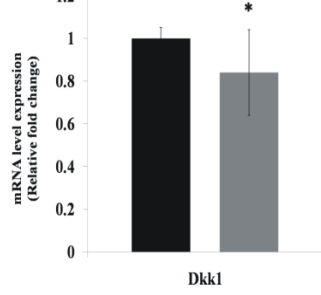
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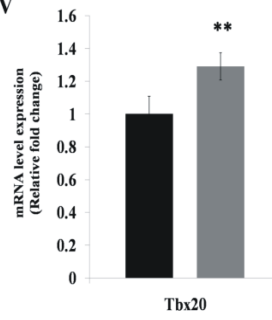
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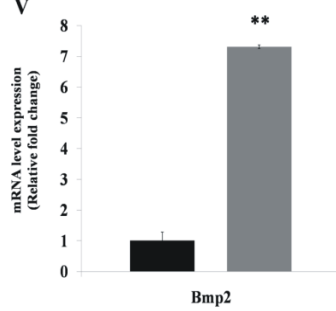
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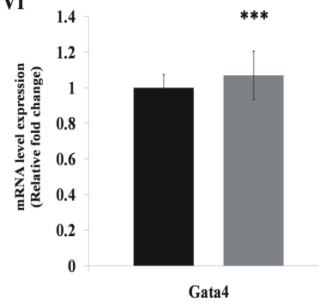
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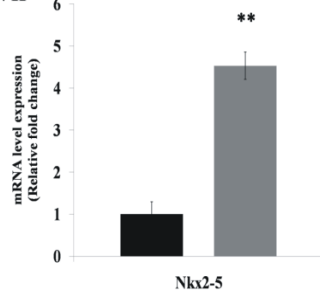
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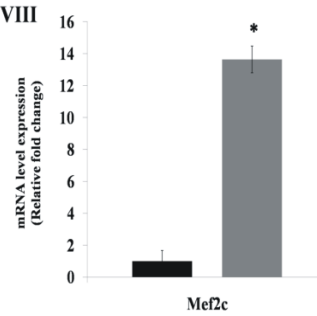
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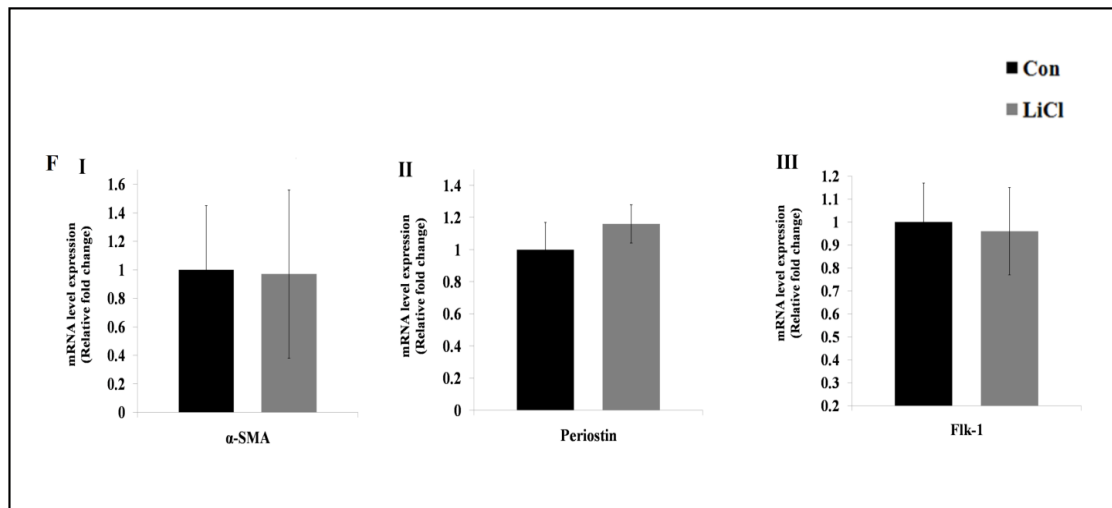
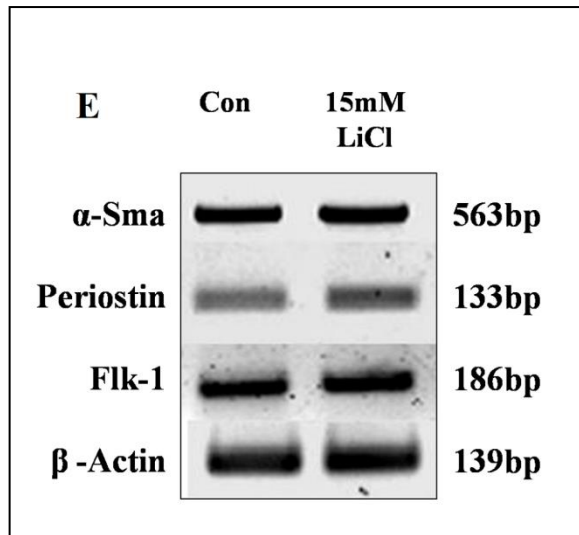


