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PhD Thesis

Generation and characterization of *in vitro* model systems to study cardiovascular specification and differentiation

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“Learn from yesterday, live for today, hope for tomorrow.

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by Albert Einstein

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Abstract

The mammalian adult heart appears devoid of endogenous reparative processes capable of compensating a massive loss of functional tissue. Hence, the discovery of heart resident cells with typical progenitor features and allegedly primed to originate cardiac lineages, hereby designated adult cardiac progenitor cells (CPCs), raised expectation in the cardiovascular area. However, a decade after their original identification, the elusive developmental origin and role of CPCs, in addition to the lack of robustness in generating cardiomyocytes are troubling the field. Conversely, the notion that a deeper knowledge on the signaling milieu and master regulators determining cardiac specification and differentiation during embryogenesis, will significantly reshape the clinical options currently available, has been strengthened.

In this doctoral dissertation original work is reported and discussed within the context of main findings and pending questions in cardiogenesis. In addition, strategies for cardiac regeneration and signaling pathways in heart formation and pathological stress are briefly reviewed, with emphasis placed on the Notch pathway, for which the role in cardiogenesis was further explored. Our research plan was primarily devoted to the generation and validation of *in vitro* model systems in which initiation and differentiation of the cardiac molecular program could be investigated.

To this end, we specifically immortalized adult $Lin^{-}Sca\text{-}1^{+}$ CPCs via overexpression of the murine telomerase catalytic subunit (mTERT), aiming to establish an *in vitro* tool to study this subset of CPCs. Extensive *in vitro* and *in vivo* characterization of an immortalized cell-clone enabled its validation as a cell line representative of CPCs expressing Sca-1, and therefore designated $iCPC^{Sca\text{-}1}$. This cell line retained the phenotypic features of primary $CPC^{Sca\text{-}1}$ but had the advantage of constituting an unlimited source of cells endowed with a stable phenotype in long-term culture. These cells engrafted the infarcted myocardium and directed improvement in cardiac performance contributing for increased neovascularization as they differentiated, although to variable extents, into cardiomyocyte-, endothelial- and smooth muscle-like cells. Importantly, this work provided the scientific community with a benchmark that can be used for functional and mechanistic studies targeting adult CPCs.

A second aim of this Thesis was the identification of early regulators involved in cardiogenesis using the robustness of embryonic stem cells (ESCs) for cardiac differentiation. Due to the role demonstrated for Notch signaling in directing cardiac fate from ESCs-derived mesodermal progenitors and hemangioblasts, we envisaged the identification of downstream effectors of Notch in the onset of cardiogenesis. For this purpose, a mES cell line expressing the Notch1 intracellular domain (NICD1) under the control of a Doxycycline (Dox)-inducible promoter was chosen. By using this inducible expression system a candidate, *i.e.* Hes5, was selected for validation in loss and gain-of-function studies. Our data fit into a model where Hes5 acts as a regulator of Isl1 and Scl levels in mesodermal progenitors, thus determining cardiac over hematopoietic cell-fate choice. Moreover, after induction of cardiac fate, Hes5 withdrawal is required to allow continued progression of the cardiomyocytic program. This work has provided mechanistic insight into how the Notch pathway specifies cardiac fate and reported for the first time the participation of Hes5 in cardiogenesis.

Finally, the role of Hes5 as a putative key factor for the regulation of cardiogenic processes in adult CPCs was investigated in a proof of concept study. The iCPC$^{Sca\text{-}1}$ cell line was used as a model system to examine the role of Hes5 in the regulation of proliferation and differentiation in adult CPCs. Hes5 had no effect in cell proliferation, yet *Hes5*-transfected iCPC$^{Sca\text{-}1}$ were able to upregulate structural cardiac genes in basal culture conditions. Interestingly, Hes5 negatively regulated *Isl1* expression, suggesting Isl1 as a putative Hes5 target also in the adult context. Moreover these results suggest that the activation of structural cardiomyocytic genes in *Hes5*-transfected iCPC$^{Sca\text{-}1}$ may be triggered by the decreased levels of Isl1, thereby identifying Hes5 as a candidate factor for enhancing cardiomyocytic differentiation in adult CPCs.

Overall, the work performed in the frame of this Thesis contributed (i) a cell line model system that will facilitate mechanistic and functional studies in need to resolve conflicting data on adult CPCs biology, (ii) the identification of a novel early regulator in the onset of cardiogenesis, *i.e.* Hes5, and (iii) the indication that Hes5 may take part, at a later time point, to reinforce a cardiogenic program in adult CPCs.

Resumo

O coração de mamíferos adultos é aparentemente desprovido de processos de reparação endógenos capazes de compensar uma perda dramática de tecido funcional. Deste modo, a descoberta de células presentes no coração com o típico fenótipo de células progenitoras e alegadamente pré-condicionadas para originar linhagens cardíacas, daí a designação de células progenitoras cardíacas (CPCs) adultas, criou expectativa na área cardiovascular. Contudo, uma década após a primeira identificação, a origem ontogénica e função imprecisas, bem como a falta de robustez na diferenciação em cardiomiócitos, têm sido alvo de controvérsia. Por outro lado, fortaleceu a noção de que um conhecimento mais aprofundado das vias de sinalização e dos reguladores fundamentais na especificação e diferenciação cardíaca durante a embriogénese contribuirá para uma reformulação das opções clínicas disponíveis.

Nesta dissertação de Doutoramento é documentado e discutido um trabalho original no contexto dos principais avanços e questões pendentes na cardiogénese. Adicionalmente, são brevemente revisitadas estratégias para regeneração cardíaca e vias de sinalização envolvidas na formação do coração e condição patológica, enfatizando a via de sinalização Notch, para a qual o papel na cardiogénese foi aprofundado. O plano de trabalhos foi essencialmente dedicado à génese e validação de sistemas modelo que permitissem o estudo da iniciação e diferenciação do programa molecular cardíaco *in vitro*.

Deste modo, foi realizada a imortalização específica de CPCs $Lin^{-}Sca\text{-}1^{+}$ adultos, através da sobreexpressão da subunidade catalítica da telomerase de murganho (mTERT), com o intuito de estabelecer uma ferramenta *in vitro* para o estudo desta população de CPCs. A extensiva caracterização *in vitro* e *in vivo* de um clone imortalizado, permitiu a sua validação como um linha celular representativa de CPCs que expressam Sca-1 e, por isso, designada $iCPC^{Sca\text{-}1}$. Esta linha celular mantém as características fenotípicas das $CPC^{Sca\text{-}1}$ primárias, com a vantagem de constituir uma fonte ilimitada de células providas de um fenótipo estável em culturas de longo termo. Após lesão do miocárdio, as $iCPC^{Sca\text{-}1}$ integraram o tecido e promoveram a melhoria da função cardíaca, contribuindo para o aumento da neovascularização e simultaneamente expressando,

ainda que em proporções variáveis, proteínas típicas de cardiomiócitos, de células endoteliais e de células do músculo liso. Fundamentalmente, este trabalho resultou na disponibilização de uma ferramenta acessível à comunidade cientifica para estudos mecanísticos e funcionais relativos às CPCs adultas.

Um segundo objetivo da Tese prendeu-se com a identificação de reguladores participantes nas fases iniciais da cardiogénese, usando a robustez do sistema de diferenciação de células estaminais embrionárias (CEEs) em células cardíacas. Dado o papel, previamente demonstrado, da via de sinalização Notch no direcionamento de progenitores mesodérmicos e hemangioblastos derivados de CEEs para linhagens cardíacas, foi antecipada a identificação de efetores da via Notch na iniciação da cardiogénese. Assim, foi escolhida uma linha de CEEs que expressa o domínio intracelular do Notch1 sob o controlo de um promotor induzido por doxiciclina. O uso deste sistema de expressão induzida permitiu a seleção de um candidato, *i.e.* o Hes5, para validação em estudos de perda e ganho de função. Os resultados enquadram-se num modelo em que o Hes5 regula os níveis de Isl1 e Scl, em progenitores mesodérmicos, determinando deste modo uma decisão preferencial pela diferenciação em linhagens cardíacas em detrimento de hematopoiéticas. Além disso, uma vez instruída a iniciação da cardiogénese, a remoção de Hes5 é obrigatória de forma a permitir a contínua progressão do programa molecular cardiomiocítico. Este trabalho contribuiu com informação mecanística sobre o controlo mediado pela via de sinalização Notch na especificação cardíaca e demonstrou, pela primeira vez, a participação do Hes5 na cardiogénese.

Por último foi investigado, como prova de conceito, o papel do Hes5 como possível fator chave na regulação de processos cardiogénicos em CPCs adultas. A linha celular iCPC$^{Sca\text{-}1}$ foi utilizada como sistema modelo para avaliar o efeito do Hes5 na regulação da proliferação e diferenciação de CPCs adultas. A indução do *Hes5* não se repercutiu na proliferação celular, no entanto, a expressão de genes cardíacos estruturais foi ativada em iCPC$^{Sca\text{-}1}$ transfectadas com *Hes5,* mantidas em condições de cultura basais. De notar que o Hes5 regulou de forma negativa a expressão de *Isl1*, o que sugere o Isl1 como provável gene alvo do Hes5 também no contexto adulto. Adicionalmente, estes resultados sugerem que a expressão dos genes cardíacos estruturais possa ser ativada

como consequência dos níveis diminuídos de Isl1, identificando assim o Hes5 como possível potenciador da diferenciação cardiomiocítica em CPCs adultos.

Em resumo, o trabalho realizado no âmbito da presente Tese contribuiu com: (i) a implementação de uma linha celular, que constitui um sistema modelo para estudos mecanísticos e funcionais, necessários para o esclarecimento da informação contraditória respeitante à biologia das CPCs adultas; (ii) a identificação de um novo regulador na iniciação da cardiogénese, *i.e.* o Hes5; (iii) a indicação de que o Hes5 poderá estar envolvido, em fases mais tardias, como potenciador do programa cardíaco em CPCs adultos.

