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REVIEW

Early cardiac development: a view from stem cells to embryos

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Abstract From the 1920s, early cardiac development has been studied in chick and, later, in mouse embryos in order to understand the first cell fate decisions that drive specification and determination of the endocardium, myocardium, and epicardium. More recently, mouse and human embryonic stem cells (ESCs) have demonstrated faithful recapitulation of early cardiogenesis and have contributed significantly to this research over the past few decades. Derived almost 15 years ago, human ESCs have provided a unique developmental model for understanding the genetic and epigenetic regulation of early human cardiogenesis. Here, we review the biological concepts underlying cell fate decisions during early cardiogenesis in model organisms and ESCs. We draw upon both pioneering and recent studies and highlight the continued role for *in vitro* stem cells in cardiac developmental biology.

Keywords Cardiac development • Cardiac cell lineages • Embryonic heart

1. Background: from embryos to stem cells

Cardiac cell fate decisions are made during early vertebrate development based on genetic and epigenetic mechanisms that remain poorly understood. While the process beginning and following the crescent stage of development is better known, the determination from epiblast to a specific cardiac cell fate is largely unclear. Defects in these early cell fate decisions deserve attention as they contribute to stillbirth in severe cases and congenital heart diseases (CHD) when milder in scope,[1] as suggested by the increased occurrence of CHDs when assisted reproductive technologies have been utilized.[2] While animal models have made important contributions to our knowledge in these developmental events, the genomic differences and the lack of adequate amounts of biological material from species such as human, mice or fish has hampered our ability to make significant progress in this area.

Initially recognized as teratocarcinoma cells,[3] pluripotent stem cells were first derived from mouse blastocysts more than three decades ago and possess qualities that are truly representative of embryonic stem cells (ESCs).[4] These cells have contributed significantly to biomedical science through generation of gene-targeted mice,[5] and have provided developmental biologists with an invaluable model *in vitro* to study normal and pathological development of early mouse and human embryos. As reported in pioneering studies,[6–8] mouse ESC (mESCs) recapitulate *in vitro* these pre-gastrulation as well as post-gastrulation cardiogenic events up to the formation of foetal cardiomyocytes. A decade later, non-human primate[9] and human[10] ESCs (hESCs) were derived and shown to give rise to most cells of the embryo, including cardiomyocytes.[11,12]

Here, we review recent advances in early cardiac development, focussing mainly on genetic studies in the mouse and briefly discussing contributions from zebrafish and chicken models. As we move from animal models towards human cardiac development, we will illustrate how stem cells have been used in combination with embryos to delineate such a genetically and epigenetically regulated complex developmental process. We also discuss how ESCs have brought additional mechanistic information to embryo studies at each important step of cardiogenesis, i.e. specification, determination and lineages segregation, and differentiation of heart-contributing cells, while also pointing out the possible pitfalls of this cell model.

2. Endoderm and mesoderm formation and segregation—recent insights from both ESCs and embryos

As the heart is the first organ to form during mammalian embryogenesis, the decisions to commit towards a cardiac cell fate are taken early in the developmental process. Studies including explant cultures, mouse/chick graft, chick/quail graft, and cell fate mapping experiments

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demonstrated that cardiac precursor cells are found before gastrulation and are located in the lateral posterior epiblast in pre-streak embryos[13] (*Figure 1A*). Gastrulation, the morphogenetic process that leads to the formation of the three germ layers (ectoderm, mesoderm, and endoderm) begins with the appearance of the primitive streak (PS). A subset of epiblast cells then moves as a sheet to the PS, and undergoes epithelial-to-mesenchymal transition (EMT), in order to ingress and transiently forms the mesendoderm.

In fish and amphibians, the mesendoderm represents an intermediate germ layer from which the endoderm and mesoderm subsequently segregate. In an amniote, the prospective 'mesendodermal' cells ingress through the PS to reach their correct topographical positions during gastrulation. The first cells to ingress give rise to the 'primitive endoderm' [visceral endoderm (VE) in mammals equivalent to hypoblast in chick].[14] Then, the second wave of ingressing cells gives rise to extraembryonic and embryonic mesodermal cells. GATA factors 4,5,6 share a common role in specification of both endoderm and mesoderm. This led to the idea that mesendoderm is an ancient germ layer that was determined early in the evolution by the same set of genes (at the bilaterians crossroad) as no GATA factors were found before this stage of evolution.[15] Mesendoderm in amniotes is thus a more time-restricted than spatially defined intermediary layer.[16]

Genetic and cellular mechanisms underlying the segregation of endoderm and mesoderm from mesendoderm have remained a key question of mammalian developmental biology and constitute a pivotal event in determining the cardiac lineages both through a cell-autonomous and a cell non-autonomous manner. Recent studies combining mammalian embryos and ESCs have begun to shed light on this process. Among signalling molecules, Nodal and Activin are members of the TGFβ family which work together with the Wnt/β-catenin pathway to determine the formation of mesendoderm in both embryos[17] (see Schier[18] for review) and mouse and human ESCs.[19–21] BMP2, another member of the TGFβ family, reprograms mesendoderm in heart forming area.[22]

BMP2 is secreted by visceral endodermal cells, extraembryonic mesodermal cells, and promyocardium and it proves instrumental for cardiogenesis as revealed by the BMP-2 deficient mouse which features severe cardiac defects.[23] Recently, the role of BMP2 specifically secreted by the VE has been further documented. BMP2 signals to epiblast-derived cells to coordinate ventral folding morphogenesis of the embryo, a process leading to invagination of the gut tube but also to the proper positioning of the heart.[24] Using VE-specific BMP2 KO mice, the same authors found that the specification of heart progenitors still occurs in the absence of VE-secreted BMP2. Later, the definitive endodermal cells intercalate with VE cells to replace them[25] in order to give rise to the foregut endoderm, a tissue still required for cardiogenesis. BMP2 is also secreted by the pharyngeal endoderm in contact with the pharyngeal mesoderm which is at the origin of the second heart field (SHF).[26]

In fact, the crucial balance between Nodal and BMP2 signalling, a process that is finely regulated by morphogen gradients and inputs from Wnt/β-catenin signalling in PS and mesendoderm, is recapitulated

