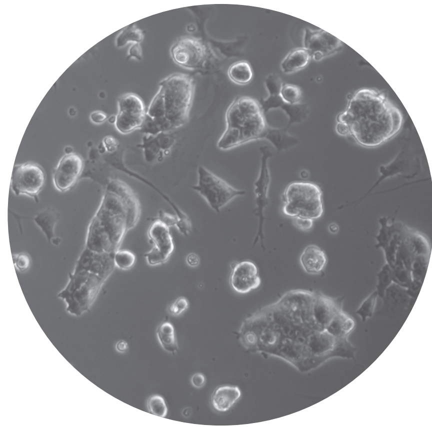

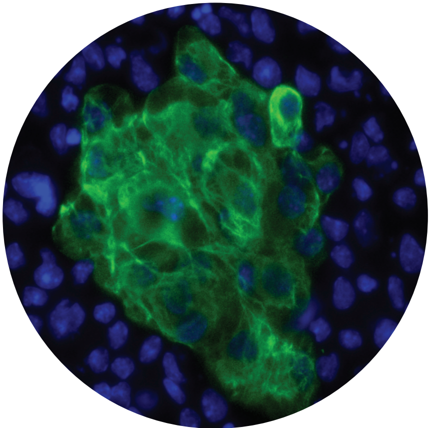

Francisca Castro Gomes Soares da Silva

Dissecting the role of Hes5 in cardiogenesis

Dissertação para a obtenção do grau de Mestre em Investigação Biomédica sob orientação científica da Doutora Ana Gonçalves Freire e co-orientação do Doutor Henrique Girão apresentada à Faculdade de Medicina da Universidade de Coimbra

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MSc Thesis

Dissecting the role of Hes5 in cardiogenesis

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“What is now proved was once only imagined.”

— William Blake

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ABSTRACT

Heart formation involves the participation of various signaling pathways that crosstalk in a temporal and context–dependent manner. The molecular events taking place from pre-gastrulation up to formation of cardiomyocytes are recapitulated *in vitro* by differentiating mouse embryonic stem (mES) cells. Importantly, by closely following the kinetics of cell fate decisions occurring in the embryo, ES cells facilitate mechanistic studies aimed at the dissection of early lineage specification. Following previous findings demonstrating the role of the Notch pathway in specifying a cardiac fate from mesodermal progenitors and hemangioblasts[1], our laboratory identified a novel function for Hes5, as a downstream effector of Notch1, at the onset of cardiogenesis (Freire, AG et al, unpublished). Loss and gain of function studies unveiled that Hes5 instructs ES cell-derived mesodermal progenitors to commit preferentially towards cardiac over a hematopoietic fate, in part by regulating the early cardiac transcription factor, Isl1. Interestingly, a short-pulsed Hes5 induction enhances cardiac specification, whereas a sustained activation impairs the emergence of contracting colonies.

The herein Thesis aimed to further dissect the role of Hes5 in cardiogenesis. To this end, we proposed to understand the role of this bHLH regulator at different stages of the cardiomyocytic program. Given the robustness of the mES cell *in vitro* model system for cardiac differentiation, a mES cell line expressing exogenous *Hes5* under the control of a Doxycycline (Dox)-inducible promoter was used. The data indicated that *Hes5* expression maintains an undifferentiated cardiac progenitor state, in part by sustaining high *Isl1* levels. These results demonstrated that after induction of cardiac fate, Hes5 withdrawal is required to allow cardiac differentiation, suggesting a confined transient temporal window for Hes5 participation in cardiogenesis.

A second aim of this Thesis was the characterization of endogenous *Hes5* expression during mES cell differentiation towards mesodermal derivatives, in a more close to physiological system. Interestingly, *Hes5* levels upregulated from day 4 to day 6 of *in vitro* differentiation, correlating to the temporal window identified for enhanced cardiac differentiation induced by transient exogenous *Hes5* overexpression.

Finally, aiming at the validation of Hes5 role in specifying cardiac fate in the developing mouse embryo, *Hes5* expression was assessed in E6.5 and E7.5 mouse embryos. These results report for the first time Hes5 expression in the nascent mesoderm of gastrulating E6.5 embryos. Interestingly, *Hes5* expression was not found or dramatically reduced in E7.5 embryos, further corroborating a transient role for Hes5 at the onset of cardiogenesis.

Overall, the work performed in the frame of the herein Thesis contributed (i) a better understanding of Hes5 role at different stages of the cardiomyocytic differentiation program (*i.e.* at the specification of cardiac progenitors and during differentiation into cardiomyocytes), (ii) the indication that endogenous Hes5 is upregulated at the time cardiac progenitors are specified during *in vitro* mES cell differentiation, and (iii) the first report of *Hes5* expression in the nascent mesoderm of E6.5 gastrulating embryos.

RESUMO

A formação do coração envolve a participação de várias vias de sinalização que interagem entre si de um modo tempo- e contexto-dependente. Os eventos moleculares que ocorrem desde a pré-gastrulação até à formação de cardiomiócitos são recapitulados *in vitro* através da diferenciação de células estaminais embrionárias (CEEs). Importante ainda é que, ao acompanharem de perto a cinética das decisões de destino celular que ocorrem no embrião, as EECs facilitam a execução de estudos mecanísticos com o objectivo de dissecar a especificação das linhagens celulares. Dado o papel, previamente demonstrado, da via de sinalização Notch na especificação de um destino cardíaco a partir de progenitores da mesoderme e hemangioblastos, o nosso laboratório identificou uma nova função para o Hes5, como efector do Notch1, na indução da cardiogénese (Freire, AG et al, não publicado). Estudos de perda e ganho de função revelaram que o Hes5, em parte por regular os níveis do factor de transcrição Isl1, determina uma decisão preferencial pela diferenciação em linhagens cardíacas em detrimento de hematopoiéticas nos progenitores da mesoderme derivados de CEEs. Interessantemente, a indução de um pulso curto de Hes5 aumenta a especificação cardíaca, enquanto que uma activação contínua diminui o aparecimento de colónias a contrair.

Esta dissertação de Mestrado teve como objectivo dissecar o papel do Hes5 na cardiogénese. Com este propósito, propusemos compreender o papel deste factor de transcrição em diferentes etapas do programa de diferenciação cardiomiocítico. Dada a robustez do modelo *in vitro* de diferenciação de CEEs em células cardíacas, foi utilizada uma linha celular estaminal embrionária que expressa Hes5 exógeno sob o controlo de um promotor indutível de Doxiciclina (Dox). Os dados obtidos indicam que a expressão de Hes5 mantém as células num estado de progenitores cardíacos indiferenciados, em parte por manter os níveis de Isl1 também elevados. Estes resultados demonstraram que após a indução de um destino cardíaco é necessária uma diminuição de expressão de Hes5 para permitir diferenciação cardíaca, sugerindo que a participação do Hes5 na cardiogénese ocorre numa janela temporal transiente.

Um segundo objectivo desta dissertação incluiu a caracterização da expressão de Hes5 endógeno durante a diferenciação *in vitro* de derivados da mesoderme, num sistema mais próximo do fisiológico. Interessantemente, os níveis de Hes5 aumentam desde o dia 4 até ao dia 6 de diferenciação *in vitro*, o que correlaciona com a janela temporal previamente identificada por indução de expressão de Hes5 exógeno na qual o Hes5 aumenta a diferenciaçao cardíaca.

Finalmente, a expressão de Hes5 foi avaliada em embriões de murganho com 6,5 e 7,5 dias com o objectivo de validar o papel do Hes5 na especificação de um destino cardíaco no desenvolvimento embrionário de murganho. Estes resultados descrevem pela primeira vez expressão de Hes5 na mesoderme nascente de embriões com 6,5 dias. Importante ainda, é o facto de a expressão de Hes5 não ter sido detectada, ou estar dramaticamente reduzida em embriões com 7,5 dias, corroborando o papel transiente do Hes5 na indução da cardiogénese.

Em resumo, o trabalho realizado no âmbito da presente dissertação contribuiu (i) um melhor conhecimento do papel do Hes5 em diferentes etapas do programa de diferenciação cardiomiocítio (*i.e.* na especificação de progenitores cardíacos e durante a diferenciação em cardiomiócitos), (ii) a indicação que a expressão de Hes5 endógeno está aumentada durante o tempo de especificação dos progenitores cardíacos durante a diferenciação *in vitro* de CEEs, e (iii) uma primeira descrição de expressão de Hes5 na mesoderme nascente em embriões com 6,5 dias.

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LIST OF NON-STANDARD ABBREVIATIONS

AV	Atrioventricular
bHLH	Basic Helix-Loop-Helix
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
Bsd	Blasticidin
CHD	Congenital Heart Disease
CM	Cardiomyocyte
CVDs	Cardiovascular Diseases
D	Day
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
Dox	Doxycicline
E	Embryonic Day
EB	Embryoid Body
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EMT	Epithelial-to-Mesenchymal Transition
ESC	Embryonic Stem Cell
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FHF	First Heart Field
GFP	Green Fluorescent Protein
Hes	Hairy and Enhancer of split
Hesr	Hes related
IMDM	Iscove's Modified Dulbecco's Medium
LA	Left Atria
LIF	Leukeamia Inhibitory Factor

LV	Left Ventricle
mESC	Mouse Embryonic Stem Cell
NICD	Notch Intracellular Domain
OFT	Outflow Tract
PBS	Phosphate Buffered Saline
PBT	PBS-Tween 20
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PS	Primitive Streak
qRT-PCR	Quantitative Real-Time PCR
RA	Right Atria
RT	Room Temperature
RV	Right Ventricle
SEM	Standard Error of the Mean
SFDM	Serum Free Diferentiation Media
SHF	Second Heart Field
siRNA	Small Interfering RNA
TGF	Transforming Growth Factor
TRE	Tetracycline Response Element

Introduction

Embryonic Heart Development

Embryonic Stem Cells
In Vitro Differentiation System

Notch Signaling Pathway

INTRODUCTION

Cardiovascular diseases (CVDs) are a burden in society and constitute the major global cause of death[2]. The combination of the worldwide increase in life expectancy, population growth and epidemiological changes in CVDs is expected to result in augmented morbidity.

The heart had been considered a post-mitotic organ[3], although in the past years evidence of generation of new cardiomyocytes throughout life has arisen[4-8]. Nonetheless, is consensual that the scarcely newly formed cardiomyocytes are unable to replace the damaged myocardium[9]. Current treatments for cardiac repair include pharmacological-based approaches or heart transplantation and, hence there is a compelling need to invest in better and more efficient strategies for cardiac repair.

Under stress conditions, signaling pathways involved in heart formation are often reactivated, resulting either in beneficial or deleterious effects[10]. Thus, the comprehension of the regulatory mechanisms underlying cardiac specification and differentiation will open the way to oversee new possibilities for therapeutic strategies.