Table of contents

List of non-standard abbreviations

5-aza	5-azacytidine
7-AAD	7-aminoactinomycin D
APC	Allophycocyanin
AV	Atrioventricular
bHLH	Basic helix-loop-helix
BM	Bone marrow
BMPs	Bone morphogenetic proteins
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumine
Bsd	Blasticidin
CDCs	Cardiosphere-derived cells
ChIP-Seq	Chromatin immunoprecipitation combined with DNA sequencing
CMs	Cardiomyocytes
CPCs	Cardiac progenitor cells
CSCs	Cardiac stem cells
D	Day
DAPI	4,6- diamidino -2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
Dox	Doxycycline
E	Embryonic day
EBs	Embryoid bodies
ECM	Extracellular matrix
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
EGM	Endothelial Growth Medium
EMT	Epithelial-to-mesenchymal transition

EPDCs	Epicardial-derived cells
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FGFs	Fibroblast growth factors
FHF	First heart field
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
FS	Fractional shortening
GFP	Green fluorescent protein
GO	Gene ontology
Hes	Hairy and Enhancer of split
hESCs	Human embryonic stem cells
Hesr	Hes-related
Hh	Hedgehog
HRP	Horseradish peroxidase
Hrt	Heart
HUVECs	Human umbilical vein endothelial cells
ICC	Immunocytochemisty
$iCPC^{Sca\text{-}1}$	Immortalized Sca-1^{+} cardiac progenitor cells
IMDM	Iscove's Modified Dulbecco's Medium
Immuno-FISH	Immunostaining combined with fluorescence in situ hybridization
IP	Intraperitoneal
iPSCs	Induced pluripotent stem cells
KD	Knockdown
LAD	Left anterior descending
LIF	Leukemia inhibitory factor
Luc	Luciferase
LV	Left ventricle

LVIDd	Left ventricle internal diameter at diastole
LVIDs	Left ventricle internal diameter at systole
LVPWd	Left ventricle posterior wall at diastole
LVPWs	Left ventricle posterior wall at systole
mESCs	Mouse embryonic stem cells
MGI	Mouse Genome Informatics
MI	Myocardial infarction
miRNA	microRNA
MSC	Mesenchymal stromal/stem cell
MT	Masson's Trichrome
mTERT	Murine telomerase catalytic subunit
NGS	Normal goat serum
NICD	Notch intracellular domain
OFT	Outflow tract
OMIM	Online Mendelian Inheritance in Man
PBS	Phosphate buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PS	Primitive streak
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
rtTA	Reverse tetracycline transactivator
SEM	Standard error of the mean
SFRPs	Secreted frizzled related proteins
sh	Short hairpin
SHF	Second heart field
Shh	Sonic Hedgehog

SMCs	Smooth muscle cells
TGF	Transforming growth factor
TRE	Tetracycline response element
TSS	Transcription start site
Y-Chr	Y-Chromosome
α-MEM	α-minimal essential medium

Chapter I

General introduction

Brief overview of strategies for cardiac regeneration

Cardiovascular diseases are a major cause of morbidity and mortality worldwide [1, 2]. Ischemic and non-ischemic cardiomyopathies eventually lead to left ventricular dysfunction and heart failure [5]. The latter is currently the most common cardiac disorder, mainly due to the increase in the average human lifespan and the progressive aging of the population in all developed countries [1, 5, 6]. Heart failure hampers quality of life, decreases life expectancy and increases medical costs considerably and, therefore, constitutes a major public health problem [5]. Heart transplantation remains as the only therapy currently available for end-stage heart failure.

Evidence reporting some capacity of the mammalian heart to generate new cardiomyocytes throughout life, although at different turnover rates, has accumulated in the past years [7-11], challenging the pre-existing dogma of the mammalian heart as a postmitotic organ [12]. It is still debatable, however, whether this newly formed cardiomyocytes result from pre-existing cardiomyocytes or from resident progenitors. While the issue of cardiomyocytic turnover remains controversial, it is widely accepted that the regenerative capacity of the mammalian myocardium is on the whole inadequate to compensate for the severe loss of heart muscle in an extensive myocardial infarction condition [13].

Ideally, restoring heart function would involve activation of endogenous regenerative processes, including recruitment of endogenous progenitors; or transplantation of pre-differentiated cardiac cells or progenitor cells capable of producing new and functional myocardium *in situ*. Alternatively, cardiogenic factors such as transcription factors, chromatin regulators or microRNAs (miRNAs) might be used for direct lineage conversion into cardiomyocytes (Figure 1).

Stem and Progenitor cell-based therapies

Stem and progenitor cell-based therapies have been explored in the last decade with variable success, and ongoing clinical trials will certainly disclose the therapeutic value of cell-based regenerative medicine for the heart. Pioneering attempts with skeletal myoblasts and bone marrow-derived cells showed some beneficial effects in cardiac

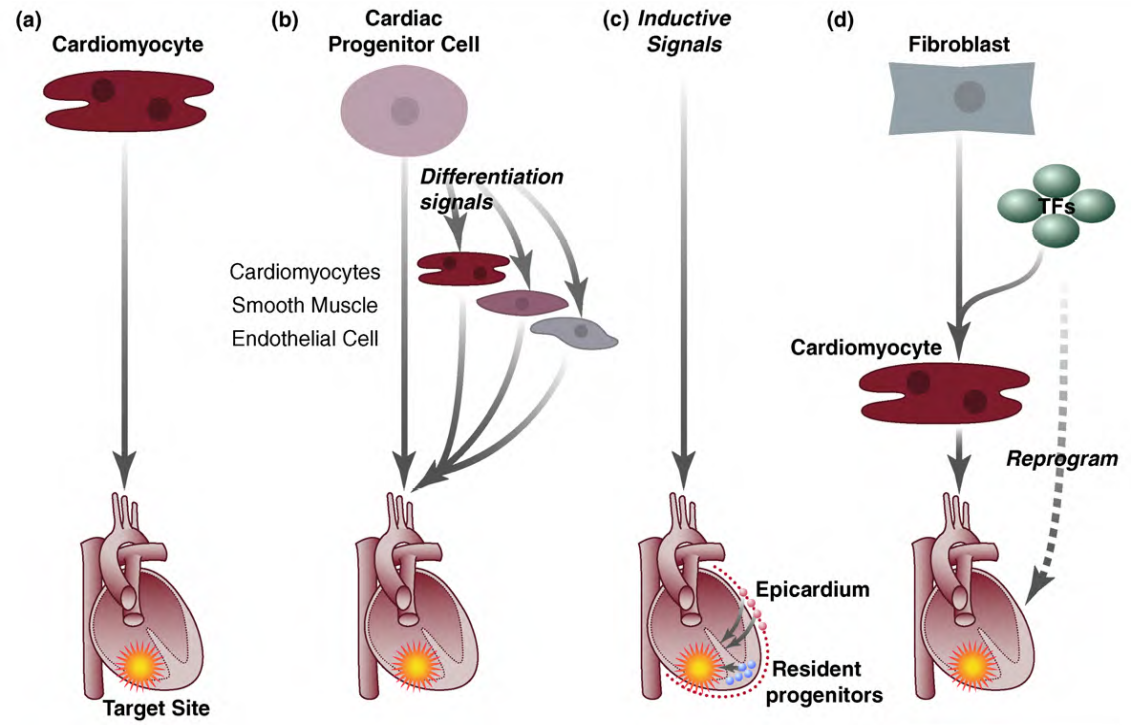

Figure 1. Strategies for regenerative cardiac treatment. Current approaches focus on the use of cardiomyocytes derived from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) for direct transplantation in the infarcted area (yellow asterisk) (a). Other strategies include the use of cardiac progenitor cells (CPCs), which can be directly injected into the infarcted tissue or guided to differentiate *in vitro* followed by transplantation of the mature cardiac cell types (b). As alternative, inductive signals can mobilize resident endogenous progenitors to regenerate/repair injured areas, overcoming the need for transplantation (c). Additionaly, transcription factors, chromatin regulators and miRNAs might be used for direct lineage conversion into cardiomyocytes (d). Reproduced from [4].

function, which correlated best with the secretion of paracrine factors opposed to a direct contribution to new cardiac cells [13]. This part of the general introduction will however focus on the current knowledge and on the uncomprehended aspects associated to the potential use of pluripotent stem cells and resident cardiac progenitor cells as sources for replacing/repairing damaged cardiac muscle.

Pluripotent stem cells

Embryonic stem cells (ESCs) are the prototypical stem cells. ESCs are generally derived from the inner cell mass of blastocysts [14, 15] and unambiguously fulfill the defining hallmarks of stem cells: clonality, self-renewal and pluri/multi-potency. Under specific culture conditions, ESCs differentiate as multicellular embryoid bodies (EBs) [16] and give rise to derivatives of the three germ layers of the embryo [17]. Indeed, ESCs are capable to initiate lineage-specific differentiation programs of many tissue/cell types *in*

vitro [18]. Based on this property, ESCs provide a unique *in vitro* differentiation culture-system to study the very early stages of development [15]. ESCs-derived cardiomyocytes have been used as an *in vitro* model to study early mouse and human cardiac differentiation, functional genomics, pharmacological testing, cell therapy and tissue engineering. The development of the cardiomyocytic lineage within the ESCs differentiation system progresses through distinct stages following closely the kinetics of the developing embryo. Strikingly, this relates not only to the sequence of expression of cardiac-affiliated proteins (*e.g.* early cardiac transcription factors, sarcomeric proteins, ion channels, connexins and calcium-handling proteins), but also to cell morphology (from round to elongated cells with well-developed myofibrils and sarcomeres) and similar mechanisms of excitation–contraction coupling and neurohormonal signaling [19]. Moreover, because ESCs are both capable to proliferate indefinitely and to differentiate into multiple tissue-cell types, they potentially represent an unlimited supply of cells/tissues for human transplantation [18]. Human ESCs-derived cardiomyocytes have been established *in vitro* using the EBs differentiation system [20, 21]. In this sense, ESCs-derived cardiomyocytes constitute a potential source for replacing tissue at the injured area. ESCs have been genetically engineered to enable specific selection of cells representing different stages of development within a given lineage. As examples, cell lines where the green fluorescent protein (GFP) is expressed under the control of a gene of interest, such as the mesodermal marker *Brachyury*, or cardiac-associated genes *Nkx2.5*, *cardiac-actin* or *myosin light chain-2v* (*Mlc2v*) were made available [19]. This is of particular interest, while aiming to isolate purified cardiomyocytic populations from undifferentiated or noncardiogenic cells within the ESCs differentiation system, limiting also the eventual risk of teratoma formation [22, 23].

Induced pluripotent stem cells (iPSCs) were firstly generated by retroviral integration of four transcription factors that reprogrammed adult fibroblasts into an ESC-like pluripotent state [24, 25]. iPSCs are used to derive cardiomyocytes which display a similar phenotype to those obtained from ESCs [26]. Furthermore, the induced pluripotent cells offer the advantages of allowing autologous cell therapies while not

involving the ethical concern of embryo destruction, and advances on safer methods for their generation are made by the day [27].

Cardiomyocytes derived from mouse and human ESCs were shown to contribute for the improvement of cardiac function in rodent and swine models [21, 28, 29], although the long-term benefits of the engraftments are still uncertain. Nonetheless, cardiomyocytes derived from pluripotent stem cells are known to display an immature phenotype, lacking the morphology, gene expression profile and functional properties characteristic of adult ventricular myocytes [30]. As the knowledge of pathways that control differentiation of cardiac lineages in embryonic development and in differentiating ESCs increases, differentiation will become more controllable, safer and will bring on higher indexes of cellular yield and lineage purity.