VII



VIII





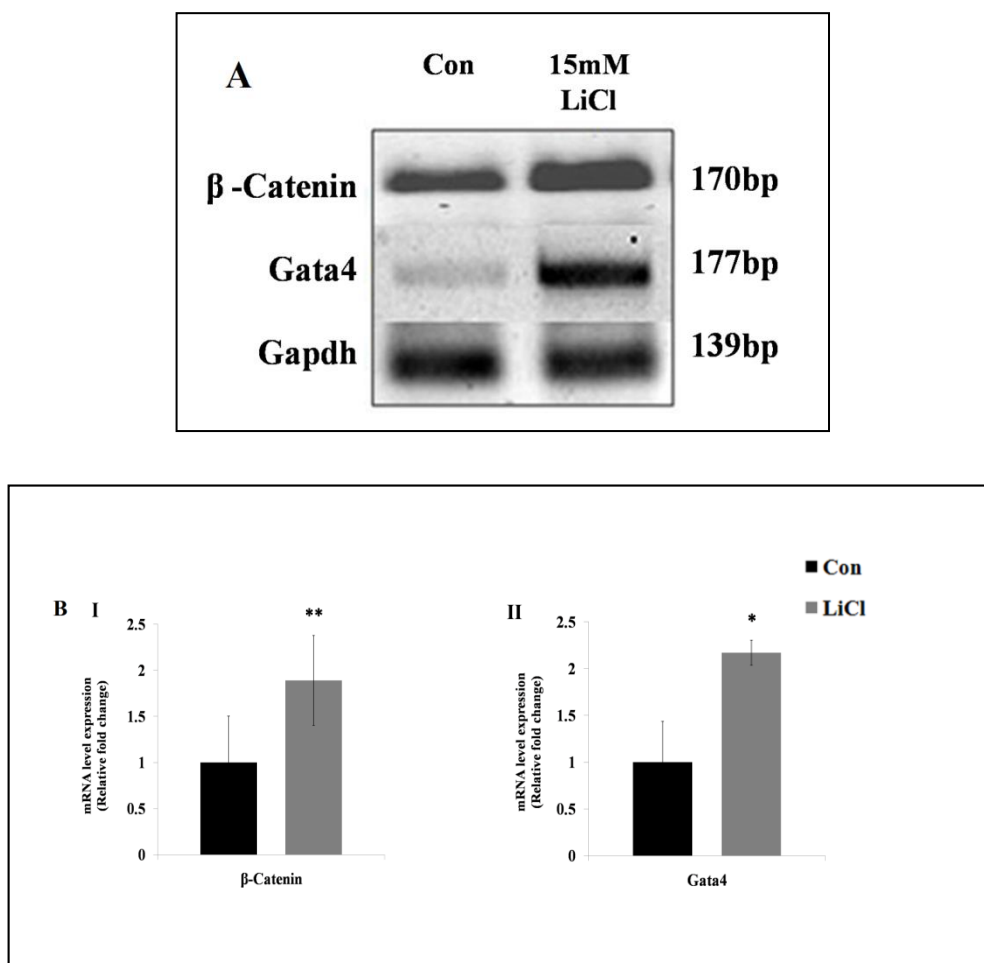
[Figure 4: Manipulation of Wnt signaling alters eEpi cell morphology with increased CM lineage specific gene expression. (A I-III): Percentage enrichment of epicardium specific cells was detected in eEpi explant culture by flow cytometric technique. Increased percentage (60.9%) of $Wt1^+$ cells was detected compared to only 2.2% unlabeled controls. In addition, only 7.0% $c\text{-Kit}^+$ cells were also detected, indicative of very few cardiac progenitor cells within the isolated eEpi explant culture with increased epicardial lineage specificity. **(B I, II):** Chick primary eEpi explant cultures were established and treated with 15mM Lithium Chloride (LiCl), the known activator of Wnt/ β -catenin signaling pathway. Bright-field images (10X) are taken from both control and treated cells. **C:** Gene expression analyses of Wnt signaling specific β -Catenin, *Wnt3A* and *Dkk1*; epicardium specific *Wt1*; *Tbx20*, *Bmp2* and CM

lineage markers (*Gata4*, *Nkx2.5* and *Mef2c*) by qRT-PCR. All these marker genes except *Wt1* and *Dkk1* have shown increased expression in LiCl treated eEpi cells compared to untreated controls. β -actin was used as loading control. **(D I-VIII):** Relative mRNA level expressions have been further validated by Real Time PCR. β -catenin, *Wnt3A*, *Tbx20*, *Bmp2*, *Mef2c*, *Gata4* and *Nkx2.5* were all increased by 2.37 fold ($p=0.01$), 2.96 fold ($p=0.04$), 1.29 fold ($p=0.005$), 7.31 fold ($p=0.005$), 13.64 fold ($p=0.03$), 1.07 fold ($p=0.0005$), 4.53 fold ($p=0.01$) respectively. Only *Dkk1* mRNA expression was decreased by 0.84 fold ($p=0.05$). Statistical significance was determined by Student's *t*-test, where * denotes $p \leq 0.05$. ($n=3$) **E:** Gene expression analyses of other cardiac lineage markers including smooth muscle specific α -Sma, fibroblast specific *Periostin* and endothelial specific *Flk-1* in control and LiCl treated eEpi cells. Here, α -Sma shows unchanged expression in both control and LiCl treated eEpi cells whereas *Periostin* and *Flk-1* show similar trend of CM marker expression in both. **F:** Relative fold change of mRNA level expression of these other lineage markers including α -Sma, *Periostin* and *Flk-1* have been quantified by Real Time PCR. All these markers show insignificant changes of their gene expression comparing between control and LiCl treated eEpi cells. ($n=3$)]

e. Wnt signaling manipulation also promotes specific marker gene expression in undifferentiated rat myoblast cells in culture

To further confirm the effects of LiCl, H9c2 (an undifferentiated rat myoblast) cells are treated with 15mM LiCl, which also indicates similar results (Figure 5 A and B I-II). Here also, increased gene expression pattern of β -catenin and selective CM specific *Gata4* expression have been analysed by semi qRT-PCR (Figure 5 A). Quantitative data have shown that in LiCl treated cells, β -catenin and *Gata4* mRNA have significantly increased by 1.89 fold and 2.17 fold respectively as compared to untreated H9c2 cells (Figure B I and II). Overall, these data depict that when β -catenin gets stabilized, it might enhance the CM lineage differentiation as marked by increased expression of *Gata4* mRNA level in myoblast cells in culture.

Figure 5



[Figure 5: Lithium chloride (LiCl) also stabilizes β -Catenin in H9C2 cells. A: Gene expression analyses by reverse transcriptase PCR of β -catenin and Gata4. Upon LiCl treatment both β -catenin and Gata4 have shown elevated expression compared to untreated control. B: This data is further validated by quantitative real time PCR. Here β -catenin and Gata4 mRNA level have been increased by 1.89 fold (Figure 5B I) and 2.17 (Figure 5B II) fold respectively. Statistical significance was determined by Student's *t*-test, where * denotes $p \leq 0.05$.]