EMBRYONIC HEART DEVELOPMENT

OVERVIEW OF MORPHOLOGICAL DEVELOPMENT

During embryonic mouse development, the three germ layers (ectoderm, mesoderm and endoderm) are formed in a morphogenetic process designated gastrulation. Cardiac and hematopoietic progenitors derive from mesodermal progenitors at approximately the same time, as they ingress through the primitive streak (PS)[11,12]. Since heart is the first organ formed during embryonic development, the molecular events underlying cardiac specification occur very early. At embryonic day 6.5 (E6.5) the cardiogenic mesodermal cells migrate from the posterior epiblast into an anterior lateral region relative to the PS forming two bilateral groups of cells which then converge to form the cardiac crescent (E7.5)[13]. This first wave of cardiac progenitors that gives rise to the crescent is denominated First Heart Field (FHF). At E8, the crescent fuses at midline giving rise to the primitive beating heart tube which then

undergoes rightward looping (E9-E12). At this point the expansion of the linear heart tube is dependent on cell proliferation and also on recruitment of Second Heart Field (SHF) cells. FHF cells will contribute to the formation of the left ventricle with small contributions to the atria, whereas SHF cells will give rise to the outflow tract (OFT), right ventricle and a large portion of the atria. Additional contribution for the cardiac structures derive from cardiac neural crest cells in the formation of the aorticopulmonary septum[14], aortic smooth muscle cells[15] and intraventricular septum[16], and from the proepicardium that will originate the epicardium[17]. At E10.5 the heart presents well-defined chambers which display fully septation and connection to the pulmonary tract and aorta at E14.5 (Figure 1).

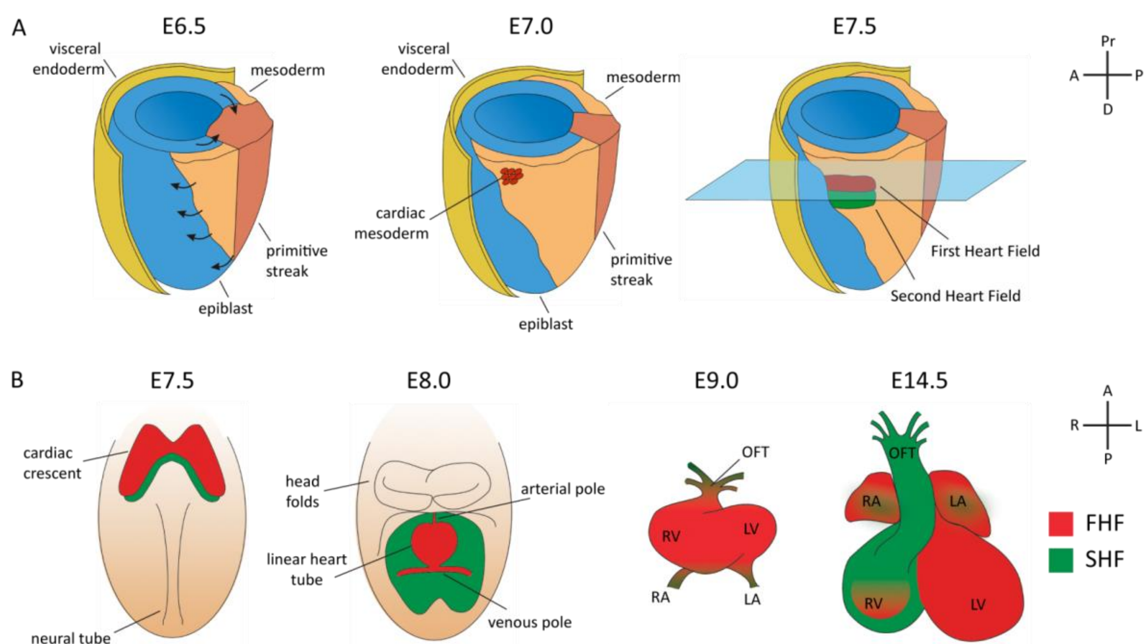

Figure 1 | Schematic Overview of Cardiac Specification and Heart Formation. (A) At E6.5 epiblast cells ingress in the primitive streak (PS) and will originate mesoderm. Cardiac progenitors migrate into a more anterior position forming the cardiac mesoderm at E7. At E7.5, the first (FHF) and second heart fields (SHF) are detectable. The FHF fuses at midline, giving rise to the cardiac crescent (B). At E8 the primitive beating heart tube is already formed. SHF progenitors gradually migrate to the linear heart tube and contribute mainly to Right Ventricle and Outflow Tract. At E9 the linear heart tube undergoes rightward looping resulting in four fully septated chambers at E14.5. FHF – First Heart Field; SHF – Second Heart Field; RV – Right Ventricle; LV – Left Ventricle; RA – Right Atria; LA – Left Atria; OFT – Outflow Tract; Pr – Proximal; D – Distal; A – Anterior; P – Posterior; R – Right; L – Left.

MOLECULAR REGULATION IN CARDIOGENESIS

Mesodermal induction is regulated by the interplay between distinct signaling pathways, including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), canonical (β-catenin dependent) and non-canonical (β-catenin independent) Wnt pathway and members of the transforming growth factor β (TGF-β) superfamily, including Nodal/Activin [18]. The mesodermal progenitors that ingress through the PS express the T-box transcription factor *Brachyury* (*T*) (also known as *Bry*)[19], a direct target gene of the Wnt/β-catenin pathway[20]. Cardiac commitment from mesodermal precursors requires the inhibition of Wnt/β-catenin pathway[21,22]. Later, Bry expression is downregulated and cardiogenic progenitors activate the expression of mesoderm posterior 1 (*Mesp1*)[23,24]. Mesp1 patterns mesoderm into distinct lineages, including cardiac and hematopoietic, in a context-dependent manner[25]. The expression of this transcription factor represents the first described molecular step in cardiac specification, being downregulated before the heart tube is formed[26]. Two sequential waves of Mesp1^{+} cells give rise to FHF and SHF precursors[27].

FHF cells are the first to differentiate mainly due to a more anterior and lateral position in the cardiac crescent which enables the exposure to BMP[28] and FGF[29] signaling and to inhibitors of the Wnt pathway[30-33]. The expression of T-box transcription factor Tbx5[34] and the homeobox protein Nkx2.5[35], although not exclusive, has been associated to FHF progenitors. Recently, the voltage-gated ion channel HCN4 was identified as FHF specific[36,37], independently from the expression observed in the cardiac conduction system and in the sinoatrial node[36].

The fate of SHF cells is regulated as well by different signaling molecules where FGF, Sonic Hedgehog (Shh) and canonical Wnt pathways promote the maintenance of proliferation (and inhibition of differentiation) prior entering the heart tube, whereas differentiation will take place as result of BMP, Notch and non-canonical Wnt signaling[38]. Unlike FHF, SHF progenitors display a characteristic marker, the LIM homeodomain transcription factor Islet1 (Isl1)[39-41], which is not found in differentiated FHF derivatives. In addition, SHF progenitors express Tbx1[42], Fgf8[43] and Fgf10[44]. Isl1^{+} cardiac progenitors originate cells of multiple cardiac lineages including myocardial, conduction system, endothelial and smooth muscle lineages[45]. Differentiation of cardiac precursors is accompanied by a downregulation of Isl1 levels, suggesting that

Isl1 expression may be incompatible with a differentiated state and/or may be crucial to maintain an undifferentiated state[39].

EMBRYONIC STEM CELLS *IN VITRO* DIFFERENTIATION SYSTEM

ESTABLISHMENT OF MOUSE EMBRYONIC STEM CELL LINES

Embryonic Stem (ES) cells are derived from the inner cell mass (ICM) of the blastocyst[46,47] and unambiguously fulfill the hallmarks of stem cells: self-renewal and pluripotency[48]. In 1981, Evans and Kaufman established a feeder-dependent pluripotent cell line isolated directly from *in vitro* cultures of mouse blastocysts that presented a normal karyotype.[46] These cells were able to differentiate both *in vitro* and *in vivo* forming teratocarcinomas in mice. This study demonstrated that pluripotent cells resembling teratocarcinoma stem cells could be isolated directly from normal early mouse embryos. In the same year, Martin[47] established a pluripotent cell line derived from ICM cells grown in conditioned medium from a teratocarcinoma stem cell line. The isolated cells had the capacity of forming teratocarcinomas when injected in mice and, when cultured at the subclonal level, differentiated into a wide variety of cell types, thereby demonstrating their pluripotency. Moreover, these cells retained their morphology in feeder-free conditions in the presence of medium conditioned by a teratocarcinoma stem cell line, suggesting the presence of a growth factor capable of maintaining pluripotency and/or inhibiting differentiation. Such soluble factor, later being identified as Differentiation Inhibitory Activity (DIA)[49], restrains the spontaneous differentiation of mouse ES (mES) cells growing *in vitro* in the absence of feeder cells. A subsequent report demonstrated that the structure and function of DIA coincided to the previously identified Leukeamia Inhibitory Factor (LIF), and that purified recombinant LIF could replace DIA in the maintenance of ES cell pluripotency[50].

DIFFERENTIATION OF EMBRYONIC STEM CELLS IN CULTURE

The establishment of ES cell lines has been an important contribution for mammalian developmental studies, as they constitute a unique tool to investigate

early cell lineage determination. ES cells have been shown to generate hematopoietic cells[51,52,53], cardiomyocytes[54-56], muscle cells[57] amongst others.

Three strategies have been used to drive ES cell differentiation: growth in suspension as three-dimensional spheroids called Embryoid Bodies (EBs)[58,59], or culture in direct contact with stromal cells[60] or as a monolayer on extracellular matrix (ECM) proteins[61]. The first method offers the advantage of enhancing cell-cell interaction in a three-dimensional structure, closely resembling what occurs in the embryo. Culture of ES cells with stromal cells has the associated problem of separating the two cell types. Despite of culture on top of specific extracellular matrix proteins can minimize the influence of other cell types and prompt the generation into particular cell lineages, the optimization of the adequate culture conditions is a more laborious process[48].

In the absence of LIF and with no additional cues mouse ES cells spontaneously and randomly differentiate into derivatives of the three germ layers[46,47]. The knowledge gathered from studies in the embryo and in the ES cell differentiation system has contributed to the optimization of defined cocktails that efficiently drive differentiation towards a desired lineage [48].