Adult Resident Cardiac Progenitor Cells (CPCs)

The observation that cardiac chimerism occurs in sex-mismatched transplantations led to the first proposal that the heart harbors cells with stem cell-like features [31]. These observations inspired the work that ultimately led to the identification and characterization of putative resident cardiac stem/progenitor cell (CSCs/CPCs) expressing c-Kit [32]. The rationale for the choice of c-Kit, the receptor for stem cell factor (SCF), reconciled both the fact c-Kit is expressed by stem cells from the hematopoietic system [33] and that bone marrow-derived c-Kit$^+$ cells were allegedly shown to ameliorate the deleterious effects after infarction while contributing to the formation of new cardiac cells [34]. This finding brought excitement to the field owing to that any heart resident CPCs would constitute the ideal source for cell therapy due to a purported “primed state” to originate cardiac lineages.

Following this pioneer study, multiple CPCs populations have been isolated based on the expression of c-Kit [32, 35] and Stem cell antigen-1 (Sca-1) surface markers [36-40], as a strategy imported from the hematopoietic field; but also based on functional properties, such as ability to efflux dyes (*e.g.* side population (SP) cells expressing the ATP-binding cassette transporter Abcg2) [41-43] and/or to migrate out of cardiac explants and form multicellular spheroids (*e.g.* cardiospheres (CS)) [44-46] (Table 1).

Table 1. Representation of distinct CPCs populations identified in the myocardium

CP cell type	Phenotype	Determined Differentiation Potential	Clonogenicity	Ref.
Sca-1$^+$ CPCs	Sca-1$^+$ c-Kit$^-$ Flt-1$^-$ Flk-1$^-$ CD31$^+$ CD38$^+$CD34$^-$ CD45$^-$	CMs	Not determined	[36]
	Sca-1$^+$ c-Kit$^-$ CD34$^-$ CD31$^-$ CD45$^-$ CD90$^+$ CD105$^+$ CD29$^+$ CD44$^+$ CD106$^+$ CD73$^+$ CD13$^+$	CMs, ECs, SMCs	+	[37]
	Sca-1$^+$ Pdgfrα$^+$ CD31$^-$ Flk-1$^-$ CD45$^-$CD90$^+$ CD105$^+$ CD29$^+$ CD44$^+$	CMs, ECs, SMCs	+	[38]
	Sca-1$^+$c-Kit^{low}CD34^{low}CD31^{low} CD45^{low} CD29$^+$	CMs, ECs, SMCs*	+	[39]
c-Kit$^+$ CPCs	c-Kit$^+$ CD45$^-$ CD34$^-$ CD20$^-$ CD8$^-$ CD45RO$^-$ Ter119$^-$	CMs, ECs, SMCs	+	[32]
	c-Kit$^+$ CD29$^+$ CD44$^+$ CD105$^+$ CD90$^+$	CMs*, ECs*, SMCs*	Not determined	[35]
SP CPCs	Sca-1$^+$ Abcg2$^+$ c-Kit^{low} CD45^{low} CD34^{low} CD31$^-$	CMs*	Not determined	[41]
	CD29$^+$ CD45$^+$ CD31$^+$	CMs, ECs†, SMCs†	Not determined	[43]
	Sca-1$^+$ c-Kit$^-$ CD31$^-$ CD45$^-$ CD44$^-$ CD34$^-$	CMs*	+	[42]
CS/CDCs	Sca-1$^+$ c-Kit$^+$ CD31$^+$ CD34$^+$ Flk-1$^+$	CMs, ECs, SMCs	+	[44]
	c-Kit$^+$ CD105$^+$ CD90$^+$ CD34$^+$ CD31$^+$ CD45$^-$ CD133$^-$	CMs, ECs†	+	[45]
	Sca-1$^+$ CD45$^-$ c-Kit^{low} CD31^{low} CD34^{low}Flk-1^{low}CD133$^-$ CD90^{low}	CMs*, ECs, SMCs	+	[46]

(+) indicates the observation of clonogenicity; (*) and (†) indicates that determination of the differentiation potential into the particular lineage was only performed *in vitro* or *in vivo*, respectively; SP: Side population; CS: Cardiosphere; CDCs: Cardiosphere-derived cells; CMs: Cardiomyocytes; ECs: Endothelial cells; SMCs: Smooth muscle cells.

Reportedly, as common ground, the distinct CPCs self-renew, are clonogenic and express key cardiac transcription factors (*e.g.* Nkx2.5, Gata4, Mef2c) while lacking mature cardiac structural genes. Moreover CPCs are multipotent, contributing for the formation of cardiomyocytes (CMs) and cells of the vasculature (*e.g.* endothelial cells (ECs) and smooth muscle cells (SMCs)) throughout life and after transplanted in the injured myocardium [12]. However, these cells do not seem capable to compensate for the loss of cardiac tissue in chronic heart failure. In the latter setting CPCs have been shown to correlate with increased growth arrest and senescence, as consequence of decreased telomerase activity [47]. The presumptive organization in discrete histo-functional units, *i.e.* clusters of stem/progenitor and early lineage-committed cells in close contact with differentiated cells and extracellular matrix (ECM), transported to the heart the "niche" concept [48], in close resemblance to well-known self-renewing systems [49].

The multiplicity of reports describing new CPCs populations isolated by different methodological procedures and displaying distinct molecular profiles and behavior *in vitro* and *in vivo*, could suggest that the adult heart harbors progenitors of unrelated lineages/affiliation. However, there is also the possibility of overlapping among the distinct subsets that might represent different developmental stages from an unique progenitor [1]. That would explain the identification of populations displaying more than one of the initially defined criteria (Table 1 and Figure 2). This suggests that defining CPCs based on pre-established markers may not be optimal for fully disclosing the identity of these cells.

Nonetheless, and despite the mentioned ambiguity, a main demand is certainly at understanding the function of these cells and their role in cardiac homeostasis and pathology. CPCs expressing either c-Kit [50, 51] or Sca-1 [52] were found in higher abundance in scenarios of cardiac injury. Moreover, Sca-1 upregulates in response to pressure overload and protects heart tissue from fibrosis and cardiac hypertrophy [53]; while *Sca-1* and *c-Kit* mutations have been shown to cause cardiac dysfunction [51, 53-55]. Interestingly, *Sca-1* deficiency affects the growth and survival of c-Kit^{+} CPCs in normal and infarcted hearts [54]. A role for Sca-1 and c-Kit in the control of proliferation and differentiation of the CPCs reservoir has been proposed [54-56].

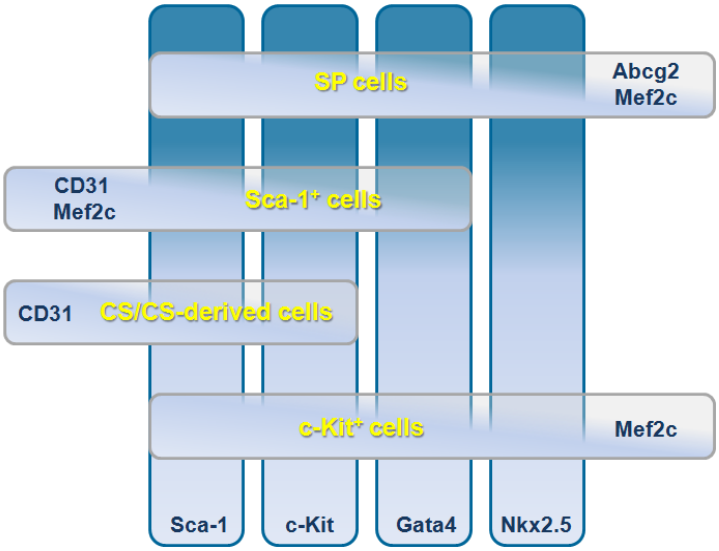

Figure 2. Representation of overlapping marker expression in distinct CPCs populations. Distinct CPCs have been characterized on the basis of expression of a particular surface marker (*e.g.* c-Kit or Sca-1) or functional properties (*e.g.* SP cells and CS/CS-derived cells). The display of overlapping marker expression might indicate the derivation from a common precursor. Adapted from [3].

Importantly, a human ortholog of the Sca-1 protein was not so far identified [57], although CPCs have been isolated from human myocardium using an antibody recognizing the rodent protein [58].

Surprisingly, although more than a decade has passed since CPCs were firstly isolated, it is still not clear to what extent these cells contribute to new myocardial tissue, either in cardiac homeostasis or after transplanted into injured hearts [3]. In one hand, there are reports of large-scale contribution for new cardiomyocytes and cells of the vasculature that integrate functionally the injured heart [32, 36, 45, 52, 59, 60]; and inclusively claims that c-Kit$^+$ cells are not only necessary, but also sufficient for cardiac regeneration and repair [61]. Conversely, there is also indication that the contribution of CPCs, although detectable, is minimal both in physiological aging and after injury [62, 63]. In fact, although CPCs are able to display phenotypic features of cardiac lineages, the *in vitro* protocols for differentiation require DNA demethylation by 5-azacytidine (5-aza) [36] and/or co-culture with cardiomyocytes [36, 42, 45], rather unusual requirements for *bona fide* stem cells. In addition, a genetic lineage-tracing study demonstrated that Sca-1$^+$ CPCs display a restricted lineage potential [63], thus contributing for the lack of certainty on the presumptive multipotency of CPCs.

Aside from the yet questionable contribution for new functional tissue, CPCs exert an overall beneficial effect on cardiac function and contribute for the attenuation of adverse tissue remodeling. These beneficial effects have been attributed to secretion of paracrine factors responsible for cardiac cell survival and neovascularization [37, 51, 52, 64, 65], or increased thickness and mechanical stabilization of the myocardial wall [66]. In accordance with a role in promoting neovascularization, *Sca-1* depletion in CPCs interfered with their capacity to improve capillary density after transplanted in the infarcted myocardium [37]. Paracrine effects; however, account for the beneficial effects of most cell-based therapies, irrespective of the cell source [67]. Finally, evidence for another mechanism has been proposed involving a synergistic effect between transplanted cells and the endogenous CPCs, in which the latter are provided with factors that stimulate their *in situ* activation, proliferation and/or mobilization [3].

Based on the reported beneficial effects, a clinical trial testing the safety and feasibility of autologous transplantation of c-Kit$^+$ CPCs (SCIPIO: NCT00474461) in heart failing patients, was conducted. The cardiac magnetic resonance results showed improved left ventricle (LV) function, reduction in infarct size, and an increase in viable tissue [68]. Sca-1$^+$ cardiosphere-derived cells (CDCs) are also under trial (CADUCEUS: NCT00893360), as they were envisioned as a consistent starting material for rapid cell expansion for transplantation, obtained from small human heart biopsy specimens. Noteworthy, preceding reports proposed that cardiac fibroblasts were the cell source of CDCs, being the latter merely a result from cell aggregation rather than clonal growth; and that contamination with original cardiac tissue would account for the spontaneous contraction observed [69].

A main controversial aspect of CPCs relates to their developmental origin, and the extent to which they constitute remnants of embryonic cardiovascular progenitors. Alternatively, these cells can also be derived from circulating cells from the bone marrow, or from the neighboring vasculature [40]. In fact, these cells share phenotypic features with circulating cells from the bone marrow, despite lacking the pan-hematopoietic marker, leukocyte common antigen (CD45). This coincides with claims that bone marrow-derived stem cells home to the injured heart and acquire a cardiac phenotype contributing to the pool of cardiac progenitors [51, 70, 71]. Furthermore, a

majority of Sca-1$^+$ cells in the heart co-expresses the platelet endothelial cell adhesion molecule, CD31 (also known as Pecam1) [36, 42, 43]; although there is a claim that cardiogenic potential is restricted to Sca$^+$CD31$^-$ cells [42].