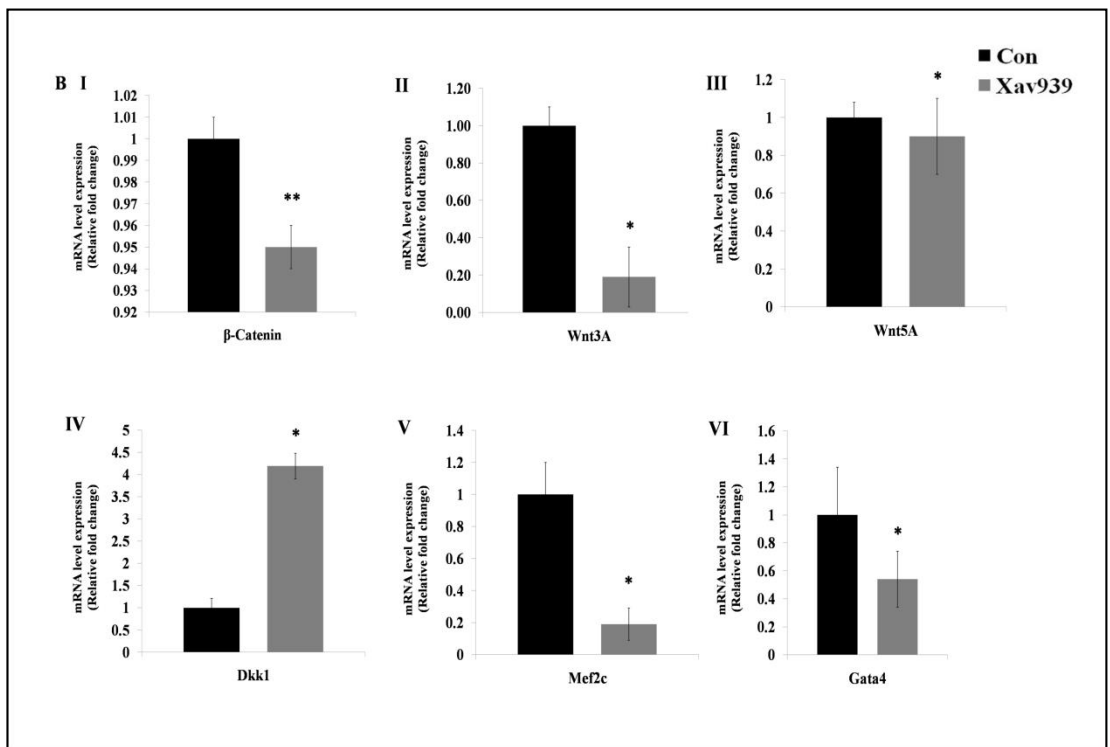
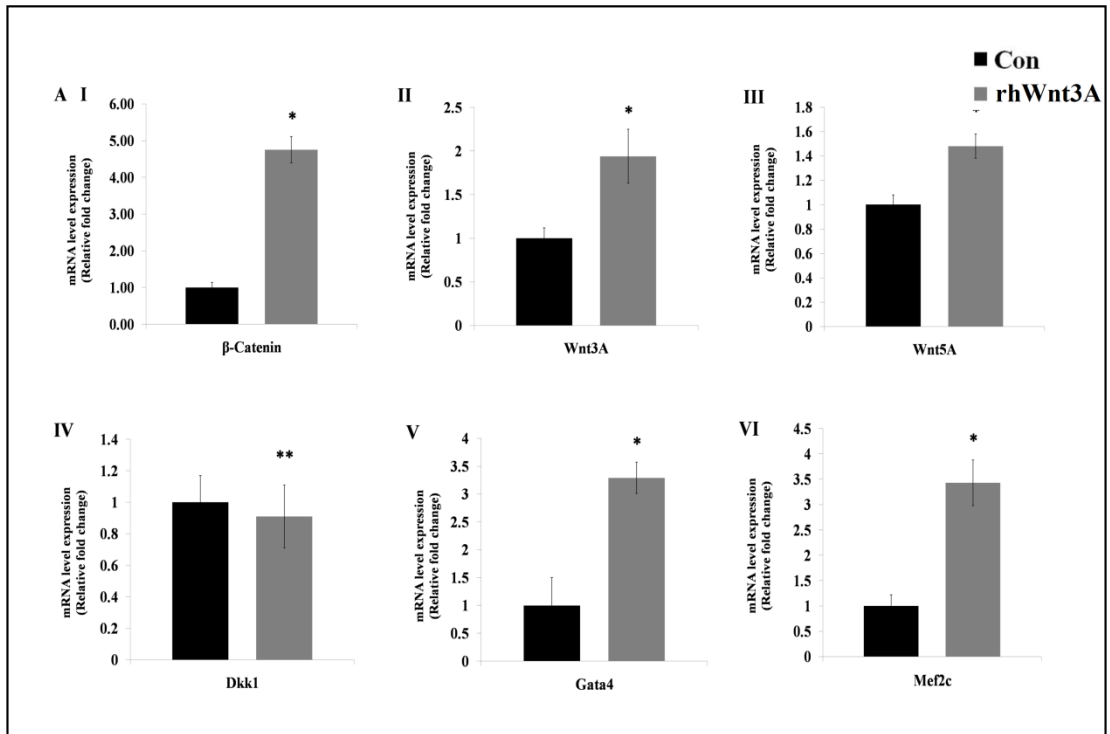
f. Wnt signaling manipulation specifically affects CM lineage specific marker gene expression

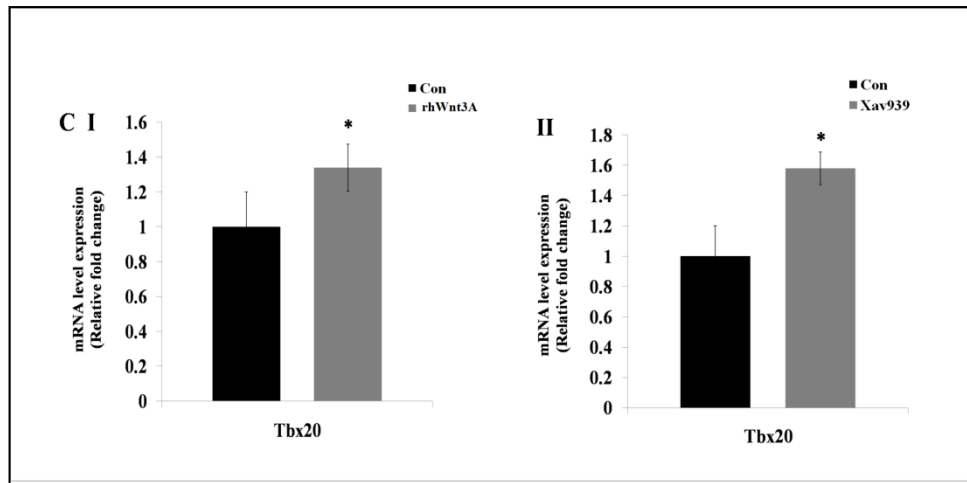
Next, to further validate the manipulation of Wnt/ β -catenin signaling in CM lineage specification, eEpi cells have been further applied with either human Wnt3A

recombinant protein (rhWnt3A) or Xav939. Then, qRT-PCR data have been analysed and these show that activation of Wnt signaling by rhWnt3A treatment, results in increased, whereas, inhibition of Wnt signaling via Xav939 treatment, results in decreased expression of selective CM specific marker genes, compared to respective untreated control eEpi cells. Likewise, in figure 5A I-VI, qRT-PCR data show that *β-catenin*, *Wnt3A*, *Wnt5A*, *Gata4* and *Mef2c* mRNA are all significantly increased by 4.75, 1.94, 1.48, 3.29, 3.43 fold respectively, compared to 0.91 fold decrease in *Dkk1* mRNA upon rhWnt3A mediated Wnt signaling specific activation. In contrast, figure 5B I-VI show the mRNA expression of *β-catenin*, Wnt targeted genes (*Wnt3A*, *Wnt5A*) and CM specific marker genes (*Gata4* and *Mef2c*) have all significantly downregulated by 0.95, 0.19, 0.9, 0.54, 0.19 fold respectively, whereas *Dkk1* is significantly upregulated by 4.19 fold upon Xav939 mediated Wnt signaling specific inhibition. In figure 5C I and II, *Tbx20* mRNA expression is also checked in both rhWnt3A treated and Xav939 treated in eEpi cells. Here, very importantly *Tbx20* get upregulated by 1.34 fold and 1.58 fold, respectively in rhWnt3A treated and Xav939 treated eEpi cells.