ES CELL-DERIVED CARDIOGENESIS

The differentiation of ES cells towards cardiac lineages progresses through different stages closely resembling the kinetics of the developing embryo. In differentiating ES cells mesodermal commitment can be assessed by the upregulation of Bry within 48 hours after the onset of differentiation[62]. Resembling mesodermal patterning of the developing embryo, ES cell-derived mesodermal population is compartmentalized in subsets characterized by differential expression of fetal liver kinase-1 (Flk1) and platelet-derived growth factor receptor-alpha (Pdgfrα)[63,64]. Balanced levels of Nodal and BMPs pattern mesoderm, as increased levels of Activin A favor $Flk1^{+}Pdgfr\alpha^{+}$ cardiac progenitors, while $Flk1^{+}Pdgfr\alpha^{-}$ hematopoietic progenitors are promoted with high doses of BMP4[65]. As in the embryo, ES cell-derived cardiac progenitors expressing early cardiac transcription factors (*e.g. Nkx2.5*, *Gata4, Tbx5, and Isl1)* differentiate into cardiomyocytes displaying structural markers (*e.g. myosin heavy chain 6* (*Myh6*, also known as *α-Mhc)*, *myosin heavy chain 7* (*Myh7*, also known as *β-Mhc*), and cardiac troponin T (*Tnnt2*, also known as *cTnT*))[66]. The sequential

expression of cardiac-affiliated proteins is accompanied by changes in cell morphology: from small and round cells with sparse and irregularly organized nascent myofibrils to elongated cells with well-developed myofibrils[67].

A major advantage of using ES cells relates to the easy genetic manipulation. Hence, ES cells have been genetically engineered to enable the isolation of cells at specific stages of differentiation. As examples, cell lines expressing reporter genes, like green fluorescent protein (GFP), under the control of *Bry*[68], *Nkx2.5*[69] or *cardiac α-actin*[70], allow the isolation of mesodermal cells, cardiac progenitors or purified cardiomyocytic populations from undifferentiated or non-cardiogenic cells.

Given the robustness in faithfully recapitulating lineage specification and differentiation, the ES cell differentiation system constitutes a unique *in vitro* model of early mammalian development. In this sense, this tool facilitates studies aiming at the comprehension of the role played by transcriptional regulators and signaling pathways in embryogenesis.

NOTCH SIGNALING PATHWAY

ELEMENTS OF THE NOTCH SIGNALING PATHWAY

The Notch signaling pathway is evolutionary conserved and regulates essential biological processes such as cell proliferation, cell apoptosis, and cell fate determination. Notch receptor was firstly described and named more than 90 years ago due to the phenotype of notched wings caused by its partial loss-of-function in flies[71].

The genome of most vertebrate species exhibit four Notch genes (Notch 1-4) encoding single pass transmembrane receptors. The extracellular domain of the receptor is composed by epidermal growth factor (EGF)-like repeats responsible for ligand binding[72]. Notch intracellular domain (NICD) contains a high-affinity binding site for CSL/RBP-Jk and tandem ankyrin repeats (ANK) essential for recruiting Mastermind-like proteins (MAML)[73-75]. Contrarily to pathways in which activation relies on secreted ligands, the canonical Notch signaling requires direct cell-cell interaction, as the ligand is also a transmembrane protein at the neighboring cell. In vertebrates, Notch ligands belonging to two families: three Delta or Delta-like (Dll1, Dll3 and Dll4, orthologues of

the *Drosophila* Delta) and two Jagged ligands (Jagged 1 and 2, orthologues of the *Drosophila* Serrate) have been identified[76,77]. After ligand binding the Notch receptor undergoes conformational changes that expose the proteolytic site for cleavage by ADAM/TACE metalloprotease[78]. To achieve complete activation of the receptor a second cleavage mediated by γ-secretase complex (containing presenilin and nicastrin subunits) is required[79]. This last cleavage releases NICD from the membrane which then translocates to the nucleus where it forms an active transcriptional complex with the DNA-binding protein RBP-Jk[80]. In the absence of NICD, RBP-Jk binds to its consensus DNA sequence and recruits co-repressors functioning as a transcriptional repressor of Notch target genes[81-83]. Activation of Notch and further binding of NICD to RBP-Jk leads to the displacement of the co-repressor complex[84] and recruitment of co-activators, such as Mastermind-like (MAML)[85], allowing transcription of Notch targets[85].

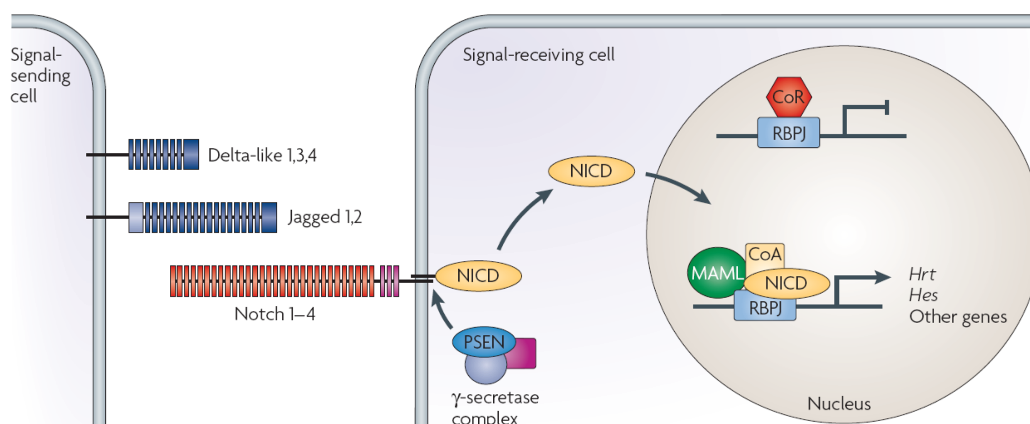

Figure 2 | Canonical Notch pathway. Mammals express four Notch receptors (Notch 1-4) and five ligands belonging to two families, Delta-like (1, 3, 4) and Jagged (1, 2). After ligand binding, Notch receptors undergoes a sequence of proteolytic cleavages, culminating in the release of the intracellular domain (NICD) mediated by the γ-secretase complex containing presenilin (PSEN) catalytic subunit. NICD translocates to the nucleus, where it forms an active transcriptional complex with RBP-J and co-activators (Co-A), including MAML. In the absence of NICD, RBP-J acts as a transcriptional repressor by binding Notch target genes and recruiting co-repressors (CoR). When Notch is activated RBP-J is converted from a transcriptional repressor to an activator. Notch targets include members of *Hes* and *Hes-related (Hrt)* family genes which encode bHLH transcriptional regulators that mediate a great part of the downstream events of Notch signaling. Reproduced from[86].

Downstream effectors of Notch signaling include members of *Hairy and Enhancer of split (Hes) and Hes-related (Hesr, also known as Hrt and Hey)* gene families, which

encode basic helix-loop-helix (bHLH) transcription factors[87-89] (Figure 2). Hes/Hey factors are important mediators of Notch activity in the regulation of cell fate determination and timing of cell differentiation. In mammals, *Hes* gene family is constituted by seven members (*Hes 1-7*), although mouse genome lacks *Hes4*. While *Hes1*, *Hes5* and *Hes7* are induced by Notch activation, *Hes2*, *Hes3* and *Hes6* are apparently Notch-independent[90]. These factors have three conserved domains: the bHLH, which is essential for DNA binding and for dimerization; the Orange domain, that regulates the selection of bHLH heterodimer partners; and the WRPW domain, which interacts with co-repressors and acts as a polyubiquitylation signal[91]. *Hey1*, *Hey2* and *Heyl* are the three mammalian members of the Hes-related family and can be induced by Notch. These proteins also possess the bHLH and Orange domains although they lack the WRPW domain, displaying instead a related YRPW peptide incapable of binding co-repressors[92].

Hes proteins repress transcription by DNA binding-dependent and independent mechanisms. DNA binding-dependent mechanisms usually involve recruitment of cofactors, such as Groucho/TLE corepressors, and independent mechanisms include prevention of DNA binding by lineage-specific bHLH activators, such as MyoD or Mash1[90].

NOTCH SIGNALING IN CARDIAC SPECIFICATION AND DIFFERENTIATION

In *Xenopus,* the activation of Notch signaling after formation of the early heart field suppresses cadiomyogenesis, as demonstrated by the downregulation of genes that encode contractile proteins (*e.g.* c*TnI, Myh6* and *cardiac actin*)[93]. Similarly in mice, activation of NICD1 in Mesp1$^+$ cells, although not affecting myocardial and endocardial cell-fate decisions, resulted in abnormal heart morphology by inhibiting myocardial maturation, with consequential death before E11.5[94]. Also supporting a suppressive role in cardiac differentiation, mES cells deficient in RBP-Jk[95] or in Notch1 receptor[96] displayed enhanced cardiomyogenic differentiation. These reports show that at specific stages of development, Notch signaling inhibits cardiac differentiation. Regardless, expression of Notch receptors in mesoderm of the gastrulating embryo[97] and at different stages in cardiovascular differentiation[98] suggests that this pathway can play important roles in cardiac development. In fact, Mesp1 activation in

differentiating mES cells[99] results in transient upregulation of multiple components of the Notch pathway. Additionally, this study has shown that blockade of Notch signaling using DAPT (a γ-secretase inhibitor) from day 2 to day 4 of *in vitro* differentiation resulted in decreased number of cardiomyocytes and endothelial cells. These observations suggested a role for this pathway early in cardiovascular lineage commitment, which has been further demonstrated in a subsequent study showing the reespecification of ES cells-derived hemangioblasts (hematopoietic/vascular progenitors) into a cardiac fate after Notch activation[1].

The different outcomes observed in cardiogenesis demonstrate that Notch signaling exerts different roles depending on the stage of development, cell context, and timing and extent of signal activation.

NOTCH SIGNALING IN HEART MORPHOGENESIS

The process of heart morphogenesis until the four chambered organ is completely formed involves a series of remodeling events, including rightward looping of the linear heart tube. During this step, Notch1 and Notch2 receptors and Dll1 ligand are required for the establishment of embryonic left-right asymmetry, and thus, proper looping of the heart[100,101]. During chamber development the atrioventricular (AV) canal is formed between the prospective atria and ventricles, separating atrial and ventricular blood flow. At the AV canal, correct valve versus chamber specification depends on restricted expression of Tbx2 and BMP2 regulated by the Notch targets Hey1 and Hey2[102,103]. In addition, as result of signaling provided by the AV canal, adjacent endothelial cells undergo epithelial-to-mesenchymal transition (EMT) forming the endocardial cushions, the precursors of the cardiac valves. Several Notch components are expressed in the mouse AV canal (Table 1) with mutations resulting in impaired EMT[104] and subsequential valve and septal defects[98,105,106] (Table 2).

Additionally, Notch signaling, mediated by endothelial Jagged1 ligand, is required for proper patterning of the OFT[107], a structure that connects the ventricles to the aorta and pulmonary arteries. Moreover, EMT mediated by Notch signaling leads to the formation of the OFT cushions, which are the primordia of the aortic and pulmonary valves[108].

During ventricular chamber development the myocardium differentiates into two layers, an inner trabecular zone and an outer compact zone[109]. Endocardial Notch signaling, mediated by Dll1[110], is indispensable for myocardial trabecular proliferation, differentiation and maturation by promoting BMP10[111] (proliferation signal), and Nrg1/ErbB and EphrinB2/EphB4 (differentiation signal) activities[94,110].