In this logic, it is important to track Sca-1- and c-Kit-expressing cells back in embryonic life, while the heart is still developing. A bipotential c-Kit$^+$Nkx2.5$^+$ myocardial and smooth muscle precursor was identified as early as embryonic day (E) 8.5 of mouse development. According to this model, c-Kit is expressed in precardiac mesodermal progenitors and subsequently downregulates once these cells commit into smooth muscle or myocardial precursors. An ancestral relationship between this progenitor and the c-Kit$^+$ CPCs isolated from the adult heart has never been clarified [72]. A following study described the presence of c-Kit$^+$ progenitors early in the developing heart and excluded an extracardiac source for these cells (*e.g.* migrating hematopoietic stem cells) [73]. Sca-1 expression is not detected in the heart before mid-gestation increasing after birth, as shown by our laboratory [40]. Interestingly, Sca-1 is found co-localizing with platelet-derived growth factor receptor alpha (Pdgfrα, also known as CD140a) in cells with mesenchymal phenotype and proepicardial/epicardial origin, which persist to the adult life [38]. The embryonic epicardium has been shown to contribute significantly to the cardiomyocyte lineage [74, 75]. In addition, a population containing 80% of Sca-1$^+$ cells in the adult epicardium has been shown to contribute to new cardiomyocytes, after re-activation of the embryonic epicardial gene, *Wilm's tumour 1* (*Wt1*), through priming by Thymosin β4 (Tβ4) [76]. Increasing evidence indicates that CPCs exhibit a mesenchymal-like phenotype, selected either in the basis of Sca-1 [37, 38] or c-Kit [35] expression. The reports demonstrating the epicardial origin of Sca-1 progenitor cells, reinforce the aforementioned idea, given that epicardial-derived cells (EPDCs) contribute to cells of myocardial stroma, *i.e.* fibroblasts, pericytes, and coronary vasculature cells (SMCs and ECs) [77]. In addition to this, the preferential commitment to smooth muscle lineage and ability to produce ECM components, have brought the question to what extent Sca-1$^+$ CPCs overlap with the tissue-resident fibroblast population, as observed in other organs [40].

All these interrogations urge to determining the developmental origin of adult CPCs and whether these cells are truly capable of a significant and equal contribution to

CMs, SMCs and ECs, a requisite for a *bona fide* cardiac stem/progenitor cell. Understanding how these lineages are determined during embryonic development is expected to contribute cues that may be translated into the modulation of the adult regulatory circuitry. The current knowledge supports that during embryonic development CMs, SMCs and ECs diverge from a common progenitor expressing Isl1, Nkx2.5 and Flk-1 [78]. This population overlaps with the designated second heart field (SHF) progenitors that contribute to the outflow tract, to the majority of the right ventricle and atria (left and right), and some regions of the left ventricle [79]. $Isl1^+$ cardioblasts were identified in the postnatal heart as developmental remnants of the fetal progenitor population [80], which persist, although scarce, into adulthood [81, 82]. Moreover, Isl1 expression is downregulated once cells differentiate into a more mature state, suggesting that Isl1 may be used to drive a cardiac progenitor cell state [79]. Interestingly, $Sca\text{-}1^+CD45^-$ cells derived from cardiospheres contain progenitors expressing Isl1 [46], and $Wt1^+$ epicardial cells (primed with Tβ4) express Isl1, while increasing its expression after myocardial infarction [76].

In summary, there is an overall lack of knowledge on the molecular signature, developmental origin and relationship between the distinct putative CPCs populations that have been described. Moreover, conflicting data in the field demand for a thorough understanding of the biology and of the role of these cells in cardiac homeostasis and repair.

(Re)programming cardiac cell fate

Following the revolutionary study of Takahashi and Yamanaka [24], the idea of reprogramming into virtually any cell type, by direct lineage conversion using a combination of transcription factors, became a hot topic of research. The strategy to reprogram into a particular cell type consists in a combinatorial approach whereby several factors are withdrawn, one at the time, until the minimum set of factors can be defined [83].

Direct lineage conversion of mouse fibroblasts into cardiomyocytes was achieved by overexpressing three developmental transcription factors (*e.g. Gata4, Mef2c* and *Tbx5,* in brief GMT) [84]. An exciting achievement, was the *in vivo* direct conversion of

cardiac fibroblasts into cardiomyocytes in the mouse, either by overexpressing a combination of transcription factors (GMT with or without *Hand2*) [85, 86] or using muscle-specific miRNAs (miRs-1/133/208/499) [87]. This is particularly important since cardiac fibroblasts are significantly abundant in the heart and key determinants in fibrosis [88]. Importantly, from a therapeutic point of view, the conversion of human fibroblasts into cardiomyocytes was also attained by using a combination of transcription factors and miRNAs (*e.g. Gata4*, *Hand2*, *Tbx5*, and *Myocardin*; and miR-1 and miR-133) [89].

The examples of cell plasticity seem to go beyond one could have ever imagined. Mesoderm was directed to heart tissue by a combination of three factors (*Gata4, Tbx5* and the cardiac-specific subunit of BAF chromatin remodeling-complexes, *Baf60*) [90]; and activation of Notch signaling in the hemato/vascular progenitor, so-called hemangioblast, redirected differentiation towards a cardiac fate [91].

Knowledge on the transcriptional regulators and signaling pathways that determine cardiac cell fate will unveil new candidates to drive efficient cardiogenesis.

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Building and repairing the heart: what can we learn from embryonic development?

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Abstract

Mammalian heart formation is a complex morphogenetic event that depends on the correct temporal and spatial contribution of distinct cell sources. During cardiac formation, cellular specification, differentiation, and rearrangement are tightly regulated by an intricate signaling network. Over the last years, many aspects of this network have been uncovered not only due to advances in cardiac development comprehension but also due to the use of embryonic stem cells (ESCs) *in vitro* model system. Additionally, several of these pathways have been shown to be functional or reactivated in the setting of cardiac disease. Knowledge withdrawn from studying heart development, ESCs differentiation, and cardiac pathophysiology may be helpful to envisage new strategies for improved cardiac repair/regeneration. In this review, we provide a comparative synopsis of the major signaling pathways required for cardiac lineage commitment in the embryo and murine ESCs. The involvement and possible reactivation of these pathways following heart injury and their role in tissue recovery will also be discussed.

Introduction

Cardiogenesis relies on early specification of cardiac myocytes from mesodermal progenitors, incorporation of exogenous sources of precursors and the spatial and timely integration of distinct signaling pathways. Genetic-based studies using the mouse embryo have uncovered regulatory crosstalks between distinct signaling pathways and a set of transcriptional cardiac regulators that control lineage commitment and heart morphogenesis. Additionally, embryonic stem cells (ESCs) derived from the inner cell mass of the embryo blastocyst, have shown to constitute a powerful *in vitro* model that faithfully recapitulates the events occurring during embryo development. Similar to the embryo, mouse ESCs (mESCs) commit into the epiblast stage and undergo differentiation as aggregates designated embryoid bodies (EBs). These are able to differentiate into derivatives of the three germ layers in a sequential set of events that mimic embryo gastrulation (reviewed in [1]). Even though there are discrepancies in the timing of lineage progenitor segregation, once the cardiac molecular program is initiated, ESCs-derived cardiac progenitors engage in the recapitulation of all cardiac cell phenotypes, though no particular spatial organization is respected (reviewed in [2]). Thus, embryos and ESCs have been used in parallel to achieve increased understanding of the complex developmental process. Knowledge withdrawn from developmental studies has been used to promote *in vitro* cardiac differentiation of ESCs and these have also brought valuable mechanistic information to embryonic studies (reviewed in [2, 3]). Additionally, several pieces of evidence have shown that detailed study of the processes regulating heart specification and formation provides important clues to attain a better comprehension of cardiogenic mechanisms and to envisage improved strategies towards cardiac regeneration. Over the following sections, we will focus on the molecular events regulating cardiac specification in both embryo and ESCs. We will also address the signaling pathways shown to be reactivated in the mammalian myocardium following injury and how they can be modulated/potentiated in order to improve cardiac repair in pathological stress.

Molecular events in cardiogenesis

In embryo development, gastrulation is a key event through which the three germ layers (endoderm, mesoderm and ectoderm) are formed. Cellular fates are specified during gastrulation by both time of recruitment to the primitive streak (PS) and perceived morphogenetic information [4, 5]. Mesodermal induction is regulated by the interaction of distinct signaling pathways including bone morphogenetic proteins (BMPs), Nodal/Activin, and Wnt (reviewed in [6]). Mesodermal cells ingressing through the PS express the T-box transcription factor *brachyury* (*Bry*, also *T*), a direct target of the Wnt pathway [7]. β-catenin, a central player in Wnt signaling, has been shown to be essential for mesoderm formation since in *β-catenin* deficient mice no mesodermal or head structures are formed and Bry is not expressed [8]. These early embryonic events are also observed in ESCs, in which mesodermal commitment is defined by the upregulation of the *Bry* gene within 48 hours after the onset of differentiation (Figure 1) [9]. Mesoderm is then patterned and specified to originate distinct mesodermal subsets, characterized by differential expression of fetal liver kinase-1 (*Flk-1*, also *Vegfr2*) and platelet-derived growth factor receptor-alpha (*Pdgfrα*, also CD140a) [10]. Concomitantly, Bry expression in these cells decreases [11] and other transcription factors are activated. One key gene in both mouse embryo and mESCs is mesoderm posterior 1 (*Mesp1*) that has been correlated with definite cardiac commitment by activating the cardiogenic transcriptional network in a context-dependent manner (reviewed in [12, 13]). The conjunction of knowledge acquired from studying embryonic development and ESCs system led to the optimization of chemically defined cocktails that efficiently drive ESCs differentiation in the absence of serum (reviewed in [1]). Different studies have demonstrated that a tight balance between canonical Wnt and members of the transforming growth factor-β (TGF-β) superfamily, including Nodal/Activin and BMP signaling pathways, regulates the specification of the anterior and posterior regions of PS in mouse [14, 15] and human ESCs (hESCs) [16]. In fact, the combination of Activin A and BMP4 has been shown to direct mESCs into a mesodermal fate [17] whereas inhibition of the Nodal/Activin pathway drives hESCs towards a neuroectoderm path [18]. Balanced levels of Nodal and BMPs determine mesoderm patterning: increased levels of Activin A favor Flk-1^{+}Pdgfrα^{+} cardiogenic

progenitors while high doses of BMP4 promote the Flk-1^{+}Pdgfrα^{-} hematopoietic reservoir [19]. Importantly, activation of Notch pathway in differentiating mESCs has been shown to block the emergence of Flk-1^{+} mesodermal progenitors [20].