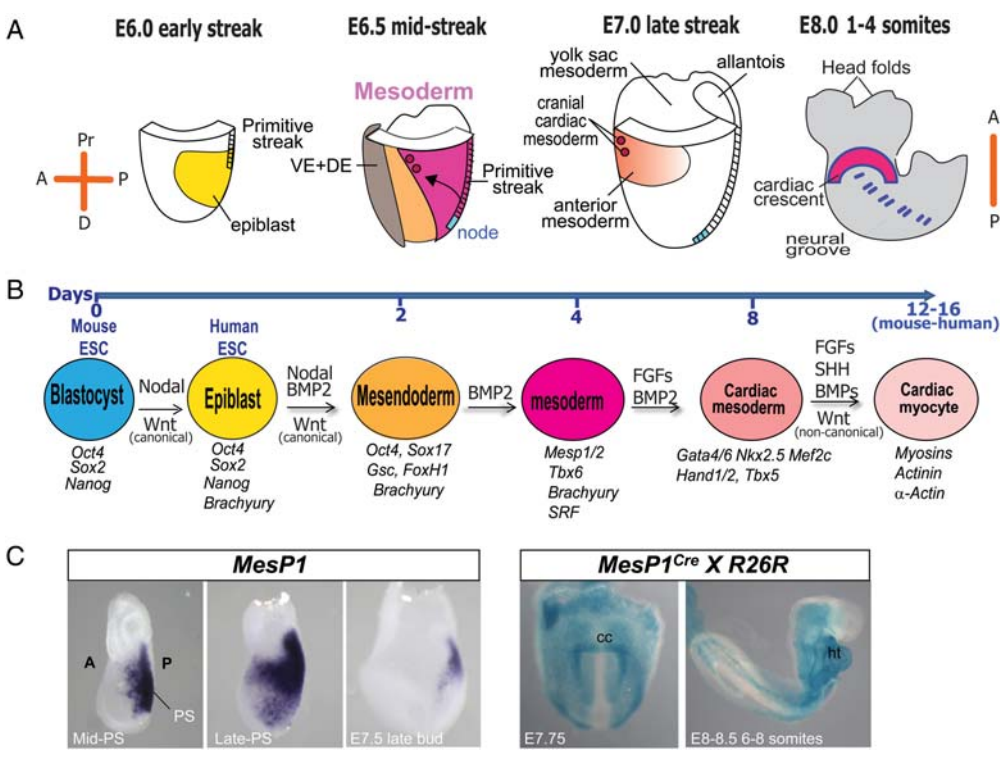

Figure 1 Comparison of cardiac ES cell differentiation and early embryonic heart development. (*A*) Time course and embryonic stages of cardiogenesis in mouse embryo. (*B*) Time course and embryonic stages recapitulated by ESC to differentiate towards a cardiac fate. (*C*) Time course and pattern of expression of MesP1 in early mouse embryo monitored by *in situ* hybridization (left panel). MesP1 Cell lineage tracing in embryos obtained from breeding MesP1-Cre with Rosa26lacZ (R26R) mice (right panel). MesP1+ cells give rise to the whole heart, as well as head and tail muscles. ExE, extraembryonic ectoderm; VE, visceral endoderm; DE, definitive endoderm.

step by step during ESCs *in vitro* differentiation (*Figure 1B*).[21] Dysregulation of the Nodal vs. BMP/Smad balance in embryos leads to a defect in the laterality of the heart-forming region,[27] while a defective Wnt pathway dramatically changes cell fate from endoderm towards cardiac mesoderm, giving rise to two linear heart tubes.[28] Thus, the proximal–distal gradient of Nodal/Smad in mouse embryos plays an important role in the segregation of endoderm and mesoderm including cardiogenic mesoderm. This gradient regulates expression of both Oct4 (encoded by *Pou5f1*) and Eomes in embryos, in the epiblast and the emerging mesendoderm, as well as in differentiating ESCs.[29–32] This morphogen gradient tunes specific transcriptional pathways segregating the mesendoderm into cardiac mesoderm and definitive endoderm along the anterior posterior axis of the embryo.

Before the emergence of the streak,[33] Oct4, which is transiently up-regulated in mouse epiblast as well as in the nascent Oct4$^+$-mesoderm in the porcine embryo, is crucial to ensure normal cardiac development in mouse embryos and ESCs.[29,30] The cardiogenic action of Oct4 is in part cell-non-autonomous and involves *Sox17*, a target gene of Oct4 and a mesendodermal/endodermal marker required for cardiogenesis.[34] Whether Eomes[32] mediates such an event or acts in a parallel transcriptional network remains to be investigated. This pathway delineated in both ESCs and embryos might be involved in a rare congenital disease (i.e. syndrome Cornelia de Lange) including a cardiac defect as recapitulated in a zebrafish model.[35]

Mesendoderm is thus a layer or a transient cell status (amniotes) at the cross-road of cell fate decisions and the actor of important decisions for cardiac cell determination. Such decisions are tightly dependent on a balance of Nodal/BMP/Smad and Wnt pathways.

A detailed understanding of the segregation of mesendoderm into separate germ layers in different animal models may help to place the interaction between the mesoderm and endoderm into a better context. This should lead to improvements in our strategies to differentiate ESCs into cardiac cells *in vitro* using growth factor supplementation. Likewise, unravelling the mechanisms underlying cell fate segregation within ESC-derived mesendoderm should help us understand better a crucial cell decision for heart development in the embryo proper, specifically when it cannot be investigated *in vivo* (i.e. human embryo).

3. Determination of cardiac cell fate among other mesodermal cells: when ESCs in culture might be a limiting model

Determination of cardiac cell phenotype begins in the late PS at E7.5 in the mouse,[36,37] when cells move from the posterior to the anterior region under the influence of instructive factors secreted by both the visceral embryonic endoderm and the pharyngeal endoderm. The mesodermal cells covering the anterior half of the PS include prospective endocardial, myocardial, and epicardial cells and express *Gata4,5,6*, *Hand1, Hand2, Wt1*, *and Nkx2–5*, a signature of heart cells. The signals that trigger the migration of cardiogenic mesodermal cells remain elusive thus far. Wnt3a was reported to guide the migration of cardiac progenitors by a mechanism involving RhoA-dependent chemorepulsion.[38] The transcription factor, mesoderm posterior 1 (MesP1) downstream of Wnt3a in the cardiogenic pathway plays a role in this process[39,40] (*Figure 1C*). Indeed, MesP1 is required for EMT, allowing mesodermal cells to ingress under the epiblast. It also mediates delamination and migration of cardiovascular progenitors from the PS.[40,41] In mouse ESCs, *MesP1* appears to serve as a master gene for cardiovascular development.[42] However, the broad pattern of MesP1 expression in mesodermal cell derivatives[43–45] and its function as a cell migratory factor in the embryo argues against such a specific role.