Table 1 | Expression of Notch pathway components during murine heart development

Notch Pathway Component	Expression Pattern	Refs
Notch1	Expressed in the cardiac crescent (E7.5). At E8.0 to E11.5 expression is limited to the entire endocardium and highly expressed in the AV canal and OFT endocardium.	112,113
Notch2	Expressed in the AV canal endocardium (E12.5) and in the OFT (E11.5 and 14.5). Expressed in atrial and ventricular myocardium (E13.5).	114-116
Notch3	Expressed in the cardiac crescent (E7.5) but not detected after heart tube formation (E8.0).	112
Notch4	Expressed in the endocardium (E10.5).	117
Jagged 1	Expressed in the AV canal and OFT endocardium and atrial myocardium (E10.5–E12.5).	98
Dll1	Expressed in the endocardium at the base of ventricular trabeculae (E9.5).	110
Dll4	Expressed in the cardiac crescent (E8.0) and the endocardium from E8.5 onwards. Expression is further restricted to the ventricular endocardium after E11.5.	105,118
Hey1	Expressed in the lateral portion of the heart tube (E8.5) and the endocardium and septum transversum (E9.5). Expressed exclusively in the atrial myocardium at E10.5.	112,119
Hey2	Expressed in the anterior portion of the heart tube (E8.5) and the AV canal and OFT endocardium (E11.0). Highly expressed in the subcompact ventricular myocardium at E10.5.	119,120
Heyl	Expressed in the AV canal endocardium (E9.5-E12.5).	106
Hes1	Expressed in the second heart field (E8.5 and E10.5).	121

AV-atrioventricular; E-embryonic day; OFT-outflow tract; EMT-epithelial-to-mesenchymal transition; Adapted from [122]

NOTCH MUTATIONS AND CONGENITAL HEART DISEASE

The tight regulation exerted by Notch components within a complex interaction with other pathways, will contribute for the proper organization, shape, cell

composition and function of the heart. Not surprisingly, mutations in different components of the Notch pathway cause congenital heart defects (CHD), including aortic valve disease and calcification, Tetralogy of Fallot and Alagille Syndrome (Table 2) [86,123,124].

Table 2 | Notch mutant mouse models with cardiovascular defects

Mutation(s)	Stage of lethality	Cardiac phenotypes	Refs
Notch1 $^{-/-}$	E9.5 to E10.5	Hypocellular endocardial cushions, impaired ventricular trabeculation	104,110
Notch2 $^{del1/del1*}$	E11.5 to birth	Pericardial effusion, thinned myocardium	104,110, 125
Jag1 $^{-/-}$		Pericardial edema	126
Jag1 $^{+/-}$, ***Notch2*** $^{+/del1}$	Postnatal to adulthood	Pulmonary artery stenosis, ventricular septal defects, atrial septal defects	127
RBP-Jk $^{-/-}$	E9.5 to E10.5	Heart looping defect, hypocellular endocardial cushions, impaired ventricular trabeculation	104,110, 127,128
Hey2 $^{-/-}$	Postnatal	Cardiomyopathy, ventricular septal defects, atrial septal defects, pulmonary artery stenosis, Tetralogy of Fallot, tricuspid valve atresia, AV valve dysfunction	129-132
Hey1 $^{-/-}$, ***Hey2*** $^{-/-}$	E11.5	Heart looping defects, impaired ventricular trabeculation, hypocellular endocardial cushions	133,134
Hey1 $^{-/-}$, ***HeyL*** $^{-/-}$	Postnatal	Ventricular septal defects, AV valve defects	106

***homozygous for a hypomorphic allele of *Notch2* with a deletion in the EGF repeat domain; AV-atrioventricular; Dll-delta-like; EGF-epidermal growth factor; Hey-Hes-related transcription factor; Jag-jagged; RBP-Jk-recombination signal binding protein for immunoglobulin J-kappa region; Adapted from [86]**

Aims

AIMS

It is currently recognized that in situations of stress, pathways involved in heart formation are often reactivated, resulting either in beneficial or deleterious effects. Therefore, the comprehension of the regulatory mechanisms underlying cardiac specification and differentiation will open the way to oversee new possibilities for therapeutic strategies. One can anticipate targeted therapies aiming at the specific modulation of the signaling environment to promote pro-cardiogenic processes in the injured heart. In particular, Notch pathway, which activity decreases during postnatal life[135], has been shown to be reactivated after myocardial infarction in association to repair and pro-survival processes[135-137]. Additionally, Notch signaling has been implicated in the specification of a cardiac fate in a context and time dependent manner[1,99]. In the interest of identifying the regulators downstream of Notch involved at the onset of cardiogenesis, previous work from our laboratory identified a novel function for Hes5, a member of the Hes family of well-known Notch targets, in the specification of a cardiac fate (Freire, AG et al, unpublished). Loss and gain of function studies demonstrated that Hes5 instructs ES cell-derived mesodermal progenitors to commit preferentially towards cardiac lineages in detriment to a hematopoietic fate, in part by regulating the levels of Isl1, an early cardiac transcription factor. Importantly, Hes5 induces cardiac specification when provided as a short-pulse whereas its sustained expression impairs the emergence of contracting colonies. These observations hinted that Hes5 may be required in a confined temporal window as a pulsed-activation to allow the progression in the differentiation program.

Following these findings, the work performed in the framework of this Thesis was conducted to further dissect the role of Hes5 in cardiogenesis.

Given the inductive role at the onset of cardiogenesis while suppressing further differentiation, we proposed to understand the role of Hes5 at different stages of the cardiomyocytic program. The experimental design consisted in employing a mES cell line that expresses *Hes5* under the control of a Doxycycline (Dox)-inducible promoter.

Next, we proposed to characterize *Hes5* expression profile, within the context of other Notch components, during mES cell differentiation towards mesodermal derivatives, in the absence of exogenous *Hes5* activation.

Aiming at the validation of Hes5 role in specifying cardiac fate in the developing mouse embryo, the final aim of the herein Thesis was to specifically assess *Hes5* expression in nascent mesodermal cells primed to become cardiac progenitors.

Materials and Methods

mES cell line

Animals

mES cell culture

mES cell differentiation

Flow Activated Cell Sorting

RNA Extraction

cDNA synthesis

Quantitative Real-Time PCR

Immunofluorescence

RNA probe synthesis

In situ hybridization

MATERIALS AND METHODS

mES cell line

The AinV/Bry-GFP mouse embryonic stem (mES) cell line[68] was a kind gift from Dr Gordon M. Keller and Dr Valerie Gouon-Evans. This cell line was previously transduced with a lentiviral pTRE-IRES-Bsd^R vector containing Flag-tagged Hes5 cDNA driven by a tetracycline response element (TRE) promoter, allowing Doxycycline (Dox)-inducible expression of exogenous Hes5.

Animals

All experiments with animals were performed in accordance with the IBMC.INEB Animal Ethics Committee and the Direcção-Geral de Alimentação e Veterinária (DGAV). Embryos from adult pregnant C57BL/6 mice with 6.5 to 9.5 days of gestation (E6.5-E9.5) were used for this study.)

mES cell culture

mES cells were maintained in the absence of feeders on 0.1% gelatin (Sigma) coated-plates in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies) supplemented with 15% Fetal Bovine Serum (FBS) (BenchMark, Cat Lot No: A00D05C,), 100 mM MEM non-essential amino acids (Gibco, Life Technologies), 100 U/mL penicillin/streptomycin (Gibco, Life Technologies), 1 mM sodium pyruvate (Gibco, Life Technologies), 2 mM L-glutamine (Gibco, Life Technologies), 0.1 mM β-mercaptoethanol (Sigma-Aldrich) and 1000 U/mL Leukemia inhibitory factor (LIF) (Esgro, Millipore).

mES cell differentiation

Mesoderm differentiation was induced as previously described[65,138] with few modifications. Briefly, at day 0 of differentiation (D0) cells were dissociated with TrypLE Express (Gibco, Life Technologies) for 4-5 minutes (min) at 37°C followed by centrifugation at 1200 rpm for 4 min in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Life Technologies) with 0,05% bovine serum albumin (BSA) (Gibco, Life Technologies) to stop dissociation. Cells were then resuspended in Serum Free Differentiation Media (SFDM) that consisted of 75% IMDM and 25% Ham's F12 medium (HyClone) supplemented with 0,5× of both N2 and B27 (Gibco, Life

Technologies), 0.05% BSA, 1× penicillin/ streptomycin, 2 mM L-glutamine, 4.5×10^{-4} M 1-Thioglycerol (Sigma-Aldrich) and 50 µg/mL ascorbic acid. Cells were plated at $1.5\text{x}10^{5}$ cells/mL in 100 mm bacterial petri dishes for 48 h to allow formation of embryoid bodies (EBs). After this period (D2) EBs were collected by centrifugation at 800 rpm for 2 min, dissociated using TrypLE Express and resuspended at $1.5\text{-}4\text{x}10^{5}$ cells/mL in SFDM supplemented with 5 ng/mL human VEGF (R&D Systems), 25 ng/mL human Activin A (R&D Systems) and 1 ng/mL human BMP4 (R&D Systems) in ultra-low attachment 6-well plates (Corning). At D3.75 of differentiation mesodermal cells were reaggregated in StemPro-34+StemPro-Nutrient Supplement (Gibco, Life Technologies) containing 2 mM L-glutamine, penicillin/streptomycin, 200 µg/mL human transferrin (Sigma-Aldrich), $4.5\text{x}10^{-4}$ M 1-Thioglycerol and 0.5 mM ascorbic acid, at $2\text{x}10^{5}$ cells/mL in ultra-low attachment 24-well plates (Corning). After 24 hours (h), aggregates were collected and replated in gelatin-coated 24-well plates in StemPro34+StemPro-Nutrient Supplement with 2 mM L-glutamine and penicillin/streptomycin. Dox (1 µg/mL; Sigma-Aldrich) was added to the medium at indicated time points. Cultures were monitored for cell contraction under light microscope and photographs were acquired using an Axiovert 200M (Zeiss) inverted fluorescence microscope.

Flow Activated Cell Sorting

D3.75 cells were dissociated with TrypLE Express and resuspended in phosphate buffered saline (PBS) containing 2% FBS, 25 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 1 mM ethylenediaminetetraacetic acid (EDTA) and sorted based on the expression of Bry-GFP using FACS Aria (BD Biosciences) cell sorter. Samples were analyzed using FlowJo software.