Migration and specification of the primitive cardiac progenitors occur during gastrulation around mouse embryonic day (E) 6.5, when cells leave the PS and acquire an anterior-lateral position forming two groups of cells on both sides of the midline [4]. The presumptive cardiac cells, which will contribute to the myocardium and endocardium, can then be detected as a crescent in the mesoderm underlying the head folds (cardiac crescent, E7.5) (Figure 1). The crescent fuses at midline forming the beating primitive cardiac tube (E8), which subsequently folds to the right creating an S-shaped structure. The folded tube then suffers a series of rearrangements and cell expansion, which ultimately lead to the formation of recognizable septated cardiac chambers (E14.5). The cellular and morphogenic events underlying mammalian heart formation have been extensively reviewed elsewhere [5, 6, 21, 22]. At least two populations of mesodermal progenitors, arising from a common origin [23], partake in heart formation. The earliest group of progenitors (first heart field, FHF) constitutes the cardiac crescent and will contribute to the left ventricle (LV) and atria. FHF expansion depends on the second heart field (SHF) and provides a platform for subsequent heart growth. Cells of the SHF will form the outflow tract and the right ventricular region. Over the last years, transcriptional regulators directing the genetic program and morphogenesis of the cardiac progenitors have been uncovered: specific markers are still lacking for FHF (although *Tbx5* has been associated with this field), whereas *Isl1* has been considered a marker for SHF (reviewed in [6, 21]).

Both lineages of progenitors are regulated by a complex signaling network, emanating from the adjacent tissues. Similarly to the embryo, evidences from the ESCs system suggest the existence of two cardiac fields or lineages with comparable molecular interregulatory networks (Figure 1) (reviewed in [2]). In the embryo, precardiac mesoderm induction and consequent FHF formation require ectodermal inhibitory Wnt signaling, as indicated by the respecification of endoderm into precardiac mesoderm in *β-catenin* depleted mice, resulting in the formation of several heart primordia [24]. These authors further observed ectopic expression of *Bmp2* following

Wnt/β-catenin inhibition, suggesting that BMP signaling activation and Wnt inhibition are required to induce cardiac mesoderm specification. A similar regulation was observed using a Notch-inducible mES cell line, in which Notch was capable of redirecting the hemangioblast into a cardiac fate through activation of BMP and inhibition of canonical Wnt pathways [25]. Accordingly in chick, zebrafish and *Xenopus*, it has been shown that heart formation is induced in embryonic regions with high Bmp2 and low Wnt activities (reviewed in [26]). The function of BMPs in the mouse appears to be more complex: *Bmp2*, *Bmp4*, *Bmp5* and *Bmp7* are expressed in the anterior mesoderm, including the heart-forming regions but deletion of BMPs seems to have a late effect on cardiogenesis: mutants present cardiac defects and are embryonic lethal but cardiac mesoderm specification still occurs [2, 27, 28]. In conditional knockouts for *Bmpr1a*, encoding the BMP type 1 receptor, progenitors fail to progress towards specific lineages and form the cardiac tube [29]. Correct tissue specification relies not only on protein interconnections but also on the time of the signaling. For example, Wnt/β-catenin signaling presents a biphasic function in cardiogenesis: it has an inhibitory effect in the FHF but plays an inductive function in SHF proliferation. β-catenin inactivation in the SHF leads to defects in development and expansion of the SHF derivatives due to decreased cell proliferation, probably owing to reduced *Fgf10* levels (which promote SHF expansion) and residual *Bmp4* expression (capable of maintaining the antiproliferative effect of BMPs) [30]. *β-catenin* gain-of-function, on the other hand, leads to increased proliferation. This work shows a clear crosstalk between signaling pathways and evidentiates the crucial role of Wnt in regulating proliferation of cardiac progenitors within the SHF and maintenance of their undifferentiated state prior to entering the heart tube. The same activity for Wnt/β-catenin was described in ESCs since the use of a Wnt3a secreting feeder layer or conditioned media promotes expansion of $Isl1^+$ progenitors and beating EBs whereas the addition of dickkopf-1 (Dkk1), a canonical Wnt inhibitor, has the opposite effect, drastically reducing $Isl1^+$ cells and beating EBs [31, 32]. Similar to *β-catenin* gain-of-function, *Notch1* deletion in embryos and ESCs leads to augmented proliferation of cardiac progenitors through increased Wnt/β-catenin activity [33]. However, the mutant embryos failed to populate the developing right ventricle (which derives from

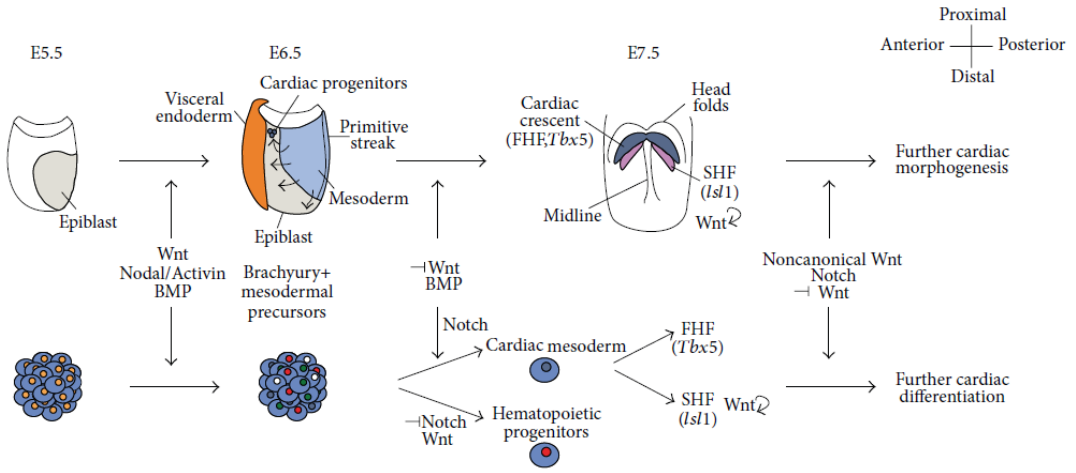

Figure 1. Signaling events in murine heart development and cardiac ESCs differentiation. In both systems, mesodermal induction from the epiblast is regulated by Wnt/β-catenin, Nodal/Activin, and BMP signaling pathways and correlates with Brachyury upregulation. Further commitment of mesodermal progenitors to cardiac mesoderm and consequent first heart field (FHF) formation require the inhibition of Wnt signaling and expression of BMPs. Similarly, in ESCs system Notch pathway inhibits Wnt/β-catenin signaling and activates BMP to specify cardiac fate. Wnt/β-catenin signaling is then activated to allow proliferation and maintenance of the SHF, both in embryo and ESCs. Further differentiation from the cardiac crescent stage to the following morphogenic phases of embryonic heart development and, in parallel, the expression of cardiomyocyte differentiation genes in ESCs require inhibition of Wnt/β-catenin. In the embryo and ESCs, this is achieved by Notch and non-canonical Wnt signaling, which inhibit the effect of Wnt/β-catenin and instruct progenitor cells within the SHF to leave the proliferative state and start differentiating. ⊣ represents inhibitory effect; ↻ represents maintenance of a proliferative state.

Isl1$^+$ cells) and genes associated with cardiomyocyte differentiation were downregulated in Notch-depleted ESCs. These observations suggest that Wnt/β-catenin inhibition is required to instruct progenitors to leave the SHF proliferative state and to start differentiating. Moreover, non-canonical Wnt pathway was also implicated in regulating SHF progenitors differentiation: loss of Wnt5a and Wnt11 affects SHF differentiation by increasing β-catenin nuclear levels [34]. The same authors [34] further showed that Wnt5a and Wnt11 are required to promote cardiogenesis and induce the expression of cardiac-associated genes in differentiating ESCs, indicating that non-canonical Wnt signaling regulates the formation of FHF and SHF associated progenitors during EBs differentiation [34]. Additionally, exogenous non-canonical Wnt2 was shown to increase cardiomyocytic differentiation from murine ESCs [35]. Together, these results indicate that Notch signaling and non-canonical Wnt are required to instruct progenitors to leave the SHF proliferative state by interfering with

Wnt/β-catenin pathway (Figure 1). Different members of the Notch pathway have been shown to be expressed along heart development regulating distinct key events in cardiogenesis. Notch proteins in the endocardium are responsible for modulating myocardial signals (*e.g.* BMPs) to regulate trabecular formation, chamber specification, and cell proliferation and differentiation (reviewed in [36, 37]). Neural crest cells and the proepicardial organ also contribute to the forming heart. Events such as cellular contribution from external sources, progenitor expansion, and differentiation towards the distinct cardiac cell types are tightly coordinated by the distinct signaling pathways, including Wnt, FGF, BMPs, Notch and Hedgehog (Hh) (reviewed in [5]). These ultimately regulate a plethora of transcription factors that constitute a combinatorial code responsible for orchestrating cardiac development and specification and differentiation of myocytes.

Reactivation of the embryonic program in cardiac pathological stress

The adult heart presents robust plasticity and it is capable of remodeling in response to distinct demands, either physiologic (normal aging or increased effort) or pathologic (*e.g.* hypertension, ischemia/myocardial infarction (MI) associated with coronary artery disease, hypertrophy, and dilated cardiomyopathies). In both cases, the first response to overcome the increased stress on the left ventricle (LV) is myocardial hypertrophic growth, which in the long-term is associated with increased risk of heart failure and sudden death (reviewed in [38, 39]).

Efforts have been made towards identifying efficient new therapies to avoid heart failure. To accomplish this, a comprehensive understanding of the biological processes and signaling pathways involved in cardiac formation and leading to heart disease is required. Distinct diseases impacting the adult myocardium have been correlated with perturbation in signaling pathways involved in embryonic heart development. On the other hand, when under pathological stress, the heart reactivates pathways traditionally associated with the developing heart and whose activity is decreased in adult hearts (reviewed in [39, 40]). In this section a general overview on the involvement of key pathways in heart disease will be presented (see also Table 1).