The lateral mesoderm includes progenitors of several cell lineages, including haematopoietic cells, endothelial cells, smooth and craniofacial muscle cells, and cardiac cells (*Tables 1* and *2*). Both mouse and human ESCs give rise to all these lineages although their segregation *in vitro* may not be equivalent to that in the embryo. In particular, spatially distinct dorso-ventral expression of genes during ingression of cells through the streak might be less faithfully recapitulated in ESC culture. In order to correctly interpret cell fate decisions *in vitro*, a deeper understanding of *in vivo* mesodermal cell specification is required.

Lineage-tracing studies in the mouse have demonstrated that the first mesodermal cell lineage to emerge is the VEGF-R2$^+$ (encoded by the mouse gene *Flk1* or human *KDR*) cell population. It originates

Table 1 Glossary of terms

Cell lineage: a series of cells derived from a stem or progenitor cell that divides to give rise to its descendant clone. Specification of cell fates might be correlated with cell division patterns, usually in primitive organisms; in other organisms, lineage patterns are variable and not always correlated with cell fates.[128]

Specification/commitment: intrinsic and acquired characteristic of a cell that leads its fate to a particular developmental state. The cell acquires the potential to differentiate autonomously when placed in an ectopic (the same embryonic) environment but not when placed in a heterotopic environment. Specification is reversible. It can be autonomous, instructed by a morphogen gradient (syncytial specification), or dependent upon neighbour cells (conditional specification). The later mode of specification is prominent in vertebrate cells.[129]

Determination: acquisition of the potential to differentiate autonomously even when placed into an embryonic region different from its original one. The process is irreversible.

Differentiation: acquisition of cellular specialization in a multi-step, time regulated process, starting from commitment and then determination of cell fate.

Field: in embryology, a morphogenetic field is a group of cells able to respond to discrete, localized biological signals leading to the development of specific morphological structures or organs. As a group, the cells within a given morphogenetic field are constrained (i.e. the cells in a cardiac field will become cardiac tissue).[130] However, it is important to note that the specific cellular programming of individual cells in a field is flexible: an individual cell in a cardiac field can be redirected via cell-to-cell signalling to replace specific damaged or missing cells. This definition is used throughout the review.

Cardiac lineages: a collection of cells that includes endocardial, myocardial, epicardial cells, conduction and pacemaker cells, which contribute to a functional heart.

Cardiogenic: with capacity to make the main cell components of the heart (myocyte, endothelial cell, fibroblasts, smooth muscle cell)

Table 2 Comparative strengths and weaknesses of embryos and stem cells

	Strengths	Weaknesses
Embryos	Possibility to study morphogenetic events; spatial organization of germ layers and specialized tissues	Limited amount of biological material
	Possibility to investigate tissue-tissue interaction	Difficult to purify cell lineages for genetic or epigenetic studies
	Possibility to study gradients of morphogens	Studies in mouse embryos are time consuming
	Possibility to delineate true morphogenetic action of growth factors (e.g. BMP2)	Early cell fate decisions difficult to study (early KO of gene often lethal)
	Possibility to study cell migration	Rare human embryonic material
ESCs	Availability in biological material	Can take differentiation roads not developmentally relevant due to their cell plasticity
	Give rise to any embryonic cell type	No controlled tissue–tissue cross-talk
	Possibility to carry out fine mechanistic (genetic and epigenetic) studies on pure cell populations	Developmental studies limited in time (pre- and post-gastrulation, up to the crescent stage)
	Delineation of early cell fate decision (such as mesendoderm segregation into endoderm and mesoderm)	Spatial organization of germ layers is limited
	Human ESC lines are available	Difficult to mimic gradients of morphogens or to reveal morphogenetic action of growth factors (e.g. BMP2)

from the most posterior mesodermal region in response to BMP4 secreted by the extraembryonic ectoderm (ExE). *Flk1*^{High} cells give rise to the visceral yolk sac mesoderm and blood islands[46] (*Figure 2*) while *Flk1*^{low} expression marks a large part of multipotent mesoderm.[47] Recently, Ishitobi *et al.*[48] reported a specific Flk1 Distal-Multipotent-Mesodermal-Enhancer that drives the *in vivo* expression of the gene in early mesodermal cells. They further found that *in vitro* these cells segregate in two types of colonies generating hemangioblasts defined as mesodermal progenitor cells committed to blood, endothelial, and smooth muscle cells[49,50] or vascular cells and some cardiac cells.

ESCs have also been used to recapitulate the *Flk1*-lineage.[49,51,52] Kouskoff *et al.*[51] showed that *Flk1*$^{+}$ ESC-derived cells first function as hemangioblasts and that FACS-purified Brachyury^{+}/Flk1^{-} re-aggregated cells give rise to contractile myocytes. The authors noticed, however, that a subpopulation (~5–10%) of the Brachyury^{+}/Flk1^{-} cells could re-express Flk1 and be further induced by VEGF to differentiate into cardiomyocytes. Thus, a subpopulation of late Flk1^{+} cells can be redirected *in vitro* towards a myocardial cell fate in mouse[51,52] and human[53] ESCs.

Interestingly, the segregation of haematopoietic and cardiac cell takes place *in vivo* with a different timing. Both haematopoietic and cardiac lineages are separated early on from the ingression of epiblast cells through the streak. There is no longer a descendant from a common progenitor at that late PS stage. Knock-out of *Wnt2*, expressed in the posterior cardiac mesoderm increases the number of Flk1^{+} and haematopoietic cells but impairs endothelial and cardiac differentiation in ESC-derived embryoid bodies (EBs)[54] (*Figure 2*). Conversely, a recent publication reported a Wnt2-induced acceleration of cardiac differentiation of ESCs. This pro-cardiogenic effect was mediated by a non-canonical pathway.[55] It appears that *Wnt2-/-* mutants exhibit decreased expression of GATA6 and display many cardiac defects including a thin atrial wall, impaired atrioventricular (AV) canal development, and a deficiency in the development of the superior AV cushion and associated myocardium.[56]