Thus, all the above data have suggested that Wnt signaling specific manipulation through biological modulators, directly implicate in the area of CM lineage differentiation of eEpi cells in cultures.

Figure 6





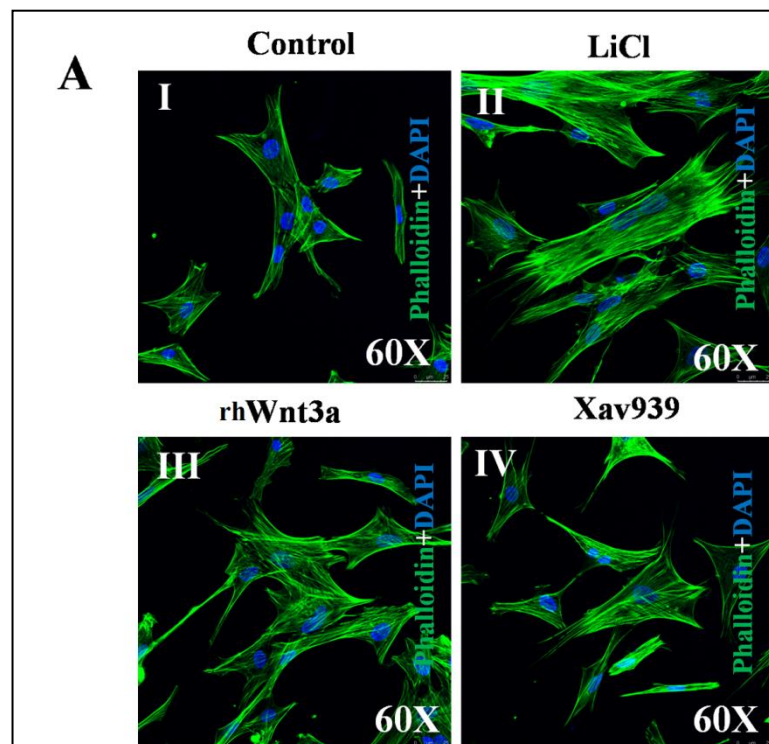
[Figure 6: *Wnt* signaling manipulation alters the CM specific marker gene expression in eEpi cells. (A I-VI): *Wnt3A* recombinant protein (rhWnt3A) treatment in eEpi cells increases CM marker gene expression: Gene expression analyses of *Wnt* signaling and CM specific markers have been performed by qRT-PCR analyses. mRNA expressions of β -catenin, *Wnt3A*, *Wnt5A*, *Gata4*, *Mef2c* were all increased by 4.75 fold ($p=0.05$), 1.94 fold ($p=0.046$), 1.48 fold ($p=0.023$), 3.29 fold ($p=0.031$), 3.43 fold ($p=0.047$), respectively. Only *Dkk1* mRNA expression was decreased by 0.91 fold ($p=0.006$). (B I-VI): In contrast, *Xav939* treatment in eEpi cells decreases CM marker gene expression: Gene expression analyses of *Wnt* signaling and CM specific markers have been performed by qRT-PCR analyses. mRNA expression of β -catenin, *Wnt3A*, *Wnt5A*, *Gata4*, *Mef2c* was all decreased by 0.95fold ($p=0.009$), 4.19 fold ($p=0.055$), 0.9 fold ($p=0.053$), 0.54 fold ($p=0.043$), 0.19 fold ($p=0.038$), respectively. Only *Dkk1* mRNA expression was increased by 4.19 fold ($p=0.048$). (C I-II): **Tbx20** expression in both rhWnt3A and Xav939 treated eEpi culture: mRNA expression of *Tbx20* was increased by 1.34 fold ($p=0.05$) and 1.58 fold ($p=0.03$) in rhWnt3A and Xav939 treated, respectively. Statistical significance was determined by Student's *t*-test, where * denotes $p\leq 0.05$. ($n=3$)]

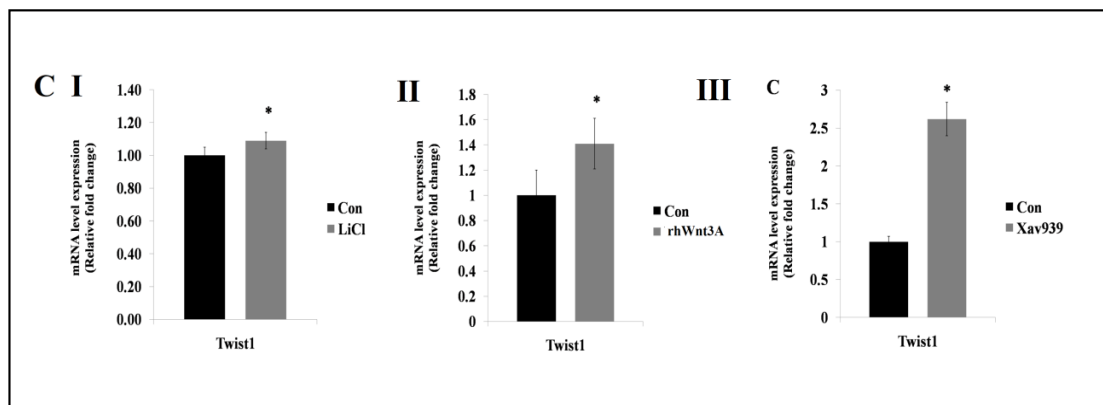
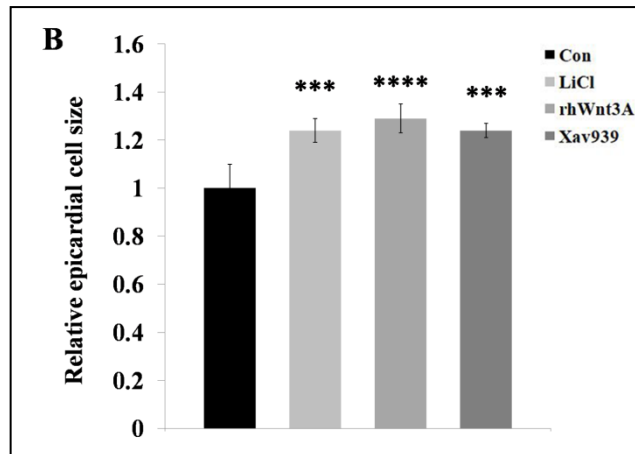
g. Wnt signaling manipulation also affects cytoskeletal rearrangements in embryonic epicardial (eEpi) cells:

Interestingly, treated eEpi cells have been found to indicate such distinguishable alteration in their typical cobblestone to elongated mesenchymal morphology. Phalloidin, which marks the cytoskeletal F-actin protein, distinctly differentiates between treated and untreated control eEpi cells. Figure 7 A and B have shown that when LiCl or rhWnt3A or Xav939 are treated to eEpi cells, then in all cases, treated eEpi cells show an increased cell size with elongated actin filaments compared to untreated control eEpi cells. Cell size difference is also detected using Image J software (NIH). Minimum 2500 cells are measured in each set of experiments in 20X. It reveals cell size increase by 1.24 (LiCl treated), 1.29 (rhWnt3A treated) and 1.24 (Xav939 treated) fold compared to untreated eEpi cells.