Notch signaling

Being such an important signaling network in distinct mechanisms, perturbation of the Notch pathway has been associated with several genetic diseases and malformations. Regarding cardiac morphogenesis, Notch plays a crucial role in regulating events such as cardiomyocytes (CMs) differentiation, atrioventricular canal development, regulation of the endocardium endothelial-to-mesenchymal transition required for valve formation, and trabeculae development (reviewed in [36, 37]). Notch pathway is active in proliferating embryonic CMs but its activity decreases after birth and declines with age coinciding with CMs maturation [41, 42]. Furthermore, Notch activation in neonatal or mESCs-derived quiescent CMs induces cell cycle reentry [43]. The involvement of Notch in mammalian cardiac response to stress has been shown to be primarily mediated by Notch1 and its ligand Jagged1, which (together with the Notch target Hes1) are upregulated in the hypertrophic heart [44]. The authors also analyzed mice lacking *Notch1* specifically in the heart, which revealed increased hypertrophy, fibrosis, and mortality. Following MI, Notch1 expression is also reactivated and detected in border zone CMs and this activation was correlated with repair and pro-survival processes, including prevention of CMs apoptosis, regulation of resident cardiac progenitor cells (CPCs) and immature CMs, and promotion of neovascularization ([41, 45, 46]; reviewed in [47]). Accordingly, delivery of the Notch intracellular domain (NICD) or of a Notch1 pseudo-ligand following MI leads to improved wall thickness and cardiac function, enhanced neovascularization, and decreased infarct area [41, 45]. Overexpression of Jagged1 in CMs restrains myocardial hypertrophy and fibrosis and promotes CPCs proliferation [48]. Importantly, blockade of Notch1 signaling with a γ-secretase inhibitor upon MI impairs the commitment of heart resident CPCs into the myocytic lineage [49]. This is of particular interest considering that stem/progenitor cells have been shown to contribute to generation of new CMs after injury, though they do not seem to actively participate in cardiomyocytic renewal during normal aging [50]. Taken together, these studies evidentiate Notch as an essential pathway with cardioprotective role in the damaged myocardium, being able to favor a pro-cardiogenic process by regulating key events in cardiac remodeling as fibrogenesis and cardiogenesis.

FGF signaling

FGFs are potent mitogens expressed from early development in the SHF, where Fgf8 and Fgf10 have been implicated in regulating progenitors proliferation and development together with other signaling pathways (reviewed in [5]). Expression of these growth factors is augmented during the onset of myocardial ischemia or infarction; their therapeutic potential has been addressed in pigs and dogs and shown to improve blood flow and preserved cardiac function in acute MI (reviewed in [51]). In rats, a combined treatment with Fgf1 and p38 MAP Kinase inhibitor following MI results in preserved wall thickness, reduced scarring, and overall improved cardiac function [52]. These effects are associated with increased proliferation and angiogenesis. Fgf1 *per se* is capable of inducing CMs cell cycle reentry and angiogenesis but the combined therapy with p38 inhibitor enhances FGF effects and cardiac regeneration [52]. The role of Fgf2, another potent angiogenic and mitogenic factor, in cardiac injury has also been extensively explored and shown to exert a protective effect against myocardial dysfunction following myocardial ischemia or infarction by increasing myocyte viability (reviewed in [53]).

Sonic Hedgehog signaling

Sonic Hedgehog (Shh) morphogens are involved in several developmental processes during embryogenesis. In the heart, Shh is involved in the establishment of the left-right asymmetry and SHF progenitors regulation (reviewed in [5]). Similar to other signaling pathways, there is evidence for Shh reactivation with concomitant upregulation of the Hedgehog patched-1 (*Ptch1*) receptor in the ischemic myocardium [54]. In this study, the authors performed intramyocardial gene transfer of naked DNA encoding human *SHH*, which resulted in successful restoration of LV function in acute and chronic ischemia, enhanced neovascularization, and reduced fibrosis and apoptosis [54]. Interestingly, in a recent study, a strategy for controlled release of Shh morphogens was developed, which enables a slow and sustained delivery of Shh-heparin complexes, maintaining a constant local concentration within the therapeutic range [55]. This approach allowed a continued exposure of the myocardium to Shh, thus promoting a prolonged beneficial effect, which includes production of survival factors and attenuation of cardiomyocytic apoptosis [55]. These studies indicate that

Table 1. Overview of studies targeting different signaling pathways in heart pathological stress

Pathway	Affected Member	Effect	Ref.
Notch	Notch1 (⊣)	Increased hypertrophy, fibrosis, and mortality; impaired adult CPCs commitment into myocytic lineage	[44] [49]
	Notch1 (→)	Improved wall thickness and cardiac function; enhanced neovascularization; decreased infarct area	[41] [45]
	Jagged1 (→)	Restraint of myocardial hypertrophy and fibrosis; increased CPCs proliferation	[48]
FGF	Fgf1 (→) Fgf2 (→)	Preserved wall thickness; reduced scarring ; improved cardiac function; increased proliferation and angiogenesis; increased CMs viability	[52] [53]
Shh	Shh (→)	Restoration of LV function in acute and chronic ischemia; enhanced neovascularization; reduced fibrosis and apoptosis	[54]
	Shh-heparin complexes (→)	Production of survival factors; attenuation of CMs apoptosis	[55]
Wnt/β-catenin	Sfrp1 (→) Sfrp2 (→)	Prevented CMs apoptosis; antifibrotic effect	[56] [57] [58]
	Dishevelled (→)	Myocardial hypertrophy; severe cardiomyopathy	[59]
TGF/BMP	Smad6 (⊣) Noggin (⊣)	Increased cell proliferation; hyperplastic cardiac cushions	[60] [61]
	Bambi (⊣)	Hypertrophy; chamber dilation; deterioration of systolic function; diastolic dysfunction	[62]
	Tgfb1(→)	Cardiac hypertrophy; increased interstitial fibrosis	[63]

(⊣) Inhibition or (→) activation of the specific pathway member.

Shh treatment offers a putative therapeutic approach in acute and chronic ischemia.

Wnt signaling

Different studies have shown that several Wnt factors are induced after experimental MI in various animal models, being involved in hypertrophy and cardiac wound healing following injury [64, 65]. Overall, blockage of Wnt signaling by targeting distinct pathway elements has a beneficial effect on cardiac remodeling (reviewed in [66, 67]). For example, the use of secreted frizzled related proteins (SFRPs) that antagonize Wnt signaling by competing for Wnt binding and preventing ligation to the frizzled receptor, reduces infarct size and improves cardiac function. This was shown either by inducing MI in transgenic mice overexpressing *Sfrp1* [56] or by Sfrp2 local secretion [57] or exogenous administration [58]. Sfrp2 was shown to increase myocardial survival after MI by preventing CMs apoptosis and exerting an antifibrotic effect through *Bmp1* inhibition, normally involved in collagen biosynthesis [57, 58]. These and other reports (reviewed in [66]) seem to indicate a reactivation of the developmental mechanisms observed in FHF, in which Wnt inhibition is required for correct formation of the LV. In accordance, mice with cardiac-specific overexpression of dishevelled (Dvl), a protein acting downstream of frizzled receptor and activator of the canonical and non-canonical Wnt pathways, present myocardial hypertrophy and severe cardiomyopathy [59]. It is worth mentioning that, although the majority of reports indicate a beneficial effect upon inhibition of this pathway, some studies have demonstrated favorable outcomes upon its activation [67]. These differences might partially be due to variations in animal models, cell type, temporal context (essential for Wnt-mediated effects, as observed in embryonic heart development) and activation of Wnt-independent mechanisms by SFRPs [64, 68].

TGF/BMP signaling

In cardiac embryo development, BMP signaling has been associated with valve formation: *Bmp2* deletion in the atrioventricular murine myocardium demonstrated that this protein is required for cardiac jelly formation and cardiac cushions development [69]. Conditional *Bmp4* mutants have profound defects in outflow tract and ventricle septation and perturbed expansion and remodeling of the endocardial cushions, resulting in abnormal valve structure [70]. In accordance with this embryonic

role, TGF-β, and BMPs in particular, have been extensively implicated in valvular heart diseases in mammals and activated BMP signaling has been detected in diseased human aortic valves (reviewed in [71]). Accordingly, perturbing the endogenous repression of the BMP signaling cascade by deleting either the inhibitory *Smad6* [60] or *noggin* [61] leads to hyperplastic cardiac cushions due to increased cell proliferation. Besides the role in valve formation, TGF-β signaling is increased in stressed myocardium, being associated with augmented fibrosis and hypertrophic growth of CMs. Smad proteins, transcription factors downstream of TGF-β/BMP, positively regulate cardiac fibrosis, a major contributor to adult heart disease and functional impairment by regulating the expression of distinct extracellular matrix (ECM) proteins (reviewed in [39]). This was demonstrated to occur both in normal aging hearts and following MI. An increase in Tgfb1, Smad proteins, and collagens was observed in infarcted rat hearts [72]. Regarding aging, 24-month-old *Tgfb1* heterozygous mice exhibited decreased myocardial fibrosis and stiffness when compared to control animals [73]. Additionally, *Tgfb1* overexpression induces cardiac hypertrophy, expression of hypertrophy-associated proteins, and increased connective tissue and interstitial fibrosis [63]. More recently, it was shown that inhibition of Bambi (BMP and Activin membrane-bound inhibitor), a negative regulator of TGF-β-mediated deleterious remodeling signals, leads to exacerbated hypertrophy, chamber dilation, deterioration of LV systolic function and diastolic dysfunction [62].

Conclusions

Heart failure is a major concern in modern society. The approaches currently taken to achieve heart function restoration aim to delay or even reverse maladaptive remodeling. Even though several advances have been made, these strategies still face challenges like preservation of the contractile function and myocyte viability. We have reviewed distinct studies showing that in response to pathologic stress there is partial reactivation of genes that promote embryonic and fetal heart development. For example, Notch signaling may be modulated to expand the resident cardiopoietic progenitor pool and reactivate cell cycle reentry of pre-existing cardiomyocytes in the adult mammalian heart in the scenario of pathological insult, limiting the extent of ischemic injury [49, 50]. Additionally, Shh holds great promise for repair/regeneration

of tissues suffering ischemic injury, even though clinical translation has been hampered by its short half-life in the body [55]. Conversely, inhibition of Wnt/frizzled signaling pathway seems also to have beneficial effect on cardiac remodeling (reviewed in [66, 67]). In this sense, learning from the embryonic development can provide important clues to understand and modulate the injury scenario. This knowledge may be used in the future to implement and adopt new therapeutic strategies for adult heart disease. Interestingly, considering that resident CPCs have been shown to contribute for the generation of new cardiomyocytes in an injury setting [50], it would be valuable to analyze whether these signaling pathways are active in adult CPCs. In fact, Notch1 has already been shown to regulate adult CPCs proliferation and commitment to myocytes [49]. Furthermore, a strategy combining CPCs delivery with FGF controlled release is currently under clinical investigation [74]. These studies suggest that important pathways for embryonic cardiac morphogenesis can be translated to the adult signaling networks. One might then predict that the manipulation of this signaling environment will bring forward insights on how to modulate/potentiate CPCs response in a disease setting by creating a more suitable environment for repair/regeneration.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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A closer look into the Notch signaling pathway

Overview of the Notch pathway

Notch was discovered and named in 1919 due to the phenotype observed in heterozygous *Drosophila* females presenting notched wings [1]. The name of the receptor baptized the entire pathway which, in a very simplistic view, includes receptors, ligands and nuclear effectors extremely conserved from the fruit fly to mammals. As both receptor and ligand are transmembrane proteins, Notch signaling is transmitted between neighboring cells and thus, by acting through mainly two mechanisms, lateral inhibition and boundary induction, Notch pathway orchestrates a multiplicity of biological processes (*e.g.* cell fate specification, cell differentiation, boundary formation, progenitor cell maintenance, apoptosis and cell proliferation). Lateral inhibition is a process by which the neighbor cell is inhibited from becoming the same cell type, whereas by boundary induction the neighbor cell will become the same type as the signaling cell. A third mechanism that controls lineage decision involve asymmetrical inheritance of Notch regulators by two daughter cells, such as the Notch inhibitor Numb, during cell division [2].