Several lines of evidence further revealed an antagonism between cardiac and haematopoietic lineages following the early segregation of the two lineages. Induction of vessel and blood specification in zebrafish represses cardiac specification and delimits the heart forming region.[57] While both hemangioblasts and part of cardiac progenitors have a common origin in the fish, the common progenitor express cardiac genes of the GATA family but not the blood or endothelial genes. The anterior lateral plate mesoderm in zebrafish is indeed a source of haematopoietic, endothelial, and cardiogenic cells, with the blood and endothelium found in the most rostral region and cardiac tissue in the adjacent more posterior region. The authors proposed that GATA5 and GATA6 are required in both the yolk sac and the endoderm for migration of cardiac progenitors to the midline, but that they are dispensable for the specification of both heart tissue and hemangioblasts. Thus, the role for GATA factors in cardiac cell specification must be allocated within the mesoderm very early in the VE and mesendoderm,[58] allowing it to respond to both blood- and cardiac-inducing signals. On the other hand, Duncan's laboratory reinvestigated the role of GATAs in cardiomyocyte differentiation after having circumvented the lethality of the GATA 4 or GATA 6 deficiency in the VE by tetraploid embryo complementation.[59] They generated a double *Gata4*$^{-}$/*Gata6*$^{-}$ mouse and showed that these embryos lack the heart, thus pointing to an essential, albeit, redundant role of GATA 4 and 6 in the cardiac transcriptional pathway. These findings are in line with the presence of many GATA sites often associated with *Smad* sites on enhancers of many cardiac genes including the early expressed *Nkx2–5*[60] (for review see Kawamura[61]). They are also in agreement with the autonomous and instrumental cardiogenic role of GATA often associated with chromatin modifiers (HDAC, Baf60) in ESCs and embryonic mesoderm.[62,63]

A recent report in zebrafish emphasized the role of FGF in favouring the cardiogenic mesoderm at the expense of the hemangioblast. In this study, Simoes *et al.*[64] show that the two lineages are mutual antagonists. Nkx2–5 in cardiogenic mesoderm prevents the hemangioblast program by repressing gene expression such as *Scl/Tal1* and *Etsrp*. Similarly, *Scl/Tal1* and *Etsrp* prevent the cardiogenic program (*Figure 2*). Using a transgenic mouse to isolate *Nkx2–5* expressing cells, Caprioli *et al.*[65] observed an induction of the erythroid molecular program, including *Gata1*, in the *Nkx2–5*-null embryos. They showed that Nkx2–5 represses *Gata1*, which further supports the antagonism between the cardiac and haematopoietic cell lineage. Similarly, Rasmussen *et al.*[66] used both mouse embryos and ESCs to track haematopoietic and endothelial lineages. The authors employed an *Ets-related factor* (*ER71*)-*Cre* mouse that marks both haematopoietic

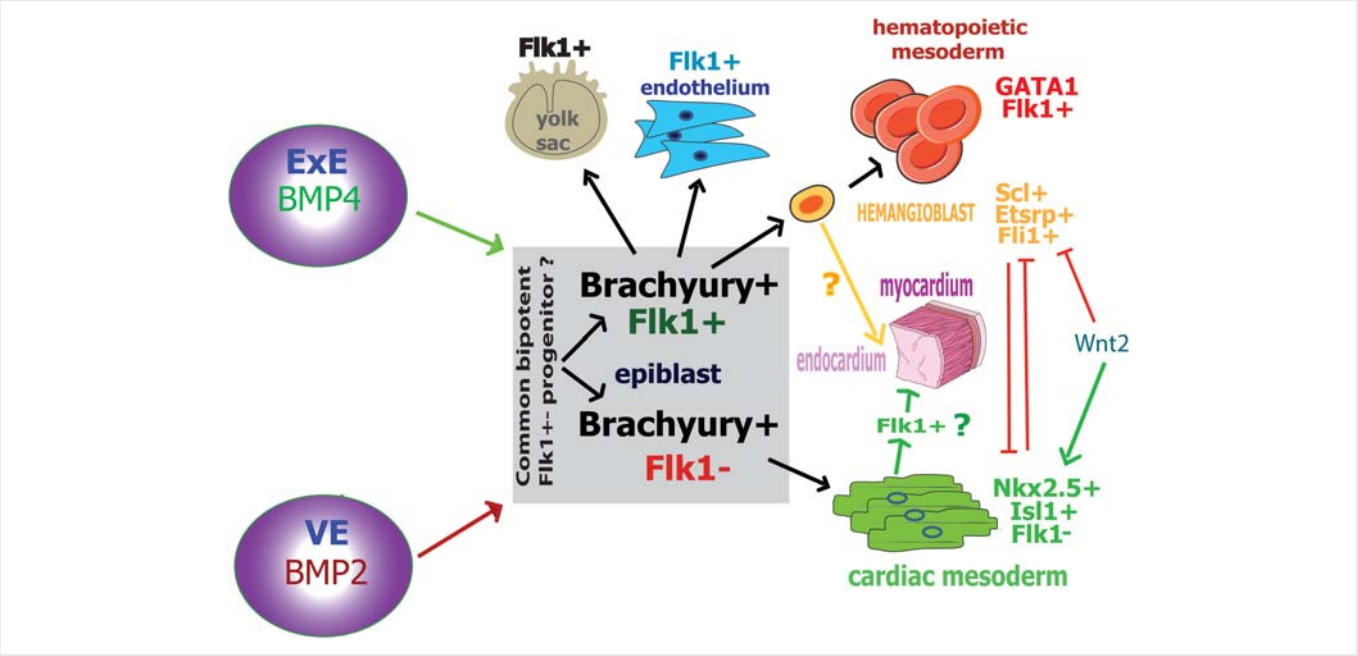

Figure 2 Early segregation of the cardiogenic and haemogenic roads. A likely existing bipotent early progenitor in the epiblast gives rise to both a Flk1+ /Brachyury+ and a Flk1-/Brachyury+ cell population, under the action of BMP4 secreted by the extraembryonic ectoderm (ExE) and BMP2 in the visceral endoderm (VE), respectively. This early event already segregates the future haemogenic and cardiogenic (i.e. myocardial) cell populations. A parallel route used by a Flk1+ lineage re-emerging from a Flk1- cell population, and also possibly part from the hemangioblast lineage leads to the endocardial cell population.

and endothelial lineages and likely the endocardial but not the myocardial lineage. However, in *ER71* null mutant, *ER71-Cre* x Rosa-EYFP-labelled cells contribute alternatively to heart lineage. Using ESCs and overexpression of *ER71* in EBs, the authors showed impairment in cardiac differentiation, thus also revealing an antagonistic action of haematopoiesis on cardiogenesis. Palencia-Desai *et al.*[67] also recently reported that the absence of *Etrsp* (i.e. *ER71*) in zebrafish leads to vascular endothelial and endocardial progenitors redirecting their fate towards the myocardial lineage. Therefore, a combination of studies using mouse and zebrafish highlighted that while sharing an early and common Flk1^{+} progenitor in the pre-streak embryo, the haematopoietic and cardiac lineages are segregated at gastrulation and are from then on mutually exclusive. ESC might retain bi-potentiality for a longer time and thus caution is required when interpreting *in vitro* data.