In consistency with changed mesenchymal phenotypes of treated eEpi cells, increased mRNA expression of *Twist1*, a mesenchymal marker associated with EMT, is also detected in all three treatment groups (1.09, 1.41 and 2.62 fold respectively upon LiCl, rhWnt3A and Xav939 treatment) (Figure 7 I-III).

Figure 7





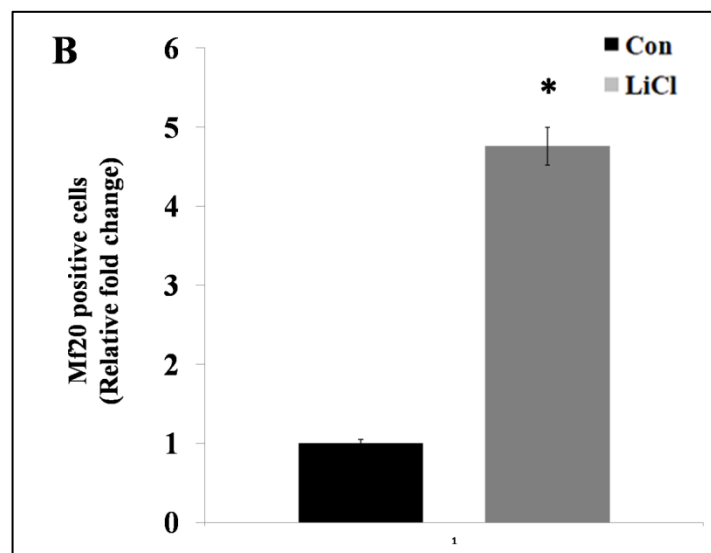
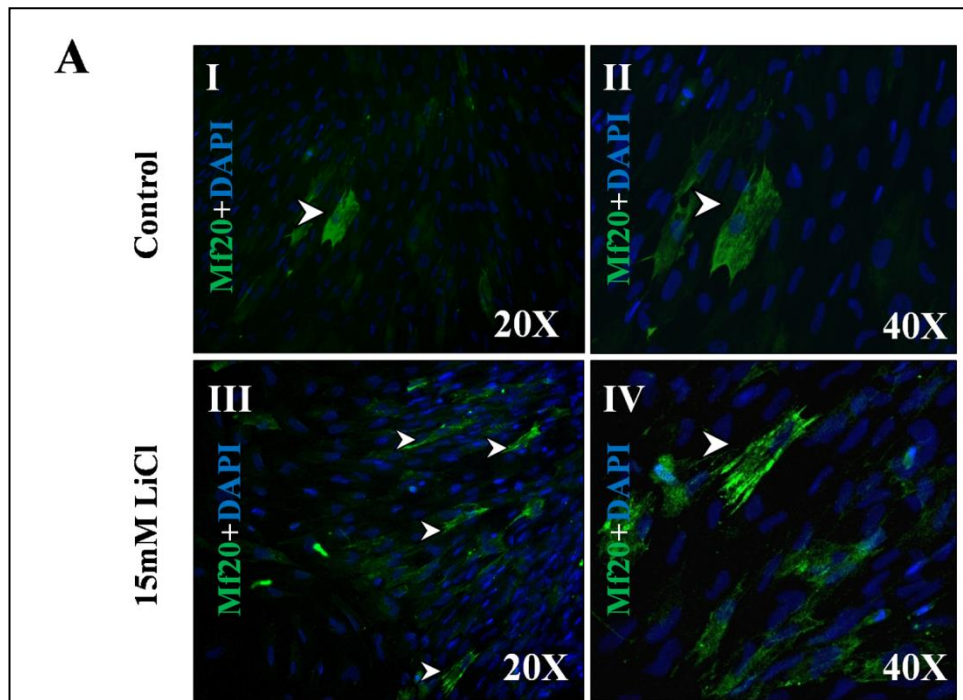
[Figure 7: Wnt signaling manipulation increases cellular size and alters morphology in embryonic epicardial (eEpi) cells. (A I-IV): Representative images are showing the increased eEpi cell sizes and altered morphology in LiCl, Wnt3A recombinant protein and Xav939 treated eEpi cells compared to untreated control cells. B: Cell size increase has been quantified by using Image J software. It is increased in LiCl, Wnt3A recombinant protein and Xav939 treated eEpi cells than in control cells by 1.24 fold, 1.29 fold and 1.24 fold respectively. Statistical significance was determined by Student's t-test, where * denotes $P \leq 0.05$. (C I-III): Gene expression analyses of Twist1 by qRT-PCR. It has been shown that Twist1 expression is increased in LiCl, rhWnt3A recombinant protein and Xav939 treated eEpi cells than in control cells by 1.09 fold, 1.41 fold and 2.62 fold respectively. Statistical significance was determined by Student's t-test, where * denotes $P \leq 0.05$.]

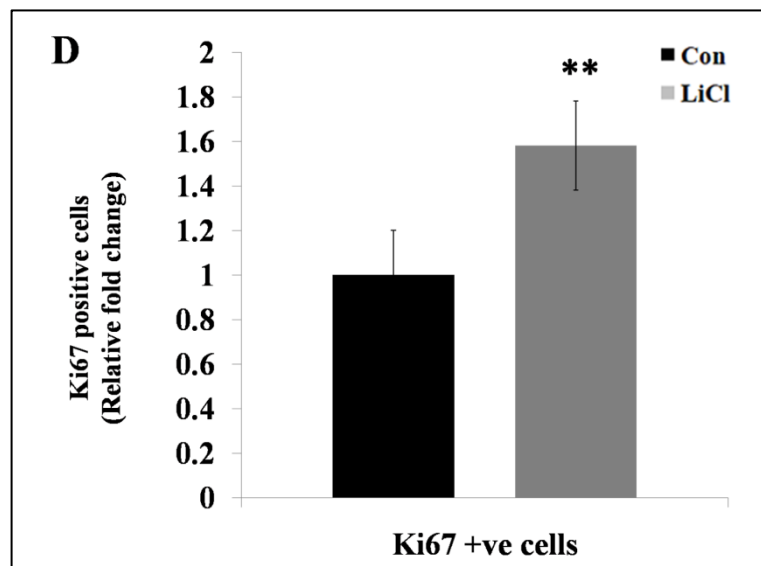
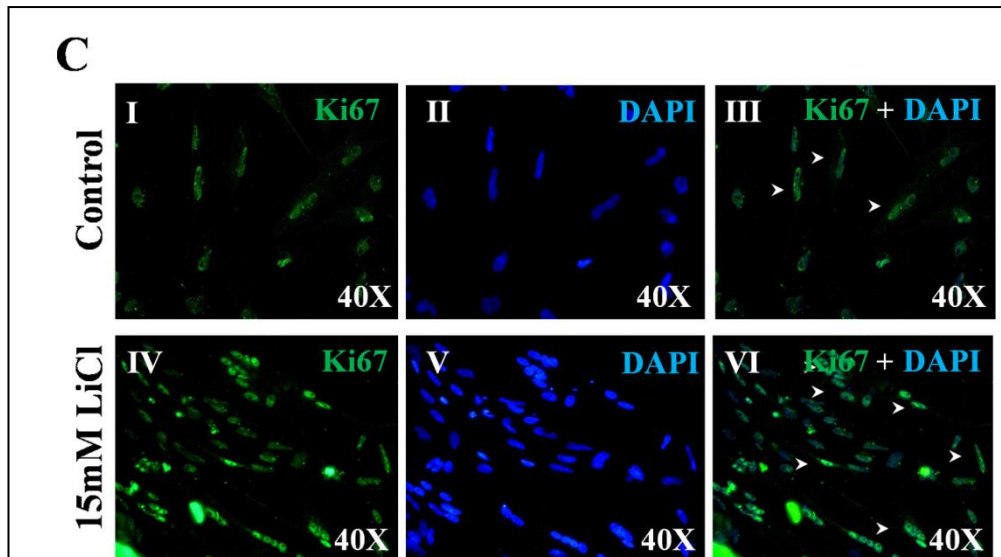
h. Increased level of β -catenin promotes increased number of proliferating cardiomyocyte in eEpi explant culture