RNA extraction

Total RNA was extracted using Trizol Reagent (Ambion, Life Technologies). Briefly, cells were lysed in Trizol Reagent and incubated for 5 minutes (min) at 15-30°C. For phase separation samples were incubated for 2 to 3 min with 0.2 mL of chloroform (Sigma-Aldrich) *per* 0.5 mL of initial Trizol Reagent. Next, samples were centrifuged at 12000 g for 15 min at 4°C. RNA from the aqueous phase was precipitated by adding 0.5 mL Isopropanol (Merck) and incubating at -20°C for at least 1 h. At this point, 1 µL of Glycogen [20 mg/mL] (Roche) was added *per* 0.5 mL of initial Trizol Reagent as it

functions as a carrier. Samples were centrifuged at 12000 g for 30 min at 4°C and RNA was washed twice with 75% ethanol at 7500 g for 5 min at 4ºC. RNA was dissolved in 15 µL RNase-free water (BioLine) and stored at -80°C.

cDNA synthesis

cDNA synthesis was performed using PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions. Briefly, up to 500 ng RNA was reversed transcribed in 1x PrimeScript Buffer, 25 pmol Oligo dT Primer, 50 pmol Random hexamers and 0.5 µL PrimeScript RT Enzyme Mix I in a total volume of 10 µL. Reaction was carried out in a thermocycler for 15 min at 37°C, followed by 5 seconds at 85°C and stopped at 4°C.

Quantitative Real-Time PCR

Quantitative real-time PCR (qReal-Time PCR) was performed by mixing 1x iQ Sybr Green Supermix (Bio-Rad), 0.15 µM of each primer, and cDNA template in a total volume of 20 µL per reaction. Reactions were carried out in triplicate on the iCycler iQ5 Real-Time PCR system (Bio-Rad) according to Table 3. Relative gene expression was calculated using the ΔC_T method using glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as a reference gene. Gene-specific primers used in this study are listed in Table 4.

Table 3 | Protocol for qReal-Time PCR

Cycle	Repeats	Step	Dwell Time (min)	Setpoint (°C)
1	1	1	3:30	95.0
2	40	1	0:20	95.0
		2	0:30	60.0
3	81	1	0:10	55.0

Relative gene expression was calculated using the ΔC_T method using glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as a reference gene. Gene-specific primers used in this study are listed in Table 4.

Table 4 | Primers used for Quantitative Real-Time PCR

Gene	Forward	Reverse
Gapdh	CGTCCCGTAGACAAAATGGT	TTGATGGCAACAATCTCCAC
Hes1	TCATGGAGAAGAGGCGAAGGGCA	GAGCGCGGCGGTCATCTGC
Hes5	GGAGAAAAACCGACTGCGGA	TGTTTCAGGTAGCTGACGGC
Hey1	GAGAAGCGCCGACGAGACCG	GGCGTGCGCGTCAAAATAACCTTT
Hey2	TGCGTTCCGCTAGGCGACAG	TGAGCTTGTAGCGTGCCCAGG
Heyl	CAGCCCTTCGCAGATGCAA	CCAATCGTCGCAATTCAGAAAG
Notch1	TCCTAAGAGCACAACCCAGGAT	TTAGGCATGGCACAGACACT
Isl1	ATGATGGTGGTTTACAGGCTAAC	TCGATGCTACTTCACTGCCAG
Myh6	GCCCAGTACCTCCGAAAGTC	GCCTTAACATACTCCTCCTTGTC
cTnT	CAGAGGAGGCCAACGTAGAAG	CTCCATCGGGGATCTTGGGT
Myh7	ACTGTCAACACTAAGAGGGTCA	TTGGATGATTTGATCTTCCAGGG

Immunofluorescence

For cardiac Troponin T staining, cells were dissociated and cytospun into slides at 400 rpm for 3 min. Following fixation with 4% paraformaldehyde (PFA) for 20 min, cells were permeabilized with 0.2% Triton X-100 (Sigma Aldrich) for 5 min and blocked for 1 h with PBS buffer containing 1% BSA and 4% FBS. Samples were then incubated with mouse anti-cardiac Troponin T (cTnT) (MS-295-P0, Thermo Scientific) at 1:500 dilution for 2 h at room temperature (RT), followed by incubation with rabbit anti-mouse 488 (A11059, Invitrogen) at 1:1000 dilution for 1 h at room temperature (RT). For nuclei staining, samples were mounted in Fluoroshield with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Images were acquired using an Axiovert 200M (Zeiss) inverted fluorescence microscope. Quantification of cTnT positive cells was performed by counting the number of DAPI stained nuclei and number of cTnT positive cells from at least 20 sections using the ImageJ software.

RNA probe synthesis

Hes5 probe was a kind gift from Dr. Domingos Henrique. Plasmid was linearized by incubation for 5-15 min at 37°C of 1 µg DNA plasmid with 0.5 U/µL *HindIII* restriction enzyme and 1x restriction enzyme buffer (Promega) in RNase free water. To stop the reaction, enzyme was inactivated at 80°C for 10 min. Plasmid linearization was assessed by running in 1x NZYDNA loading dye (Nzytech) on a 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer at 90 Volts (V). For synthesis of digoxigenin (DIG) labeled Hes5 antisense RNA probe, 1 µg linearized plasmid was mixed with 0.875x transcription buffer (Promega), 0.01 M DTT (Roche), 0.5x DIG RNA labelling mix (Roche), 0.92 U/µL T3 RNA polymerase (Roche) and 2 U/µL RNasin (Promega) in RNase free water. Incubation was performed at 37°C for 3 h and placed at 4°C to stop reaction. To degrade DNA, the obtained mixture was incubated for 30 min at 37°C with 1 U/µL RQ1 RNase free DNase (Promega) and 2 U/µL RNasin (Promega). For RNA precipitation, the solution was incubated overnight at -20°C with 2.5 mM Tris-Base and 0.25 mM EDTA, 0.1 M LiCl and 600 µL EtOH 100%. The supernatant was discarded after centrifugation for 30 min at 14000 rpm and the pellet was washed with 1 mL 70% ethanol followed by a centrifugation at 14000 rpm for 15 min. All centrifugations were performed at 4°C. After discarding the supernatant, the pellet was left to dry on ice. The RNA pellet was resuspended in 50 µL RNase free water, and the probe was stored at -20°C. Probe obtained before and after precipitation was assessed for integrity, by running in 1x loading buffer on a 1% agarose gel in TAE buffer at 90 V.

In situ **hybridization**

Embryos were fixed overnight in 4% PFA at pH 7.5 at 4°C. *In situ* hybridizations were performed as previously described[139]. Briefly, embryos were dehydrated in a graded series of methanol: phosphate buffered saline – Tween 20 (PBT) for storage at -20°C. Prior to use, embryos were rehydrated in a graded series of methanol:PBT, treated with 10 µg/mL proteinase K (Sigma) according to the developmental stage (7 min (E6.5 and E7.5) or for 17 min (E9.5)), and post-fixed for 20 min in 4% PFA and 0.1% glutaraldehyde. Embryos were incubated for 1 h at 65°C with 1:1 solution of PBT:Hybridization (1:1) solution (Hybmix) (Table 5). Following incubation overnight at 65°C with Hes5 antisense probe diluted in Hybmix, tissue was sequentially washed in Hybmix, Hybmix/MABT and finally in MABT (Table 5). Blocking was performed by

incubating the embryos first for 1 h in MABT containing 2% blocking reagent (BL) (Roche), then 1 h in MABT containing BL and 20% sheep serum (Sigma-Aldrich), both at RT. Immunodetection of DIG, was performed overnight at 4 °C with anti-digoxigenin antibody (Roche) (1:2000). Embryos were washed 4 times with MABT for 1 h and enzymatic development of the signal was performed by incubation of the embryos at 37 °C with 4-Nitro blue tetrazolium chloride (NBT) (Roche)/ 4-toluidine salt (BCIP) (Roche) in NTMT (Table 5). Embryos were observed and photographed using an Olympus SZX10 Binocular Magnifying Glass acoplated with a DP21 Olympus camera.

Table 5 | Reagents used for *in situ* hybridization

Reagents	Constitution
Hybridization Solution (Hybmix)	1.3x SSC (Invitrogen), , 5 mM EDTA (Sigma Aldrich) 50 % formamide (Q-Biogene) 50 µg/mL Yeast RNA (Roche) 0.2 % Polyoxyethyl 20 Enesorbitan Monolaurate (Tween 20) (Sigma Aldrich) 0.5 % CHAPS (Roche) 100 µg/mL Heparin (Sigma Aldrich)
MABT	0.5 M Maleic Acid (Sigma Aldrich) 744 mM Sodium Chloride (BDH Prolabo) 5% Tween 20
NTMT	0.1 M Sodium Chloride 0.1 M Tris-HCl (Sigma Aldrich) 50 mM $MgCl_2$ (Merck) 1% Tween 20

Data and Statistical Analysis

Data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA with post hoc Tukey's test. $p<0.05$ was considered statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

Results

Differentiation of mouse Embryonic Stem Cells (mESC) towards mesodermal derivatives

Sustained Hes5 expression maintains an undifferentiated cardiac progenitor state by promoting high Isl1 levels

Endogenous levels of Hes5 upregulate from D4-D6 of in vitro differentiation

Hes5 is expressed in the nascent mesoder of E6.5 gastrulating embryos

RESULTS

Differentiation of mouse Embryonic Stem Cells (mESC) towards mesodermal derivatives

To dissect the role of Hes5 in cardiac specification we used the AinV/Bry-GFP mES cell line[68] that contains the GFP cDNA targeted to the *Brachyury* (*Bry*) locus, a mesodermal marker. Also, this cell line was transduced with a lentiviral cassette containing Flag-tagged *Hes5* cDNA driven by a tetracycline response element (TRE) promoter. In this system, exogenous *Hes5* is expressed only in the presence of Doxycycline (Dox) (Figures 3E and 3F). To direct cells towards mesodermal derived lineages, pluripotent cells (Figure 3B) were differentiated as Embryoid Bodies (EBs) in the absence of LIF and serum as previously described[65,138], with some modifications (Figure 3A). Briefly, mES cells were cultured in low-attachment plates in serum-free differentiation media (SFDM) in the presence of 50 µg/mL ascorbic acid and 4.5×10^{-4} M 1-thioglycerol (MTG) for 48 h to allow Embryoid Body formation (Figure 3C). At day 2 (D2) of *in vitro* differentiation cells were dissociated and reaggregated in the previous media supplemented with 1 ng/mL BMP4, 25 ng/mL Activin A and 5 ng/mL VEGF. Fluorescence microscopy (Figures 3C and 3C') and flow cytometry (Figure 3D) analyses demonstrated that cells successfully differentiated into mesodermal cells expressing Bry-GFP. At D3.75 mesodermal progenitors (> 90%) (Figure 3D) were sorted based on Bry-GFP expression and cultured in StemPro-34+StemPro-Nutrient Supplement containing 0.5 mM ascorbic acid, $4.5\text{x}10^{-4}$ M MTG and 200 µg/mL Transferrin and allowed to reaggregate for 24 h, followed by seeding in gelatin-coated plates in StemPro-34+StemPro-Nutrient Supplement (D4.75). Cells were maintained in this media up to 6 days to allow differentiation into mesodermal derivatives and cardiogenesis was assessed by emergence of contracting *foci*. To induce exogenous *Hes5* (*iHes5*) expression specifically in mesodermal progenitors Dox was added at D3.75 and maintained for specific periods of time. Efficient *Hes5* induction was confirmed by analysis of mRNA levels at 24 h (850 fold increase) and 48 h (1050 fold increase) after treatment with Dox (Figure 3F).