4. Cardiac lineages segregation, cell differentiation, and maturation

4.1 Differentiation of cardiogenic mesoderm: interactive and inductive cross-talk between germ layers

By the late PS stage, the prospective heart mesoderm is located in the intermediate and anterior proximal regions of the mesodermal layer underneath the cephalic neural plate.[68,69] Mesodermal lineages, including both the cardiac mesoderm and the emerging definitive endoderm progenitors remain in tight proximity between the most anterior and posterior regions of the streak, within the mesendoderm.[36,70] Using embryonic explants, the same authors confirmed the requirement of the visceral embryonic endoderm for the cardiac progenitors of the late streak stage embryo to acquire a cardio-myogenic cell fate. The endoderm also instructs the mesoderm by facilitating migration of bilateral heart fields towards the embryonic midline, through a mechanical event.[71]

In vitro, commitment, determination, and differentiation of ESCs towards a cardio-myogenic lineage also require cues from endodermal cells. Weitzer and colleagues showed that mESC-derived EBs cannot differentiate into beating cardiomyocytes without the endodermal external layer which imitates the extraembryonic or primitive endoderm.[72] Mummery's group reported that visceral (primitive) endodermal cells (i.e. END-2 cell line) improve cardiac differentiation of hESC.[73] The paracrine cardiogenic property of endodermal cells was further demonstrated in two publications by Anne Foley's laboratory. First the authors analysed the transcriptome of extraembryonic endodermal cells (XEN, PHYS2 cells) and END-2 cells (visceral endodermal cells).[74] Then, the authors revealed that the cell lines mimicking the heart-inducing embryonic anterior visceral endoderm (AVE) also featured a cardiogenic action on mESC. Conditioned media from the three endodermal cell lines increased beating activity of EBs while the PYS2-CM and XEN-conditioned medium, but not END2-medium expands the size of the pool of cardiac progenitors in EBs.[75] These data suggest that the cardiogenic effect of the conditioned medium is mediated by BMP2, indeed secreted by post-PS AVE. Thus, *in vitro*, BMP2 exerts a cardiac inductive action. These findings suggest a dual and time-dependent role of BMP2 secreted by AVE: an early (i.e. early streak stage) instructive role at the onset of gastrulation, mimicked by ESC and a late (late streak stage) morphogenetic role in ventral folding, is required for the right positioning of

the cardiac progenitors.[24] Such a dual action might be difficult to mimic by cells in culture.

4.2 Endomyocardium as an early segregated lineage: how ESCs might help in delineating this lineage

Using replication-defective retroviral-mediated gene transfer to trace cells, Mikawa's laboratory showed that cells in the rostral half of HH stage 3 chick PS generate a daughter population that migrates into the heart field. Their subsequent differentiation into either endocardial or myocardial cells, but not both[76] suggesting an early segregation of endocardial and myocardial progenitors. In the mouse, differentiation of pre-cardiac mesodermal cells in the bilateral heart prospective region also give rise to both endocardial and myocardial progenitors.[77]

The endocardial progenitor cells are quite difficult to track in the mouse embryo proper as they arise from different origins. Genetic lineage tracing studies in the mouse[47] suggested that endocardial and myocardial cells could arise from a common *Flk1*$^+$ progenitor when migrating epiblast cells exit the PS. Baldwin's laboratory confirmed that these *Flk1*$^+$ cells are distinct from hemangioblasts since they express *lacZ* under the control of the endocardial-specific *NFATc1* promoter/enhancer regions and thus are endocardial endothelial cells.[78] This suggests that *Brachyury*$^+$/*Flk1*$^-$ cells can subsequently express *Flk1* that gives rise to endocardium. Interestingly, hemangioblast program, as represented by the expression of *Scl/Tal1,* has been reported in zebrafish and is required for early endocardial morphogenesis[79] (*Figure 2*).

Using mESCs, Kattman *et al.*[80] reported that a subset of GFP-*Bry*$^+$ cells that are initially *Flk1*$^-$ can be induced to express *Flk1* when stimulated by VEGF and become both endothelial cells and myocytes. This and other mouse ESC studies are in general agreement with experiments performed in embryos showing that both *Isl1-Cre*[81] and *Mef2c-AHF-Cre* labelled cells give rise to both myocardium and endocardium.

In the cardiac crescent, the Ets-family protein Etv2 has been identified as an Nkx2−5 target and a key gene for endothelial−endocardial specification,[82] confirming that endocardial cells arise from a *de novo* process of vasculogenesis. In a recent paper,[83] the authors used live imaging of quail embryo and lineage tracing in the mouse to show that the endocardium derives from vascular endothelial lineage also suggested by Rasmussen *et al.*[66] *Flk1*$^+$ mesodermal cells are therefore instrumental in generating the endocardium, which can originate from both an *Isl-1*$^-$ and an *Isl-1*$^+$ lineage. ESCs specifically engineered to express reporter genes under the control of late specific marker of the endocardium such as a specific *Nfatc* enhancer[84] will be helpful in identifying new endocardial specific genes. In parallel, the use of retrospective clonal analysis in mice will be complementary to the approaches using either ESCs or *Cre-lox* mice, to further delineate the embryonic origin specifically the likely diversity of the endocardial lineage(s).

4.3 Separation of epicardial and myocardial cell fates

The epicardium is formed by the outgrowth of pro-epicardial cells in the pro-epicardial organ (PEO). The PEO is thought to arise from the transverse septum and migrates towards the sinus venosus into the pericardial cavity when the heart tube elongates. Subsequently, migration and cell replication (i.e. EMT) along the surface of the heart tube results in the formation of the epicardium. Epicardial-derived cells (EPDCs) then migrate into the myocardium and differentiate into smooth muscle cells and fibroblasts.