Next, to see the effect of Wnt signaling activation on CM differentiation, immunostaining has been performed on both LiCl treated and untreated eEpi cells using cytoplasmic sarcomeric myosin specific Mf20 antibody. Surprisingly, greater numbers of Mf20⁺ cells (arrowhead in figure 6A III) are determined compared to less number of Mf20⁺ cells in untreated controls (arrowheads in figure 8A I). Likewise, higher magnification images have been also shown in figure 8A II and IV, respectively. Now, quantitative data show 4.76 fold upper of Mf20⁺ eEpi cells in LiCl treated compared to untreated control eEpi cells (figure 8B). Minimum 500 cells as DAPI⁺ are considered for analyses in each set of experiments (n=3). Overall, LiCl mediated Wnt signaling activation enhances increased number of Mf20⁺ cells in eEpi explant culture. In addition, proliferative indices have also been analysed in both LiCl treated and untreated eEpi explant cultures. Ki67, known marker of proliferating cells, has been used for immunoreactivity and to detect proliferating eEpi cells in explant cultures. Here also, greater number of Ki67⁺ nuclei are detected in LiCl treated eEpi cells (Figure 8sC, IV-VI) compared to untreated controls (Figure 8C, I-III). Next, quantitative data shows around 1.58 fold upregulated Ki67⁺ cells in LiCl treated eEpi cells (figure 6D). Minimum 400 cells as DAPI⁺ are counted in each set of experiments (n=3).

Overall these all data demonstrate that LiCl mediated Wnt signaling activation via stabilization of β -catenin upsurses the generation of greater number of cycling CMs.

Figure 8





[Figure 8: Lithium chloride (LiCl) treatment promotes proliferation with increased number of Mf20 positive cells in eEpi explant culture. (A I-IV): Chick primary epicardial explant cultures were established and treated with 15mM Lithium Chloride (LiCl). Fluorescent images (10X) were taken from both control and treated cells. These control and LiCl treated eEpi cells were immunostained by Mf20 antibody to mark sarcomeric myosin (green) counterstained with DAPI (Blue) positive nuclei. Mf20 positive cells were marked by arrowheads. This data depict increased number of Mf20 positive cells in LiCl treated eEpi cells (III, IV) compared to untreated control (I, II). **(B):** Increased number of Mf20 positive cells were represented statistically which is around 4.76 fold ($p \leq 0.05$). **(C I-VI):** Proliferative indices were detected using

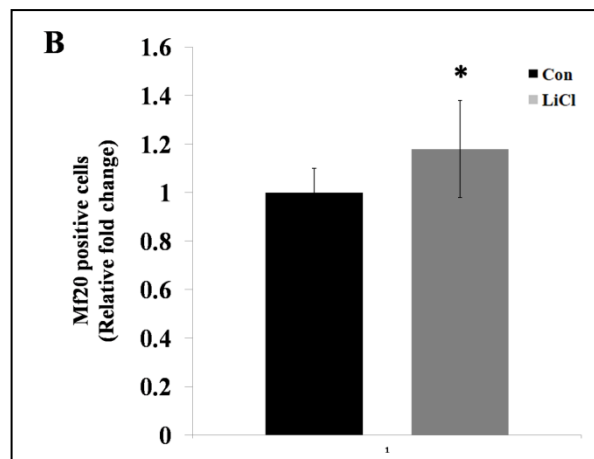
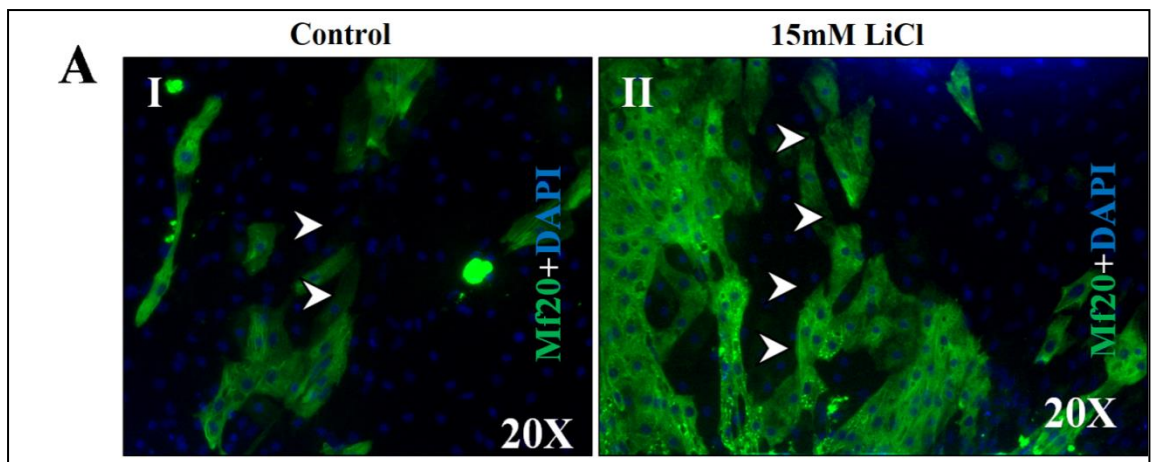
Ki67 specific antibody (Green) counterstained with DAPI (Blue) positive nuclei. Ki67+DAPI stained cells were marked by arrowheads in C (III) and F (VI). (D): Here, quantification of the data demonstrated that LiCl treated cells show increased cell proliferation as detected by 1.58 fold increase ($p \leq 0.05$) compared to untreated control eEpi cells. (n=3)]