Most vertebrate species exhibit four receptors (Notch1 to Notch4), being Notch1 and Notch2 the most similar to the Notch receptor described in *Drosophila*. Notch receptor is a heterodimeric molecule composed by two fragments linked by disulfide bonds: the extracellular domain and a second composed by the transmembranar and intracellular domains. Vertebrate ligands, also transmembranar proteins, belong to two families: Delta (Delta or Delta-like (Dll) 1, 3 and 4) orthologues of the *Drosophila* Delta, and Jagged (Jagged 1 and 2) orthologues of the *Drosophila* Serrate [3]. Binding of the ligand triggers the proteolytic cleavage of the receptor, firstly by ADAM/TACE-family of metalloproteases, and secondly by the γ-secretase complex (containing presenilin and nicastrin subunits) which releases the Notch intracellular domain (NICD) [4]. The distinct ligands have equal ability to interact with the receptor and trigger proteolytic cleavage. However differences are found in their expression pattern and some context-specificity is observed, as deletion/inhibition of specific ligands results in different effects. In the canonical Notch pathway, NICD translocates to the nucleus and

associates with the DNA-binding protein RBP-J (also known as CSL from the orthologue proteins: CBF1, Supressor of Hairless and Lag1) and with the co-activator Mastermind-like (MAML), activating the transcription of the Notch target genes by displacement of co-repressors (Figure 1) [5-7]. Notch effectors 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) proteins and mediate great part of the Notch pathway effects, such as cell proliferation, differentiation, apoptosis and binary cell-fate decisions. In fact, regulation by Hes and Hesr proteins has been associated to the most important effects of the Notch pathway. Usually acting as transcriptional repressors, these factors antagonize the effects of bHLH activators, thereby regulating the timing of cell differentiation and cell-fate decision that will result in correct organ formation [8]. Yet, the expression of *Hes*/Hesr genes, although being often induced following NICD nuclear translocation, it is not exclusively driven by Notch signaling activation, as for example Shh pathway has been also shown to activate *Hes1* expression [9, 10].

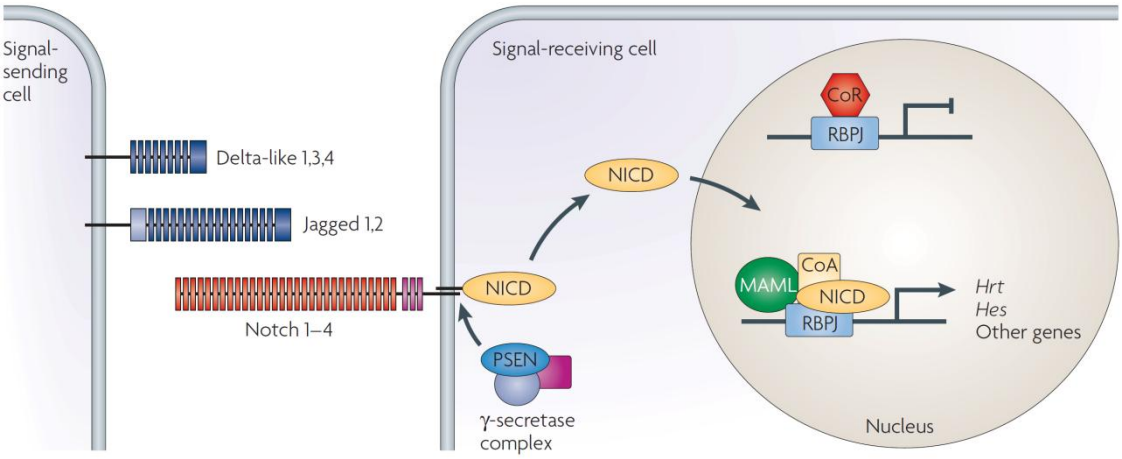

Figure 1. Canonical Notch pathway. Mammals express four Notch receptors (Notch1-4) and five ligands belonging to two families (Delta-like 1, 3 and 4 and Jagged 1 and 2). As ligand and receptor are transmembranar proteins Notch signaling is transmitted between neighboring cells. Binding of a ligand activates a sequence of proteolytic cleavages of the receptor that culminates in the release of the Notch intracellular domain (NICD) mediated by the γ-secretase complex containing presenilin (PSEN) catalytic subunit. Cleaved NICD translocates to the nucleus where it forms an active transcriptional complex with the DNA-binding protein RBP-J and with co-activators (CoA), including MAML. In the absence of activated Notch, RBP-J binds Notch target genes and recruits co-repressors (CoR) acting as a transcriptional repressor. In the presence of NICD the co-repressor complex is displaced and RBP-J is switched to a transcriptional activator of Notch target genes. 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 [11].

An intrinsic aspect of the Notch pathway, and also associated to Hes/Hesr proteins activity, relates to its context-specific nature, in which the cellular-context, timing, duration and dose of the signal will determine different outcomes. These features often reverberate in contradictory findings, in which a dual effect on a particular biological process or pathology is reported. In consequence, the Notch signaling is often associated to controversy in the most varied fields of research.

Notch signaling in cardiac specification and differentiation

Cardiac fate is specified during gastrulation (E7) in mesodermal progenitors emerging from a distal part of the primitive streak [12]. Cardiac progenitors undergo then epithelial-to-mesenchymal transition (EMT) and migrate bilaterally from the primitive streak to form the left and right heart fields (primary heart field) that will fuse to form the cardiac crescent. EMT and migration from the primitive streak depends on the expression of Mesp1 transcription factor [13]. Importantly, Mesp1 has also been demonstrated to regulate cardiac cell fate and EMT in differentiating ESCs [14]. Also in differentiating ESCs, Mesp1 has been shown to transiently upregulate directly components of the Notch pathway, while Notch blockade by treatment with a γ-secretase inhibitor from D2-D4 of *in vitro* differentiation compromised cardiovascular differentiation in *Mesp1*-induced cells. These observations suggested that Notch acts at the specification or early in cardiac lineage commitment [15]. The demonstration that transient Notch activation specifies mesodermal progenitors towards a cardiac fate using the ESCs differentiation system was soon to appear. Interestingly, in the same study, Notch induction in hematopoietic/vascular progenitors, the so-called hemangioblasts, redirected the cell fate towards cardiac commitment in detriment of the expected hematopoietic lineages [16]. In correlation with these observations, in the mouse embryo, Notch1 expression is found in nascent mesodermal cells at primitive streak stage (E7), what may indicate the participation in mesodermal specification towards its derivatives [17]. However, these findings apparently contradict the previous knowledge that Notch exerts a suppressive role on cardiac differentiation. Indeed, in mouse embryo enforced NICD1 activation in Mesp1-expressing cells, although not affecting myocardial and endocardial cell-fate decisions from $Mesp1^{+}$ cells, induced heart abnormalities by impairing myocardial differentiation

[18]. Likewise, Notch activation in *Xenopus* embryos after formation of the early heart field suppressed the expression of genes encoding contractile proteins, indicating impairment of myocardial differentiation [19]. Interestingly a similar regulation was documented in *Drosophila* [20], suggesting a conservative mechanism throughout the animal kingdom evolution. Supporting an inhibitory role in cardiogenesis, mESCs deficient in RBP-J [21] or in Notch1 receptor [22] showed enhanced cardiogenic potential, while Notch activation favored neuroectodermal fate [22, 23]. Yet, there is a possibility that cardiac induction is promoted by a non-canonical Notch pathway which is always independent of RBP-J and may occur either dependent or independently of ligand interaction and/or cleavage by γ-secretase [24]. This hypothesis would reconcile with the demonstration that mice lacking RBP-J show lethality at E8.5, and thus presumably have normal cardiac development up to primitive heart tube stage [25]. These evidences demonstrate that Notch signaling can interfere with cardiac lineage differentiation distinctly, depending on the specific time of induction and duration of the signal.

At later stages in heart development, loss-of-function mutations in Notch pathway members result in visible cardiac abnormalities, *e.g.* random heart looping (double *Notch1* and *Notch2* mutant; *Delta-like1* (*Dll1*); *RBP-J*) [26, 27], septal and valve defects (*Hey1*, *Hey2*, double *Hey1* and *HeyL* mutant, *Notch2*, *Jagged1*, *Hes1*) [28-34], and impaired trabeculation (*Notch1*, *RBP-J*) [35] are some of the described phenotypes. The effects of defective Notch signaling have revealed a function for several Notch pathway components in the initiation of EMT, required for formation of the cardiac septum and valve structures [31]; and in the regulation of myocyte proliferation and differentiation, essential for trabeculation and myocardial development [35] (Table 1).

Thus, Notch signaling plays a crucial role in cardiogenesis and in cardiac repair, previously explored in this dissertation [36]. The time and context-specific intervention of different components of the Notch pathway will determine the effect in cardiac lineage determination and further progression in the differentiation program. This tight regulation by Notch signaling will contribute, within a complex crosstalk with other pathways, for making the heart of the correct size, organization, shape and cell composition.

Table 1. Expression of Notch pathway members during heart development

Pathway member	Expression pattern
Notch1	Expressed in the cardiac crescent (E7.5). From E8.0 to E11.5 detection is limited to the endocardium and highly expressed in the AV canal and outflow tract endocardium [17, 37, 38].
Notch2	Expressed in the AV canal (E12.5) and the outflow tract endocardium (E11.5 and E14.5). Expressed in atrial and ventricular myocardium (E13.5) [39-41].
Notch3	Expressed in the cardiac crescent (E7.5) but not detected after linear heart tube formation (E8) [37].
Notch4	Expressed in the endocardium (E10.5) [42].
Jagged1	Expressed in the atrium (E10.5) and in the AV canal endocardium and outflow tract (E12.5) [43].
Delta-like1	Expressed in the endocardium at the base of ventricular trabeculae (E9.5) [35].
Delta-like4	Expressed in the cardiac crescent (E8.0) and restricted to endocardial cells and endothelial cells onward [44].
Hey1	Expressed in the endocardium (E8.5-E9.0). Restricted to atrial myocardium at E10.5. [38, 45].
Hey2	Expressed in the primitive ventricle of the primordial heart tube (E8.5). Restricted to ventricular myocardium at E10.5 [45, 46].
HeyL	Expressed in the AV canal endocardium (E9.5-E12.5) [31].
Hes1	Expressed in the second heart field (E8.5 and E10.5) [34].

AV, atrioventricular; E, embryonic day; OFT, outflow tract; EMT, epithelial-to-mesenchymal transition. Adapted from [47].