In the recent years, genetic lineage tracing studies using *Tbx18*^{Cre} or *Wilms'tumor 1* (*Wt1*) *Cre*[85,86] and floxed *R26R*^{lacZ} *Cre reporter* mice suggested that EPDCs also give rise to myocardial cells. However, this concept has been challenged[87] as both *Tbx18* and *Wt1* may be expressed earlier in myocardial precursor cells prior to the formation of the PEO, pointing to the limitation of the *Cre-lox* technology. An elegant study[88] examining both *Wt1* epicardial specific knock-out mice and *Wt1* null ESC-derived EBs revealed a *Wt1*$^+$ mesodermal cell population at the origin of post-EMT of *Nkx2−5*$^+$/*Isl1*$^+$ cardiac progenitors. *Wt1* null ESC-derived EBs did not express *Kdr*, *Nkx2.5*, *Hand1*, and *Isl1* suggesting that Wt1$^+$ epicardial prospective cells could be part of the MesP1+ cell population. FGF signalling via MEK1/2 can overcome BMP/Smad signalling and was proposed to be mandatory for the early separation of the epicardial lineage from pre-cardiac mesoderm (*Figure 3*) that will eventually give rise to the developing myocardium.[89] These early Wt1+ cell population are the precursor cells to the eventual adult epicardium.[88] However, FGF is not required to induce or maintain expression of epicardial markers such as Tbx18 or Wt1.[90] Thus, it could be interesting to investigate whether the timely manipulation or alteration of the balance between FGF and BMP signalling in mESC- and hESC-derived mesodermal cells could allow early segregation of the epicardial from the myocardial lineage. Alternatively, the ESCs could be helpful to investigate other signalling and genetic pathways important for such a cell decision.

5. Embryonic cardiac 'fields' and lineages: ESCs as a potential investigation

The first identifiable cardiomyocytes are found in the splanchnic mesoderm, situated in the cardiac crescent. As the embryo grows, the crescent fuses to form the primitive heart tube. The primitive heart tube gives rise to the left ventricle, AV canal, sinus venosus, and major parts of the atria. The looping and elongation of the heart tube depends upon a second source of cardiac progenitor cells lying medially and dorsally to the crescent. These progenitors, lying within the pharyngeal mesoderm, contribute to right ventricular and outflow tract (OFT) myocardium and a minor sleeve of smooth muscle cells at the base of the great arteries. The identity of these cells was subsequently revealed by the expression of *Fgf8, Fgf10*, and by the *Fgf10-lacZ* transgene.[91] The expression of *Isl1* in the pharyngeal splanchnic mesoderm has been associated with the presence of an SHF in such region. Further studies revealed its contributions to both arterial (anterior) and venous (posterior) poles of the heart tube.[81] Perturbation of SHF development through conditional mutagenesis in the mouse, or ablation of subpopulations of progenitor cells in the chick, results in partial extension of the heart tube and alignment defects during cardiac septation.[92,93] Such defects correspond to CHD, including conotruncal anomalies such as overriding aorta, tetralogy of Fallot, and double outlet right ventricle.[94]

While the existence of SHF was actively debated, studies employing retrospective clonal analysis clearly showed the contribution to the OFT and the right ventricle from the pharyngeal mesoderm.

Therefore, the left-ventricular free wall is exclusively populated by cells of the first lineage while the OFT is predominantly colonized by cells of the second lineage.[95] Although this analysis cannot predict the spatial location of the progenitors, it does predict that these two lineages segregate early around the onset of gastrulation and share a common progenitor.

However, the idea of fields (not lineages) was challenged as several previous reports argued against the existence of several morphogenetic fields *per se* as described by embryologists and defined as a region of an embryo that gives rise to a distinct morphological structure, e.g. the heart, regardless of the subdivision of this structure.[96] As heart fields have been marked by growth factors (*Fgf10*, *Fgf8* for the SHF) or gene expression (*Tbx1*, *Isl1* for the SHF, *Tbx5* for the FHF), the question arises about the definition of heart field as region of morphogenetic signalling, a region with a defined pattern of gene expression, or even a region with a defined epigenetic or a higher order chromatin structure signature. This debate has been documented earlier in more detail by Van den Berg and Moorman.[97] In fact, at the early days of this new concept, the existence of the 'SHF' was linked to expression of specific marker such as *Isl1*.[81] However, Isl1 protein has been detected earlier and transiently in the cardiac crescent[98] denoting the difficulty of tracing embryonic fields or lineages based only on expression patterns of transcription factors at a given time. Of note, *Isl1* is also expressed in very early BMP2-induced cardiac committed mESCs or hESCs,[99] reflecting a pre-gastrulation stage before the segregation into one or the other supposed heart fields *(Figure 3)*. Recent data also point towards a pattern of expression of a transcription factor that is regulated not only by specific enhancers but also by epigenetic events. Without one or the other type of regulation, transcription factor enhancer like the one from Tbx5 will be broadly activated in both left and right ventricle (i.e. FHF and SHF)[100] and thus cannot be used as a strict marker of one specific lineage.

In vitro studies with ESCs have also suggested the existence of two cardiac cell lineages. ESCs can differentiate within EBs without any spatial organization but they are able to give rise to all cardiac cell lineages including nodal, ventricular, atrial,[101] and early pacemaker cells relying on $InsP_3$-induced Ca^{2+} oscillations and in turn membrane spontaneous depolarization, in a study using both ES cells and *in vivo* approaches,[102] late pacemaker,[73,103] endocardial,[104] and epicardial cells.[88] Knocking down 'heart field-enriched' transcription or growth factors in ESCs within a precise time-window is needed to determine whether progenitors in the FHF also give rise to cells of the second cardiac lineage *in vitro*.

A recent study specifically combining Wnt5a and Wnt11 null embryos and ES cells reported the requirement of both Wnts to promote both heart fields in a time- and signalling-dependent-manners. Wnt5a and Wnt11 signal through a non-canonical β-catenin pathway but repress the later, in order to favour the SHF. This effect is preceded by an induction by the same Wnts of the FHF before determination of the SHF.[105] That points out the complex orchestration of both heart fields by the same growth factors. In conclusion, it is more appropriate to define two main cardiac cell lineages originating from a common progenitor[106] that is committed in the unique prospective heart region defined in the epiblast. The fact that *MesP1*$^{+}$ hESC-derived mesodermal clonal cells could segregate into the first (*Isl1*$^{-}$, *Tbx5*$^{+}$) or second (*Isl1*$^{+}$, *Tbx1*$^{+}$, *Raldh2*$^{+}$, *Hes1*$^{+}$, *FoxH1*$^{+}$) cardiac lineage[107] under the action of FGF8,[108,109] suggests the presence of a common progenitor for the two