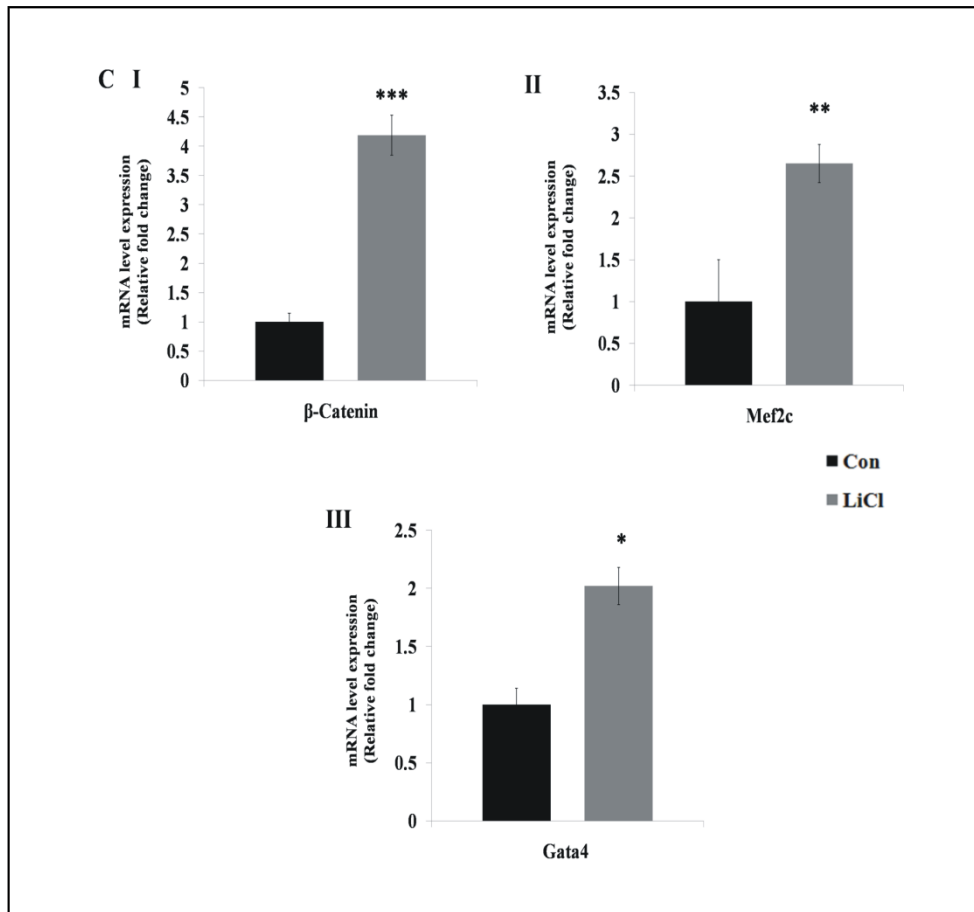
i. Increased level of β -catenin also promotes more number of CMs in PEO explant culture

Previously, in figure 3, Semi qRT-PCR data have shown the increased expression of CM lineage specific markers in isolated PEO compared to eEpi. Therefore, to check, if PEO explant culture shows similar and/or better cardiomyogenic potential following LiCl treatment, PEO explant cultures are also experimented with LiCl. Likewise, Mf20 immunoreactivity has also been applied to find out CM number in 15mM LiCl treated PEO explant culture for 24 hrs (arrowhead in figure 9A II) compared to untreated controls (arrowheads in figure 9A I) and quantified as 1.18 fold upregulation in treated cells (figure 9B). In consistency with the increased number of Mf20⁺ eEpi cells, increased expression of β -Catenin and two CM lineage specific marker genes (*Gata4*, *Mef2c*) have also been analysed. In 15mM LiCl treated PEO explant culture, expression of β -Catenin (4.19 fold), *Gata4* (2.02 fold), *Mef2c* (2.51 fold) get increased compared to untreated PEO cells (figure 9C I-III). Therefore, due to increased basal level of CM lineage marker gene expression in PEO derived cells compared to eEpi cells, LiCl mediated CM differentiation effect is much more pronounced in treated eEpi cells (4.76 fold) compared to treated PEO derived cells (1.18 fold), although both cell populations show cardiomyogenic potential upon LiCl treatment in culture.

Overall, upon LiCl treatment, PEO derived cells also show cardiomyogenic potential as evidenced by increased number of Mf20 labeled CM along with increased level of CM marker gene expression compared to untreated control cells.

Figure 9





[Figure 9: Lithium chloride treatment promotes increased number of Mf20 positive cells and CM marker gene expression in PEO explant culture. (A I-II): Chick primary PEO explant cultures were established and treated with 15mM Lithium Chloride (LiCl). Fluorescent images (10X) are taken from both control and treated cells. Control and LiCl treated PEO cells were immunostained by Mf20 antibody to mark sarcomeric myosin (green) counterstained with DAPI (Blue) positive nuclei. Mf20 positive cells are marked by arrowheads. This data depict increased number of Mf20 positive cells in LiCl treated PEO cells compared to untreated control. **B:** Increased number of Mf20 positive cells were represented statistically which is around 1.18 fold ($p \leq 0.05$). **(C I-III): LiCl treatment in PEO derived cell upregulates CM marker gene expression:** Gene expression analyses of CM markers have been performed by qRT-PCR detecting mRNA level. β -catenin, Gata4, Mef2c was all increased by 4.19 fold ($p=0.0009$), 2.02 fold ($p=0.015$), 2.51 fold ($p=0.040$), respectively. ($n=3$)]

D. Discussion

During vertebrate cardiogenesis, epicardium is derived from the extracardiac precursor organ, proepicardial organ or PEO, which is situated at the venous pole of the embryonic heart (Asli et al., 2014; C. M. Braitsch et al., 2013). PEO is known to be differentiated from the splanchnic mesoderm at HH stage 20. PEO can be defined as a group of multipotent mesothelial cells, located at the atrio-ventricular (AV) junction between liver and cardiac sinus venosus (Jenkins et al., 2005). Epicardial development occurs when Proepicardium derived cells start to migrate over the whole myocardium and gradually form the outermost epicardium (Schlueter & Brand, 2013). At later stages in utero, embryonic epicardium (eEpi) as epicardial derived cells or EPDCs differentiate into various cardiac lineage specific cells directly. EPDCs widely contribute to give rise to important cardiac lineage specific cell types, including smooth muscles, cardiac fibroblasts, coronary endothelial cells and even cardiomyocytes (CMs) as represented schematically in the proposed model in Figure 8 (Kennedy-Lydon & Rosenthal, 2015; Smart et al., 2011). A couple of reports have distinguishably revealed many theories about EPDC differentiation and their association, involvements and also contribution in cardiac repair and regeneration (Austin et al., 2008; C. L. Cai et al., 2008; Srivastava, Saxena, Dimaio, & Bock-Marquette, 2007; Wessels & Pérez-Pomares, 2004). From the developmental aspects, EPDCs are originated from PEO, thus, there must be interrelated complex molecular mechanisms and regulatory pathways between PEO derived cells and EPDCs, which needs further exploration.