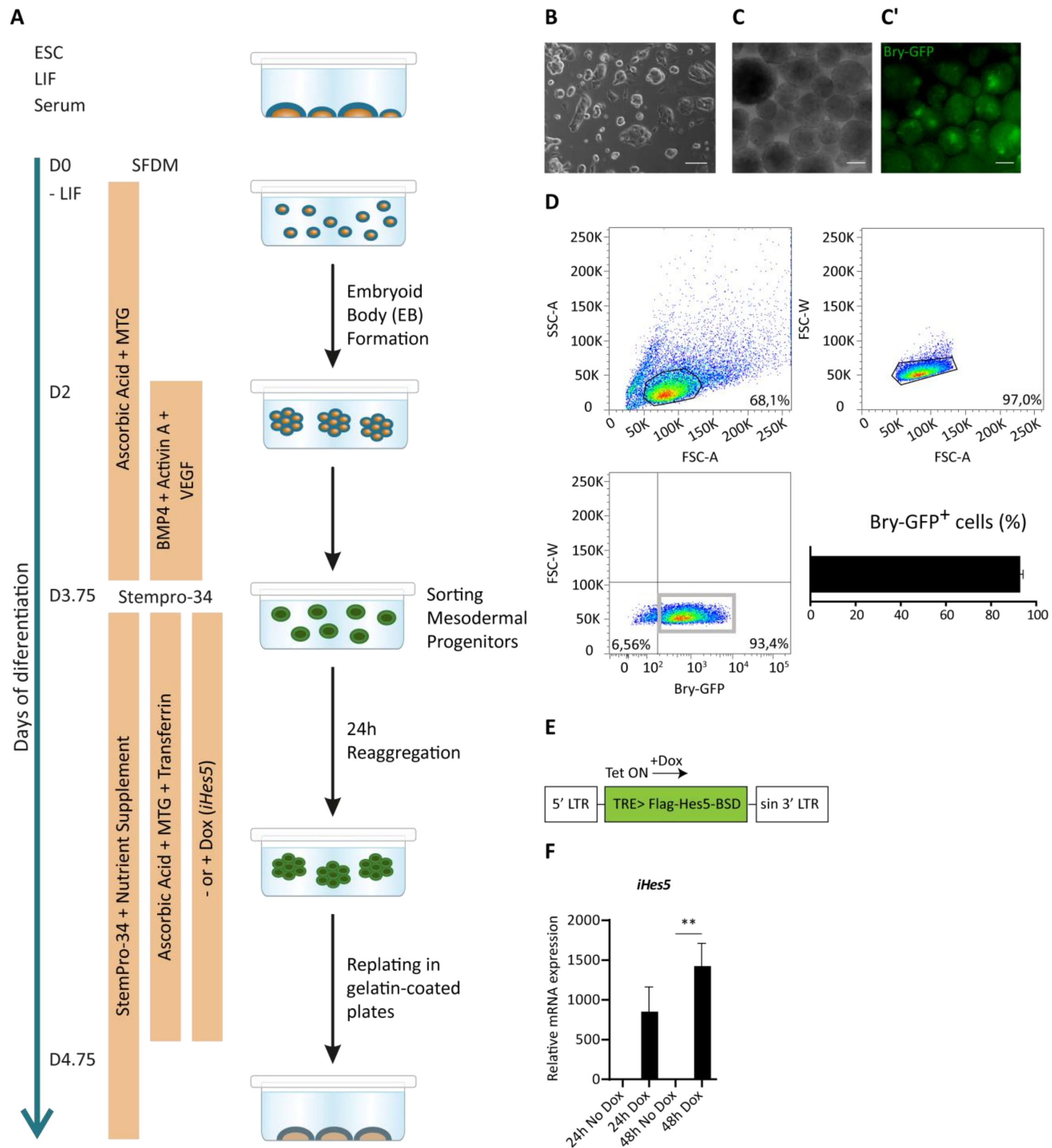

Figure 3 | (A) Schematic overview of the experimental procedure used to differentiate mES cells towards mesodermal derivatives. Generation of Embryoid Bodies (EBs) was promoted in serum-free conditions. At D2 cells were dissociated and reaggregated in the presence of BMP4, Activin A and VEGF. At D3.75 Bry-GFP mesodermal progenitors were sorted, allowed to aggregate for 24 h in Stempro-34 media and plated in gelatin-coated plates at D4.75. To induce *Hes5* overexpression Doxycycline (Dox) was added at D3.75. and maintained for specific periods of time. (B) mES cell colonies on gelatin-coated plates. Scale bar: 100 µm. (C-C') Phase contrast image of Embryoid Bodies (C) and Bry-GFP expression (C′) at D3.75 of *in vitro* differentiation. Scale bar: 100 µm. (D) Flow cytometry profile at D3.75 of *in vitro* differentiation and quantification of Bry-GFP^{+} cell percentage (n=11). (E) AinV/Bry-GFP mES cells were transduced with a lentiviral cassette containing a tetracycline response element (TRE) promoter driving the expression of exogenous Flag-tagged Hes5 and Blasticidin (BSD) in the presence of Dox. (F) Quantification of relative mRNA expression demonstrating *Hes5* overexpression at 24 h and 48 h after Dox treatment (n=3-4). Expression is normalized to No Dox condition at each time point. Data are represented as mean ± SEM. **p < 0.01.

Sustained Hes5 expression maintains an undifferentiated cardiac progenitor state by promoting high Isl1 levels

Previous findings in our laboratory demonstrated that a 24 h pulse-activation of *Hes5* in D3.75 mesodermal progenitors resulted in enhanced cardiac commitment, while its sustained expression compromised the emergence of contracting colonies (Freire, AG et al, unpublished). Given the inductive role at the onset of cardiogenesis while suppressing further differentiation, we proposed to understand the role of Hes5 at different stages of the cardiomyocytic program. To determine the period of time in which *Hes5* expression enhances cardiac differentiation, D3.75 mesodermal cells were subjected to different pulses of Dox treatment (No Dox, 24 h, 48 h, 96 h and 144 h) and the number of contracting foci was counted at D10. Only *Hes5* activation for 24 h and for 48 h promoted a significant increase in the number of contracting *foci,* whereas induction for 96 h and 144 h resulted in similar or decreased number, respectively, when compared to non-induced control (Figures 4A and 4B). As expected, sustained *Hes5* induction (*i.e.* 144h) resulted in almost abrogation of contracting cells, indicating that *Hes5* expression is detrimental for the progression in the cardiac differentiation program.

To investigate whether *Hes5* expression is incompatible with a differentiated state, the mRNA levels of cardiac troponin T (*cTnT*), myosin heavy chain 6 (*Myh6*) and myosin heavy chain 7 (*Myh7*), that encode structural proteins indicative of differentiation into cardiomyocytes, were analyzed. At D10 of *in vitro* differentiation the mRNA levels of *cTnT*, *Myh6* and *Myh7*, that encode proteins of the contractile machinery, were upregulated when a 24 h pulse was applied (3-, 6- and 2-fold, respectively) and further increased in 48 h-induced cells (6-, 23- and 9-fold, respectively), comparatively to non-induced control (Figure 4C). These observations are in agreement with the increased emergence of contracting *foci* in 24 h- and 48 h-induced cultures. In addition, *Hes5* activation in a sustained manner (*i.e.* 144 h) resulted in decreased *cTnT*, *Myh6* and Myh7 mRNA levels when compared to shorter periods of induction, possibly accounting for the diminished number of contracting *foci* (Figure 4C).

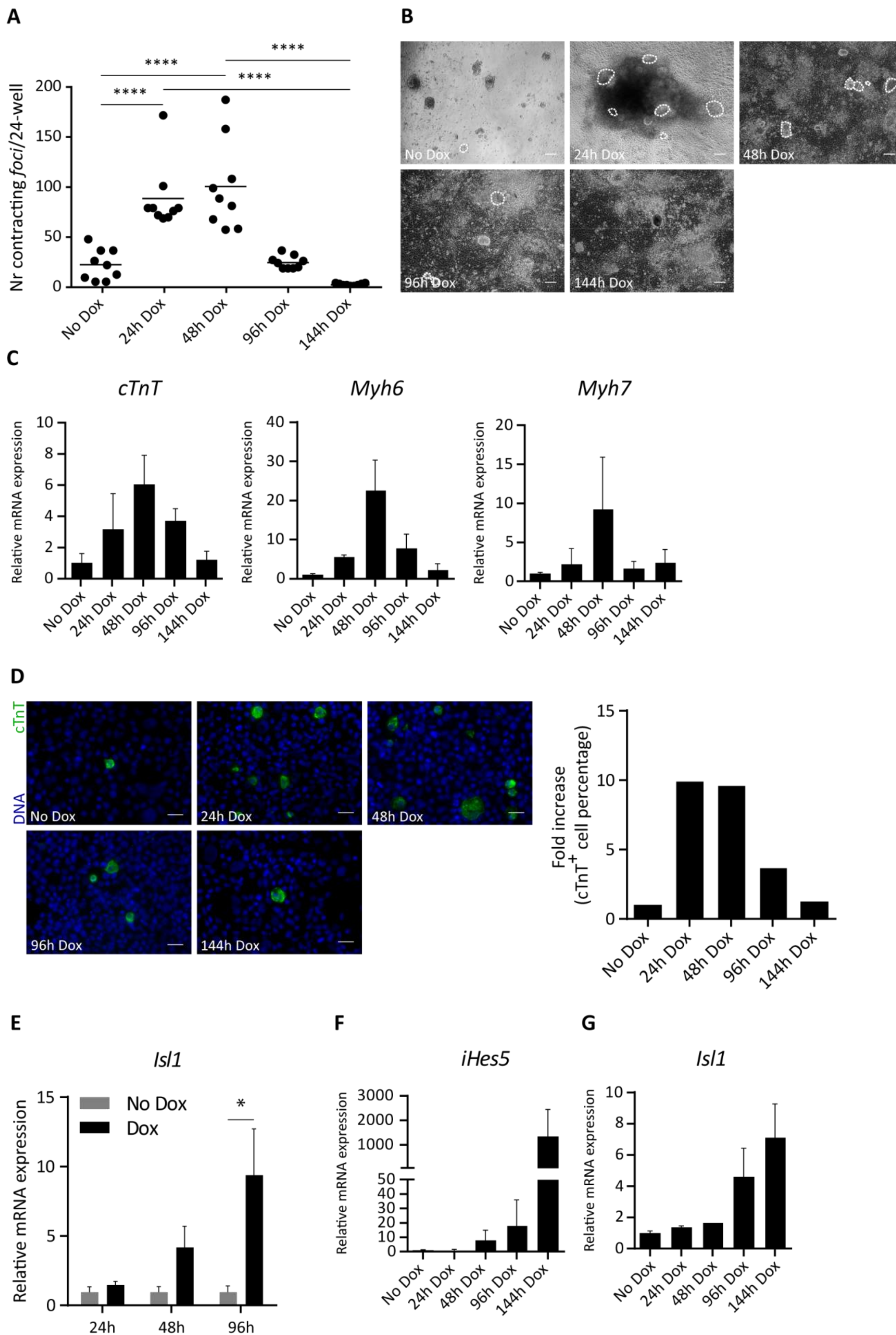

Figure 4 | (A) Quantification of contracting *foci per* well (3 wells *per* biological triplicate) show significantly higher number of contracting cells as a result of 24 h and 48 h *Hes5* induction.