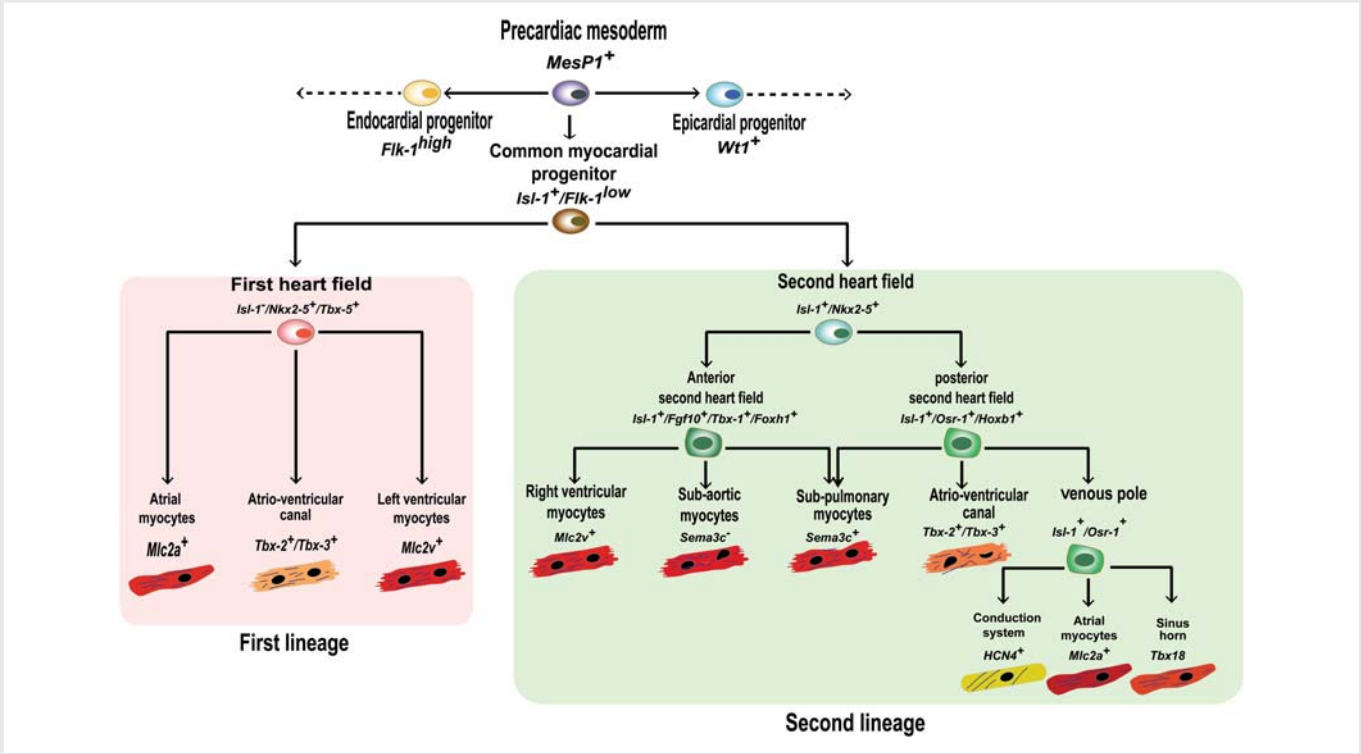

Figure 3 Cardiac fields and lineages. The cartoon depicts the cardiogenic tree with specific fields and lineages as described in the last decade literature.

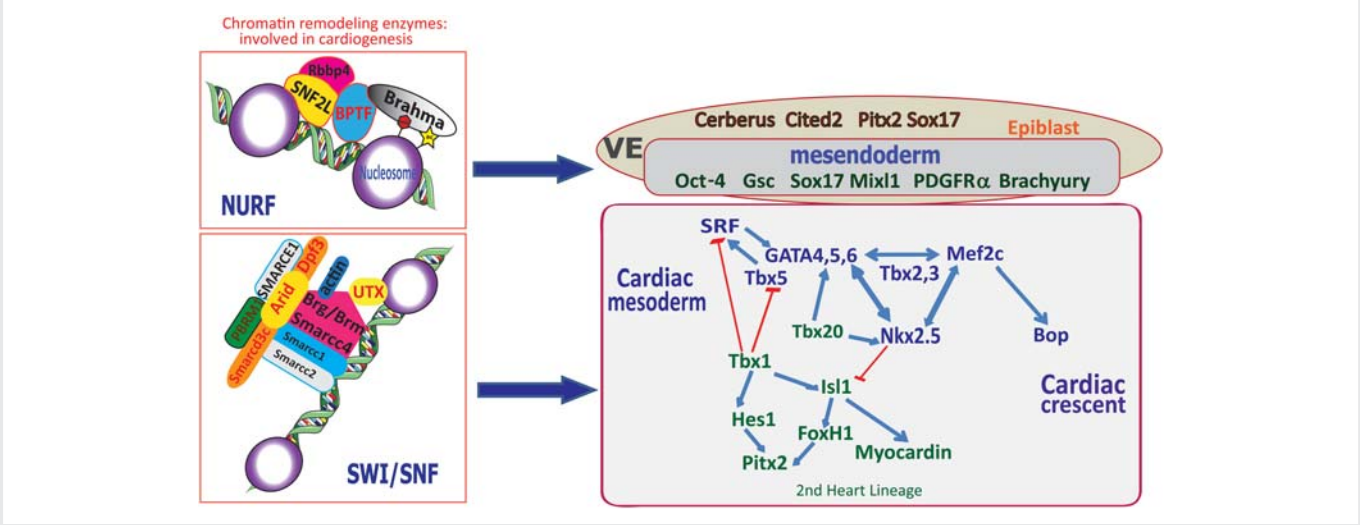

Figure 4 Genetic and epigenetic regulation of the cardiogenic transcriptional network. Both the NURF and the SWI/SNF complexes participate in the modulation of expression of genes required for cardiogenesis. The figure briefly summarizes the key stages through which the embryo develops to generate its heart and the major genes participating within networks in cardiogenesis. The enzymes written in red have specifically been reported to regulate expression of genes important for normal cardiogenesis.

cardiac lineages as previously predicted in embryos.[95] Such a predetermined cardiac progenitor present before gastrulation might originate from the bi-potential mesendoderm or ectomesoderm as identified by retrospective clonal analysis[110] and/or guided by signals (both chemical and mechanical) from both endoderm and ectoderm during ingression of cells through the primitive streak. Epigenetic regulation of gene transcription is also expected to further tune the specificity of cell lineages.