Significant number of reports in cardiac repair and regenerative biology, unveiled that EPDCs are involved in CM differentiation (C. L. Cai et al., 2008; Smart et al., 2011; Wessels & Pérez-Pomares, 2004). Strikingly, another report has also demonstrated the CM differentiation potential of EPDCs due to great changes in gene expression profile during PEO to epicardium development, termed as 'Epicardial lock' (Buermans et al., 2010). Genome Ontology (GO) based gene expression data clearly reveal that selective gene clusters, including cell cycle progression (*AurkA*, *cdc2*), DNA maintenance (*Msh2*, *Exo1*), ECM composition and maintenance (*Col1a2*, *Col3A1*) and associated signaling pathways (Tgfb β , Ras) are significantly altered between PEO and eEpi development. Likewise, in the support, our data also detected *Tbx20*; a T-box transcription factor gene, increasingly expressed in PEO derived cells compared

to eEpi cells in developing avian embryo. Many other investigators have also described about EPDCs' role and contribution in cardiac differentiation but less information is available on differentiation potential of PEO derived cells and their repair/regenerative capacity. CM differentiation mechanisms might have been directed through Wnt/ β -catenin signaling from PEO derived cells (Buermans et al., 2010). Likewise, we have got the data which indicate better CM differentiation potential of PEO derived cells than eEpi cells in explant culture, as generated in this study that basal level expression of CM specific markers get increased including *Tbx20* (Figure 3). But, due to technical limitation of PEO isolation and cell culture, we have mostly targeted on eEpi explant culture and their differentiation mechanisms. In this study, we have attempted to exhibit differentiation capacity of eEpi cells by manipulating Wnt/ β -catenin signaling. We have also successfully detected about 61% enrichment of avian *Wt1*⁺ eEpi cells showing flow cytometry derived data (Figure 4A I-III). Also, as expected, only 7% *c-Kit*⁺ enriched cell population are also detected, suggestive of differentiated phenotype beyond early cardiac progenitor markers like *c-Kit* in eEpi cells in culture (Figure 4 AIII). According to previous reports, researchers have successfully applied Lithium Chloride (LiCl), a classical activator of Wnt/ β -catenin pathway via stabilization of β -catenin protein through GSK3 β inhibition (Sinha, 2004). Likewise, Lithium chloride mediated β -catenin stabilization promotes the number of *Mf20*, sarcomeric myosin positive cells in eEpi cells in culture with increased proliferation compared to untreated eEpi cells (Figure 8). At the early stages of heart development, CM differentiation can be promoted by *Bmp2*/*Smad* pathway and inhibited by Fibroblast growth factor-2 (E. C. Svensson, 2009). Activation of *Fgf* signaling in PEO cells diminishes *Bmp*-induced *smad* phosphorylation and nuclear localization, which is mediated by mitogen activated protein kinase (MEK) or extracellular signal regulated kinase (ERK) dependent pathway. Myocardial differentiation also relates ERK activation and *Fgf2* mediated inhibition of *Bmp*/*smad* pathway, differentiating CM derivatives from PEO cells (Kruithof et al., 2006; Wijk et al., 2010). Therefore, it is poorly understood whether *Bmp*/*Fgf* pathway is also regulated by *Tbx20* or not, as *Tbx20* is already known as an upstream molecule of *Bmp2* and increased *Bmp2* mRNA expression is analysed in LiCl treated eEpi cells along with increased mRNA level of *Tbx20* (Figure 4 C and D-IV). *Tbx20*, being an important promoting factor of cardiac cell proliferation in heart

morphogenesis, likewise it might promote proliferation of eEpi cells treated with LiCl (Figure 8 C). Overall, Wnt signaling upregulation along with increased level of *Tbx20*, are associated with CM lineage specification and proliferation in avian eEpi cells in culture. Additionally, this study also reveals similar increased level of CM differentiation capacity in PEO derived cell in culture treated with LiCl (figure 9), although the technical limitation of PEO isolation and culture restricted further functional analyses in PEO compared to eEpi cells in culture. But nevertheless, manipulation of Wnt/ β -catenin signaling might be a significant strategy to stimulate CM differentiation from both the PEO and eEpi derived cell in culture. In addition to LiCl experiments, we have also validated on eEpi cells by treating with Wnt3A recombinant protein to induce and Xav939 to inhibit Wnt signaling to show similar results (Figure 6 A-C). Moreover, expression of selective Wnt agonist genes (*Wnt3A*, *Wnt5A*) and antagonist gene (*Dkk1*) are also analysed to further add implications on the involvement of Wnt pathway for CM lineage differentiation of eEpi cells *in vitro*.

Adult mammalian heart has very limited regenerative capacity after a significant CM damages due to myocardial injuries. Hence search for novel approaches to generate more CMs from resident non-CM lineages are innovative and significant in cardiac regenerative therapies. Recently considerable research interests on EPDC progenitors are greatly focused and are also directed towards identifying and isolating EPDCs having capability of CM differentiation that could be used to repair or regenerate the injured myocardium. But, according to our data, the indication of PEO and/or eEpi cells as a better source for CM is worth finding to enrich more CM renewal strategies after adult cardiac injury. Zebrafish hearts have been shown to be regenerated through epicardial activation and it also suggest that the epicardium is a potent source of mitogenic factors and instructive signals for CM cell division (van Wijk et al., 2012). Wnt/ β -catenin signaling involvements have also been reported to trigger the epicardial cells for better cardiac function after ischemic injuries (Duan et al., 2012; Sasaki et al., 2013; E. M. Winter & Gittenberger-De Groot, 2007). Likewise, also Notch signaling pathway is associated with reactivation of adult mouse epicardium contributing new resident cell types to decrease fibrosis after myocardial injury (Karantalis, Balkan, Schulman, Hatzistergos, & Hare, 2012; Russell et al., 2011). Previous report states that loss of GSK3 β in mice leads to *β -catenin* stabilization and increased CM proliferation during development, while conditional loss of GSK3 β in

adult cardiomyocytes enhances cardiomyocyte proliferation after injury (Sinha, 2004). Moreover, inhibition of GSK3 β followed by *β -catenin* stabilization in Wnt signaling might be a useful target to discover a novel approach in EPDC based cardiac cell therapies. Overall, our data bear strong indications to draw a novel insight into epicardial biology, where manipulation of Wnt/ β -catenin signaling in the PEO and/or eEpi cells might play critical functions in CM differentiation in the field of cardiac repair and regenerative medicine.