Cardiac troponin T protein levels were also assessed by immunocytochemistry (Figure 4D). As expected, Dox treatment for 24 and 48 h resulted in a dramatic increase in the number of cTnT expressing cells when compared to No Dox. In contrast to shorter periods of activation, *Hes5* induction for 96 h and, in particular for 144 h, resulted in decreased percentage of cells expressing this protein (Figure 4D).

Taken together, these results show that enhanced cardiogenesis from mesodermal progenitors mediated by Hes5 is confined to a temporal window from D3.75-D5.75 (*i.e.* 24 h to 48 h of induction).

Given these observations and the described role for Hes factors in maintaining undifferentiated progenitors in other organ systems[91], we hypothesized whether Hes5 maintains an undifferentiated cardiac progenitor state. In the embryo, after cardiac specification and migration of SHF progenitors into the heart tube, Isl1 levels downregulate as progenitors differentiate into cardiomyocytes[39]. Given the previous results in the laboratory showing that Hes5 binds and putatively regulates Isl1 (Freire, AG et al, unpublished), we questioned if Isl1 is a downstream effector of Hes5 in the maintenance of a cardiac progenitor state. Thus, *Isl1* mRNA levels were evaluated at 24 h, 48 h and 96 h after *Hes5* activation in D3.75 mesodermal progenitors. The results demonstrated that *Isl1* levels increasingly upregulated with longer exposure to Dox (1.5-, 4- and 9-fold, respectively) (Figure 4E), indicating that *Hes5* positively regulates *Isl1* expression. Next, the mRNA levels of *Hes5* and *Isl1* were analyzed at D10 following treatment with Dox for different time periods (No Dox, 24 h, 48 h, 96 h, and 144 h). As expected, the results showed high *Hes5* mRNA levels when Dox was still present in the media (*i.e.* 144 h; 1400-fold increase) (Figure 4F).

(B) Phase contrast images representing cultures treated with Dox for different pulses (No Dox, 24 h Dox, 48 h Dox, 96 h Dox and 144 h Dox). Dashed lines delimit contracting *foci*. Scale bar: 100 µm. (C) Relative mRNA quantification of cardiac structural genes (*cTnT, Myh6* and *Myh7*) demonstrating particular upregulation in cells induced for 48 h (n=2). (D) Immunofluorescence detecting cTnT protein and quantification indicating superior fold increase in cell percentage when a 24 h and 48 h-pulse were applied (n=1). Scale bar: 20 µm. (E) Relative *Isl1* mRNA expression at 24 h, 48 h and 96 h after Hes5 induction showing upregulation with longer exposure to Dox (n=3-4). (F) Assessment of *Hes5* mRNA levels at D10 of differentiation after treatment with Dox for different pulses. Sustained Dox treatment resulted in higher *Hes5* mRNA levels (n=2). (G) Quantification of *Isl1* mRNA levels at D10 demonstrating upregulation in response to 96 h and 144 h of *Hes5* activation (n=2). Data are represented as mean ± SEM. *$p < 0.05$; ****$p < 0.0001$. Relative mRNA expression is normalized to No dox condition.

Interestingly, and supporting a direct response to Hes5 activation, *Isl1* mRNA levels showed increasing values the longer Hes5 expression was maintained (1.4-, 1.7-, 4.7- and 7-fold increase, respectively to 24 h, 48 h, 96 h and 144 h of Dox treatment) (Figure 4G). These data demonstrate that Hes5 positively regulates *Isl1* levels and suggests that the continuous promotion of Isl1 upregulation may account for the impairment in cardiomyocyte differentiation.

Endogenous levels of Hes5 upregulate from D4-D6 of *in vitro* differentiation

Our previous *Hes5* gain and loss of function studies demonstrated that Hes5 acts at the specification of cardiac lineages as a downstream effector of Notch1 (Freire, AG et al, unpublished). To investigate the role of Hes5 in specifying a cardiac fate in a most close to physiological condition, mES cells were differentiated, according to the protocol described above, in the absence of exogenous *Hes5* activation. Thus, the expression profile of *Hes5*, as well as *Notch1* and other known downstream effectors of Notch pathway, was evaluated during differentiation into mesodermal derivatives. Given the demonstration that Hes5 regulates the cardiac progenitor marker Isl1, the expression profile of this gene was also assessed at the same stages of *in vitro* differentiation. *Notch1* showed an oscillatory pattern, with two increased peaks of expression at D4 and D6 relatively to control levels in ES cells (ESC) (3.8- and 4.3-fold increase, respectively) (Figure 5A). Importantly, *Hes5* levels highly upregulated from D4-D6 (ranging from 6- to 11-fold increase), comparatively to the control. This profile of expression correlates with the temporal window identified for enhanced cardiac differentiation induced by transient exogenous *Hes5* overexpression (D3.75-D5.75) (Figure 5B and Figures 4A-4D). *Hey1* was significantly upregulated at D3 and D3.75 (10- and 11-fold increase, respectively) when compared to control (Figure 5C); whereas *Hey2* levels were increased from D3 onwards, with significant upregulation at D4.75 and D6 (10.5- and 11-fold increase) (Figure 5D). *Heyl and Hes1* maintained a regular expression overtime, though *Heyl* demonstrated a trend to increase at D4 (Figures 5E and 5F). *Isl1* mRNA levels were significantly increased from D3.75 to D6 (varying from 50- to 80-fold increase), approximately the same time period demonstrated by *Hes5*, and presumably, at the time cardiac progenitors are specified.

These data are in agreement with the previous results suggesting a confined temporal window (D3.75 to D5.75) for cardiac specification *in vitro* mediated by Hes5.

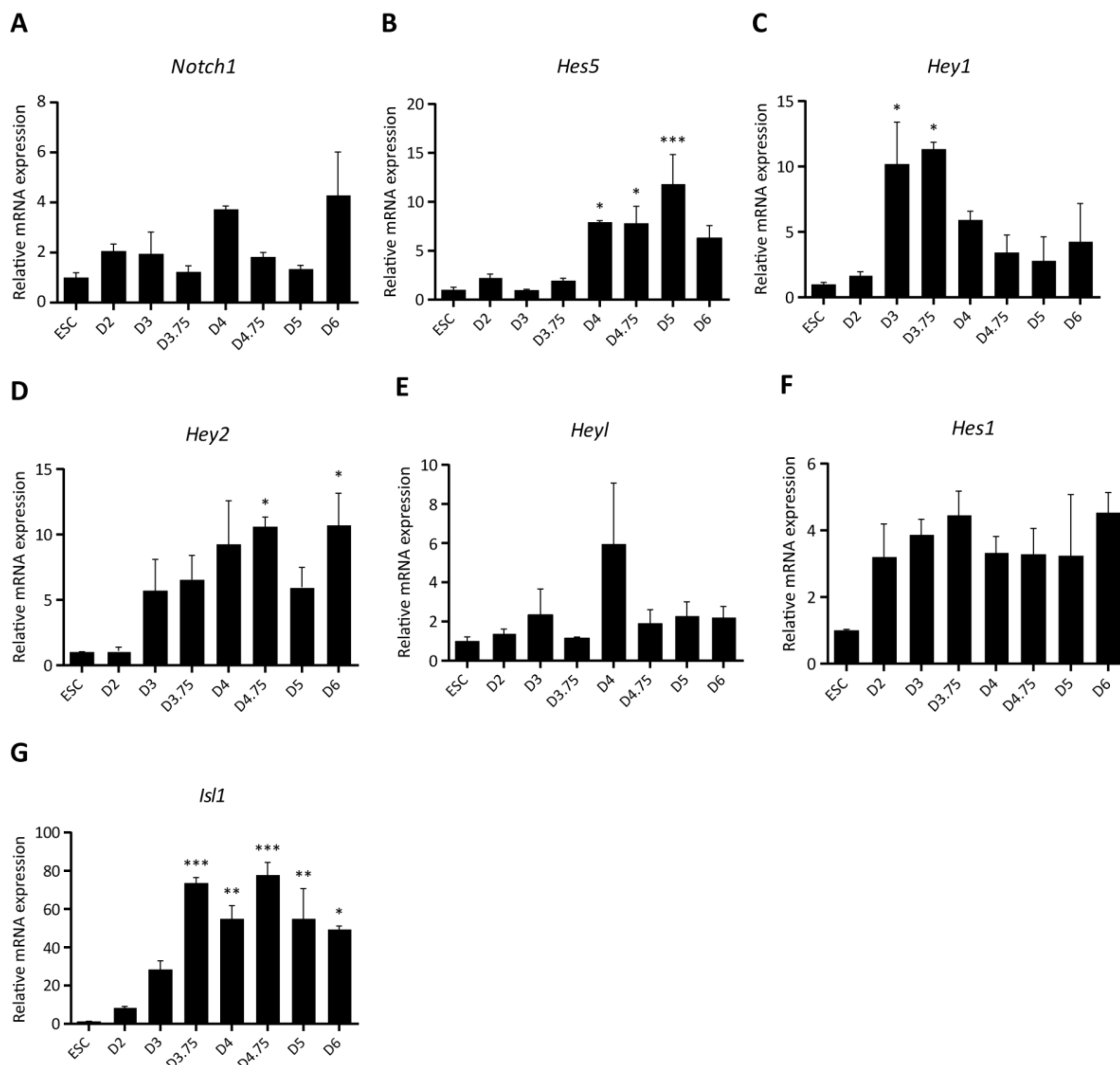

Figure5 | (A-F) Expression profile of Notch1 and downstream effectors of the Notch pathway during *in vitro* mES cell differentiation towards mesoderm derivatives (ESC, D2, D3, D3.75, D4, D4.75, D5 and D6) (n=3). (B) *Hes5* upregulates from D4-D6 of differentiation (n=3). (G) Relative *Isl1* mRNA expression during *in vitro* differentiation showing a significant upregulation from D3.75 onwards (n=3). Data are represented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. Relative mRNA expression is normalized to ES cells (ESC).