6. Epigenetic regulation of early cardiogenesis: a role for stem cells?

During embryonic development, a carefully orchestrated interplay between transcription factors and epigenetic modifiers are required to instruct multipotent mesoderm to differentiate into cardiac progenitor cells. The genetic pathways underlying early cardiac development have been recently reviewed.[111,112] We will thus focus on epigenetic mechanisms for which ES cells could provide significant insights.

Among the different mechanisms involved in epigenetic modification (e.g. DNA methylation, nucleosome positioning, histone methylation/acetylation, etc.), we chose to focus on chromatin remodelling here since there has been growing interest in this area recently. Chromatin remodelling is an energy-dependent process that utilizes ATP to alter nucleosome position and change chromatin structure to either a euchromatic (transcription-permissive) or a heterochromatic (transcription-prohibitive) state. Furthermore, modifications on histones by methylases, demethylases, acetyltransferases, and deacetylases can provide additional modulation to gene expression.

For early cardiac development, published studies that specifically address epigenetic mechanisms have centred on the role of ATP-dependent chromatin remodelling factors that regulate both intra-and inter-chromosomal interactions (*Figure 4*). Until recently, it was unclear whether components of any of the chromatin-modifying enzyme complexes such as SWItch/Sucrose Non-Fermentable (SWI/SNF), Imitation SWItch (ISWI), Chromodomain Helicase-DNA binding (CHD), and INOsitol requiring 80 (INO80) are essential for early cardiac development (for review see Ho and Crabtree[113]). The vertebrate SWI/SNF complexes, including Brg1/Brahma-associated factor (BAF) complexes, are multimeric protein complexes that change their composition as cells progress from undifferentiated progenitors to fully mature cells.[114] Mouse embryos that are homozygous deficient for *Brg1*, the core component of the BAF complex, exhibit hypoplasia of the ventricular myocardium and die at E11.5.[115]

Recent studies have also shown that other components of the BAF complex are necessary for proper cardiac development (for review see Chang and Bruneau[116]). Genetic deletion of Smarcd4/BAF60c results in defective development of heart and skeletal muscle, suggesting a shared requirement of chromatin remodelling factors in myogenesis.[117] Given its early and more restricted expression in the heart during embryonic development, BAF60c may provide the link for the interaction between the ubiquitously expressed macromolecular BAF complex and the enhancer regions of cardiac-specific early genes. The cardiac specificity and the regulation of epigenetic state by BAF60c are underscored by the recent demonstration that the over-expression of Gata4, Tbx5, and BAF60c is able to convert multipotent mesodermal cells into cardiomyocytes.[63]

While these pioneering studies have begun to shed light on some of the epigenetic mechanisms in cardiac development, it should be pointed out that the limited tissue material available from an early embryo has hampered our ability to understand the role of epigenetics in early cardiac lineage commitment. To circumvent this problem, ESCs have been employed to examine the role of the chromatin remodelling complex during early embryonic development. As a few examples, Gao *et al.*[118] showed that the loss of BAF250a in ESCs results in defective mesodermal and cardiac cell differentiation from murine ESCs. Furthermore, Landry *et al.*[119] identified a key role of Bptf, a component of the ISWI complex, in regulating the expression of mesendodermal, mesodermal, and endodermal genes such as

Sox17, Cerberus, Wnt3a, and Brachyury using Bptf null ESCs *(Figure 4)*. A recent report using ESCs describes the involvement of an ubiquitin ligase TRIM33 in regulating Nodal-induced expression of mesendoderm-enriched genes *Goosecoid* (*Gsc*) and *Mix-like 1* (*Mixl1*). Nodal receptors trigger the formation of complexes including Smad4−Smad2/3 and the ubiquitin ligase TRIM33/TIF1γ (ectodermin)−Smad2/3. TRIM33 silencing in mESC and hESCs blunts expression of *Gsc*, *Mixl1*, *brachyury Scl/Tal1*, and *Nkx2−5* as well as *Sox17* and *Foxa2,* respectively.[120] Thus, this epigenetic mechanism could underlie or regulate the formation of the mesendoderm.

Dovey *et al.*[121] used a cre/lox strategy in ESC and ESC-derived EBs to investigate the specific role of HDAC1 and 2 in cell differentiation. Of note, specific deletion of HDAC1 favours both neuronal and cardiac differentiation of ESCs as monitored by a significant upregulation vs. wild-type of GATA4, Nkx2−5, Mef2c, and beating activity of EBs.[121] Another recent study combining the use of ESCs and embryos both deficient in UTX, a demethylase acting on the meH4K27 mark showed UTX potentiation of the SRF and Tbx5, Nkx2−5 and GATA4 transcriptional core *(Figure 4)* thus pointing to this protein's important role independent of its demethylase activity in early cardiac gene expression. UTX promotes the recruitment of Bgr1 to cardiac specific genes,[122] thus UTX/Brg1 (acting on H3K27me) together with Bptf (acting on H3K4me) are instrumental in turning on a genetic cardiac program.

With the discovery of human hESCs, we are now able to directly study the role of epigenetic modifiers in human embryonic development using hESCs as a surrogate. Recent studies in human ESCs have demonstrated potential epigenetic regulatory mechanism of enhancers of developmental genes.[123] Given that enhancers are likely to work in a tissue-, cell lineage-, and species-specific fashion,[124,125] the generation of purified mesodermal or cardiovascular progenitors from hESCs would enable us to obtain a much higher level of precision in our understanding of the role of these enhancers.

7. Conclusions

Throughout this review, we attempted to illustrate emerging concepts in cardiac developmental biology as described by recent as well as pioneering studies performed in the past few decades. We provided specific examples of complementarities between studies using embryos and pluripotent stem cell. We believe that ESCs from either mouse or human origins can be a powerful tool for uncovering new pathways in which new transcription factors and signalling molecules such as the Retinoblastoma protein Rb,[126] or p63[127] participate. It is possible that ESCs may also enable the discovery of previously unrecognized genes in cardiac development. The emerging role of epigenetics in early cardiac development will benefit from both embryo-based as well as ESC-based studies and is likely to advance with the improvements in novel tools and technologies such as ChIP-sequencing. We believe that stem cells *in vitro* and embryology *in vivo* are complementary to one another and can both help us understand better early cardiac developmental events and the associated cardiac congenital diseases. We foresee an increase in laboratories using both of these models and expect greater collaborations between stem cell biologists and cardiac embryologists for the benefit of both communities. Ultimately, these efforts will enable us to achieve significant advancements in the field of cardiac developmental biology.

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