Hes5 is expressed in the nascent mesoderm of E6.5 gastrulating embryos

Following the identification of a new role for Hes5 in cardiac specification in the mES cell differentiation system, we aimed at the validation of Hes5 role in specifying cardiac fate in the developing mouse embryo. Thus, RNA i*n situ* hybridization using Hes5 antisense probe was performed in E6.5 and E7.5 mouse embryos to specifically assess *Hes5* expression in nascent mesodermal cells. As Hes5 expression in the embryo

has only been reported at E9.5 onwards in non-cardiac systems[140,141], RNA i*n situ* hybridization was performed in E9.5 embryos as a positive control (Figure 6A).

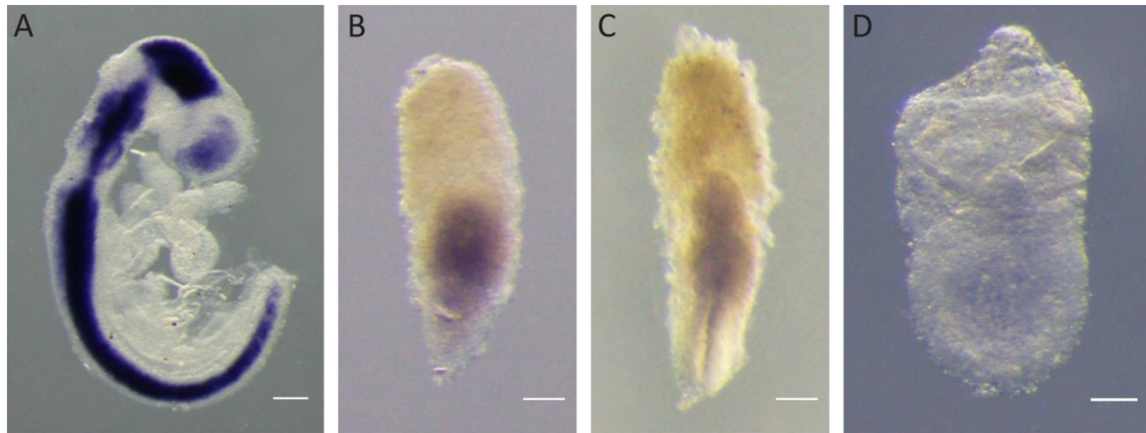

Figure 6 | (A-D) *In situ* hybridization using DIG-labeled Hes5 antisense probe in E9.5, E6.5 and E7.5 embryos. (A) Positive control of *Hes5* expression in the head, eye and neural tube of E9.5 embryos. Scale bar: 400 µm. (B-C) E6.5 gastrulating embryos demonstrating *Hes5* detection in the presumptive nascent mesoderm (5/7 embryos). (D) E7.5 embryo showing faint *Hes5* expression (1/3). Scale bar: 100 µm.

Interestingly, *Hes5* expression was observed in the nascent mesoderm of E6.5 embryos (5/7 embryos), displaying a pattern that apparently co-localizes with the region described for cardiogenic mesoderm and cardiac progenitor markers[142] (Figure 6B and 6C). This profile of expression at this developmental stage correlates with the timing observed during *in vitro* differentiation. Interestingly, *Hes5* expression was no longer observed (2/3 embryos) or dramatically reduced (1/3 embryos) in E7.5 embryos (Figure 6D), suggesting a transient temporal window for Hes5 activity, as demonstrated *in vitro*. Although further studies are required to analyze in more detail the specific location of Hes5 in E6.5 embryos, these results strongly hint a role for Hes5 in instructing mesodermal cells towards a cardiac fate in the developing embryo.

Discussion

DISCUSSION

Embryonic stem (ES) cells are pluripotent cells with the remarkable ability to maintain an undifferentiated state indefinitely in culture, while capable of originating all cell types of the organism. Due to these properties, the ES cell differentiation system has brought valuable mechanistic insights to developmental studies, particularly in the comprehension of early lineage determination. Previous findings at the laboratory disclosed a new role for Hes5, as an effector of Notch1, in favoring cardiac over hematopoietic fate from ES cell-derived mesodermal progenitors (Freire, AG et al, unpublished). Moreover, these findings demonstrated that Hes5 enhanced cardiac commitment when provided as a 24h-pulse, whereas its sustained expression compromised the emergence of contracting colonies. These observations suggested that Hes5 is required for cardiac induction in a confined temporal window. Hes family of bHLH transcriptional regulators govern embryogenesis by regulating binary cell decisions and the timing of cell differentiation, normally as antagonists of bHLH activators[91]. Hes5 has been described as a Notch effector in non-cardiac systems, as are examples the role in preventing premature neuronal differentiation[143-145,146], regulating differentiation of hair cells in the inner ear[147] or in favoring B-cell over T-cell fate in the thymus[148]. Yet, to our best knowledge, the participation of Hes5 in cardiac development has been neither suggested nor demonstrated.

This Thesis had as main objective to further dissect the role of Hes5 in specifying cardiac lineages. Having in mind that sustained Hes5 expression upon cardiac induction impaired the emergence of contracting colonies, we questioned whether Hes5 maintains an undifferentiated cardiac progenitor state. Indeed, Hes5, in combination with Hes1, maintains neural stem cells in the embryo telencephalon in an undifferentiated state[144]. Here, we present evidence supporting that enhanced cardiogenesis from mesodermal progenitors mediated by Hes5 is confined to a temporal window from D3.75-D5.75. During heart formation, constitutive Notch expression was shown to inhibit cardiomyocytic maturation by compromising the expression of cardiac muscle proteins[93,149]. Our results are in agreement with this previous knowledge, as sustained Hes5 induction resulted in decreased mRNA levels of structural genes (*i.e. cTnT*, *Myh6* and *Myh7*) and reduced number of $cTnT^+$ cells, when

compared to shorter periods of activation. These observations suggest that cells in which sustained *Hes5* expression is induced have committed into a cardiac fate but are unable to progress in the cardiomyocytic differentiation program. In accordance with the described role for Notch and its effectors in the maintenance of undifferentiated cells in neuronal development[150,151], we hypothesize that these cells are kept in a cardiac progenitor state.

Interestingly, Hes5 positively regulated Isl1 expression, being the levels of this cardiac progenitor marker maintained high when Hes5 was induced for longer periods of time. In the embryo, after cardiac specification and migration of SHF progenitors into the heart tube, Isl1 levels downregulate as progenitors differentiate into cardiomyocytes[39]. In line with this evidence, we suggest that the continuous promotion of Isl1 expression may in part explain the maintenance of a cardiac progenitor state. Further studies are required to confirm this hypothesis. It would be interesting to address whether the non-contracting colonies observed in cultures subjected to sustained Hes5 induction correspond, in fact, to reservoirs of $Isl1^+$ cells. Additionally, Isl1 silencing using siRNA could be performed to evaluate if a decrease in the levels of Isl1 would rescue cardiac differentiation. Furthermore, our findings bring new insights into how the Notch pathway acts as an inducer or suppressor of cardiac differentiation depending on the cell context, timing and duration of signal activation. Still, studies are underway to fully comprehend the mechanism underlying cardiac specification mediated by Hes5.

Although these findings are of great importance, they represent nevertheless effects of gain-of-function mutations. Thus, we next evaluated whether, in a close to physiological condition, *Hes5* is upregulated during ES cell differentiation within the temporal window identified for enhanced cardiac induction by exogenous Hes5. Remarkably, *Hes5* upregulates from D4 to D6 of *in vitro* differentiation, correlating with the temporal window identified by performing gain-of-function studies (D3.75-D5.75). Moreover, endogenous *Hes5* upregulation coincides with the time of emergence of cardiac progenitors during *in vitro* differentiation, as inferred by increasing expression of Isl1. Future work is required to ultimately demonstrate whether Hes5 expression instructs mesodermal cells to become cardiac progenitors in

a cell-autonomous manner. One can foresee, for instance, that clonal analysis would contribute valuable information.

In fact, a role for Hes5 in cardiogenesis had never been demonstrated. *Hes5* null-embryos have apparently no heart defects[145,146], though to the best of our knowledge, there are no specific studies addressing cardiovascular malformations in these embryos. There is evidence for compensatory mechanisms between Hes proteins, as the example of Hes1 and Hes5 in the nervous system[145] or arterial cell fate specification[152]. It is likely that Hes5 function in specifying cardiac fate from mesodermal cells could be compensated by another Hes/Hey protein. The expression profile of the different Notch effectors hints Hey2 as a candidate for further studies addressing compensatory effects, as it upregulated in the same time period as Hes5. Contrarily to the evidence in neuronal development, it is less likely that Hes1 compensates Hes5 function in cardiogenesis, as a stable expression was maintained overtime. Compensatory processes between Hey transcription factors have also been suggested in later stages of heart morphogenesis, since although *Hey1* and *Heyl* are participants, only the double mutant presents valve and ventricular septal defects[106].

Yet, Hes5 expression in the embryo has only been reported at E9.5 onwards in non-cardiac systems[140,141]. The use of the ES cell differentiation system enabled us to capture a transient role for Hes5 at the time of cardiac specification, that otherwise would be challenging and laborious to visualize in the developing embryo. Mesodermal progenitors are specified during gastrulation, while epiblast cells ingress through the primitive streak (PS)[13] and give rise to cardiac progenitors at the early-mid streak stage (E6.5-E7)[11]. This work reports for the first time Hes5 expression in the nascent mesoderm of E6.5 gastrulating embryos. Given the evidence we have collected in the ES cell differentiation system, it is likely that this pattern of expression in the embryo correlates to a role for Hes5 in specifying a cardiac fate from mesodermal progenitors ingressing through the PS. Although a more detailed analysis is required, the observed pattern of Hes5 expression resembles the one described for cardiogenic mesoderm and cardiac progenitor markers[142,153]. Double *in situ* hybridization for Hes5 and Mesp1 and/or whole mount *in situ* hybridization combined with immunohistochemistry for mesodermal or cardiac progenitors will allow a more precise characterization. Interestingly, *Hes5* expression was not detected or dramatically reduced in E7.5

embryos, further suggesting a transient role at the nascent mesoderm, as hinted by the observations in differentiating mES cells. Moreover, following our previous findings suggesting that Hes5 specifies cardiac fate as a downstream effector of Notch1, it would be interesting to address whether Notch1 precedes the expression of Hes5 in the gastrulating mesoderm. Genetic lineage tracing studies will unveil the fate of the progeny originated from Hes5-expressing mesodermal cells and thus, confirm the participation of Hes5 in determining cardiac lineages.

This work contributes further insights into how Hes5 favors cardiac fate from ES cells-derived mesodermal progenitors and constitutes the first attempt aiming at the validation of the role played by this factor in the determination of cardiac progenitors in the developing embryo.

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