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Chapter II

Aims and structure of the Thesis

Aims and structure of the Thesis

In the last decade cardiovascular research has developed great efforts to implement cell-based strategies to restore myocardial tissue and improve cardiac function. Therapies based on the transplantation of cells of different origins have shown variable rates of success and, importantly, have revealed the importance of selecting the ideal cell source for heart repair/regeneration. The ideal cell-based therapy would involve the use of cells whose differentiation program is restrained to the generation of the cardiac lineages. Thus, the original discovery of cells displaying stemness features within the myocardium, so-called cardiac progenitor cells (CPCs), ignited enthusiasm in the field. However, advances have mostly been driven by a clinical urge for heart repair, and thus emerged from more applied studies on cell culture and cell transplantation. Consequently, a considerable number of clinical trials, very early initiated, have not yet gathered a support that can only be granted by robust data on the basic biological aspects concerning endogenous CPCs. In good truth, there are, undoubtedly, particular challenges to the *ex vivo* analysis of these cells, *e.g.* the scarcity within the myocardium, rapid senescence and phenotypic alterations due to long-term culture. There is also a propensity for variability, related to the heterogeneity of the distinct populations under study in different laboratories. This is, by large, a result of the distinct methodologies in use, and among others to mention, the choice of a single marker for isolation [1]. In a few words, this field of study is diseased of a lack of standardization.

It is currently recognized, that pathways that control embryogenesis are functional or reactivated in situations of stress, resulting in either beneficial or deleterious effects. Therefore, the comprehension of the regulatory networks orchestrating cardiac specification and differentiation will open a window to oversee new possibilities for therapeutic strategies; as it would be, for example, the specific modulation of the signaling environment to promote pro-cardiogenic processes. The knowledge gathered from the embryonic heart development and differentiating ESCs have contributed valuable candidates with a central role in cardiogenesis. As examples, master transcription factors and miRNAs have been used to reprogram cardiac fibroblasts into

cardiomyocytes *in vivo* [2, 3, 4]. Likewise, it is legitimate to anticipate candidates withdrawn from studies in ESCs and in the embryo might show as key factors to enhance cardiogenic potential of adult heart-resident CPCs.

In the framework of this Thesis collaborative work between Pinto-do-Ó & Di Nardo and Pinto-do-Ó & Lemischka laboratories was established to come forth with potential tools and answers for some of these questions.

The first objective of this work aimed at the production of a source of unlimited replicates of $Lin^{-}Sca\text{-}1^{+}$ progenitors endowed with a stable function and phenotype in long-term culture. The experimental design consisted in the isolation and clonal selection of heart-resident $Lin^{-}Sca\text{-}1^{+}$ cells immortalized by overexpression of mTERT. An extensive *in vitro* and *in vivo* characterization of an immortalized $Lin^{-}Sca\text{-}1^{+}$ clone was carried out in order to validate the newly generated line as a model system representative of the native cellular counterparts. The detailed experimental work and results are reported in the Chapter III.

Next, in recognition that knowledge on the early determination of cardiac fate during embryonic development might provide information on how to elicit/enhance cardiomyogenic potential in adult CPCs; a screen to identify early regulators in cardiogenesis was performed using the ESCs differentiation system. This *in vitro* model system offers the unique advantage of allowing mechanistic studies intended to dissect early lineage specification, and overcome technical constraints inherent to studies with embryos in very early developmental stages [5]. Chen et al, have demonstrated, using the mESCs differentiation system, that transient NICD activation directs mesoderm, and remarkably, respecifies the hemangioblast (a hematopoietic/vascular progenitor) towards a cardiac fate [6]. Thus, we proposed to identify effectors downstream of Notch playing a role in the onset of cardiogenesis. Our experimental design consisted in employing a mES cell line that expresses NICD1 under the control of a Doxycycline (Dox)-inducible promoter to identify the members of Hes/Hesr families playing a role in cardiac specification. The detailed experimental work and results are reported in the Chapter IV.

The final aim of the herein Thesis was to investigate whether the identified developmental factor(s) could also play a key role in the modulation/regulation of a cardiac molecular program in adult CPCs. A preliminary study was performed aiming at the examination of the role of Hes5 (the candidate identified in the Chapter IV) in the regulation of proliferation and differentiation in adult CPCs, using the iCPC$^{Sca\text{-}1}$ cell line (established in the Chapter III) as a model of endogenous adult CPCs. The experimental work and preliminary results are reported in the Chapter V.

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Chapter III

Stable phenotype and function of immortalized $Lin^{-}Sca\text{-}1^{+}$ cardiac progenitor cells in long-term culture: a step closer to standardization

Stable phenotype and function of immortalized Lin$^-$Sca-1$^+$ cardiac progenitor cells in long-term culture: a step closer to standardization

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Abstract

Putative cardiac progenitor cells (CPCs) have been identified in the myocardium and are regarded as promising candidates for cardiac cell-based therapies. Although two distinct populations of CPCs reached the clinical setting, more detailed studies are required to portray the optimal cell type and therapeutic setting to drive robust cell engraftment and cardiomyogenesis after injury. Owing to the scarcity of the CPCs and the need for reproducibility, the generation of faithful cellular models would facilitate this scrutiny. Here, we evaluate whether immortalized $Lin^{-}Sca\text{-}1^{+}$ CPCs ($iCPC^{Sca\text{-}1}$) represent their native-cell counterpart, thereby constituting a robust *in vitro* model system for standardized investigation in the cardiac field. $iCPC^{Sca\text{-}1}$ were established *in vitro* as plastic adherent cells endowed with robust self-renewal capacity while preserving a stable phenotype in long-term culture. $iCPC^{Sca\text{-}1}$ differentiated into cardiomyocytic-, endothelial-, and smooth muscle-like cells when subjected to appropriate stimuli. The cell line consistently displayed features of $Lin^{-}Sca\text{-}1^{+}$ CPCs *in vitro*, as well as *in vivo* after intramyocardial delivery in the onset of myocardial infarction (MI). Transplanted $iCPC^{Sca\text{-}1}$ significantly attenuated the functional and anatomical alterations caused by MI while promoting neovascularization. $iCPC^{Sca\text{-}1}$ are further shown to engraft, establish functional connections, and differentiate *in loco* into cardiomyocyte- and vasculature-like cells. These data validate $iCPC^{Sca\text{-}1}$ as an *in vitro* model system for $Lin^{-}Sca\text{-}1^{+}$ progenitors and for systematic dissection of mechanisms underlying CPC subsets engraftment/differentiation *in vivo*. Moreover, $iCPC^{Sca\text{-}1}$ can be regarded as a ready-to-use CPCs source for pre-clinical bioengineering studies toward the development of novel strategies for restoration of the damaged myocardium.

Introduction

Cardiac cell therapy was shown to be a very complex endeavor for which a completely innovative vision and novel technologies are necessary. Indeed, in spite of intensive scientific and economic efforts, the technological exploitation of the stem cell potential has generated frustrating results owing to the unsystematic and simplistic approaches considered so far. Myocardial cell delivery in experimental animals and patients results in a very low number (~ 3-10%) of cell persistence in the myocardium due to significant cell death and/or loss to the bloodstream with subsequent entrapment into the lungs [1, 2]. Although some beneficial effects have been reported, the improvement of cardiac function has been greatly ascribed to the release of paracrine factors rather than to an increased presence of functional cardiomyocytes (CMs) in the injured myocardium [3-5]. Regardless, in the last decade, adult cardiac progenitor cells (CPCs) have gathered abundant attention [6-18], paving the way to a quick transition from the bench to the bedside [19, 20]. In fact, two independent clinical trials, each addressing a different subset of progenitor cells (c-Kit-expressing cells for SCIPIO [19] and cardiosphere-derived cells for CADUCEUS [20]), were carried out to assess their therapeutic potential in the myocardial infarction (MI) setting. Although the short-term results on a reduced patient number have shown a partial amelioration of the heart condition, most aspects of the CPCs biology are still undefined. Hence, only an indisputable demonstration of the beneficial effects of these strategies can suppress the innumerable doubts regarding protocols based on harsh manipulations of stem/progenitor cells [21]. Among many others, the factors governing the admirable equilibrium of the myocardial *in vivo* complexity, such as the interactions between progenitor cells and environmental components, remain largely undefined. Therefore, the only possibility to mimic the dynamics of myocardial homeostasis in order to establish safe and reliable repair procedures is a stringent ‘‘standardization’’ of the different aspects of myocardial complexity [22].

A clear understanding on CPCs biology is impaired by the scarcity of progenitors present within the myocardium as well as by time-consuming isolation procedures, and, importantly, by their phenotypic variability when isolated and expanded in different laboratories [23]. Consistently, progenitor cells that apparently partake in the

same population can display non-comparable features. To circumvent these limitations, the aim of this study was to create a benchmark to be used as a reference for progenitor cell populations isolated from the myocardium in different laboratories on the basis of a plethora of non-specific cell-surface markers [6-15, 17, 18]. For this purpose, lineage negative/Sca-1$^+$ (Lin$^-$Sca-1$^+$) CPCs have been isolated from murine hearts and immortalized via murine telomerase catalytic subunit (mTERT) overexpression. Recently, the role of telomerase activity in maintaining CPCs viability and regenerative potential was demonstrated [24]. Aging leads to telomeric shortening in CPCs, thus leading to a senescent phenotype, as shown by the expression of p16INK4a. Such events have been correlated to cardiac function impairment, suggesting that CPCs loss could be a main determinant in heart failure. Moreover, the overexpression of the human telomerase reverse transcriptase (TERT) catalytic subunit in various cell lines resulted in extended cellular lifespan [25-27] without detectable changes characteristic of malignant transformation [28].

In the present study, a cell line of immortalized Sca-1$^+$ cardiac progenitor cells (iCPC^{Sca-1}) has been specifically generated for the first time. iCPC^{Sca-1} were extensively characterized to assess whether after immortalization these cells had preserved the hallmarks of their native-cell counterpart and the recognized capability to engraft and differentiate when transplanted in an MI murine model. Cells grew *in vitro* as adherent cells with a typical spindle-shape morphology and displayed robust self-renewal capacity, while preserving a CPC tri-lineage potential (*i.e.*, differentiated in CMs-, endothelial- and smooth muscle like-cells). iCPC^{Sca-1} transplanted into the MI border zone significantly reduced the MI-induced left ventricle (LV) anatomical and functional alterations. Importantly, the cells engrafted, established functional connections, and differentiated *in loco* into cardiomyocyte- and vasculature-like cells, as previously shown for their native counterparts. Thus, the iCPC^{Sca-1} line constitutes an unlimited source of Sca-1$^+$ CPCs replicates and can be used as a model system for *in vitro* high-throughput studies and for the dissection of the *in vivo* role of adult heart-resident progenitors.

Methods

Animals

C57BL/6 mice aged 6-12 weeks were used. Procedures were approved by Instituto de Biologia Molecular e Celular – Instituto de Engenharia Biomédica Animal Ethics Committee and National Direção Geral de Veterinária (permit no: 022793), and are in conformity with the European Parliament Directive 2010/63/EU. Humane endpoints were followed according to the Organization for Economic Co-operation and Development Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000). Mice were anesthetized by an intraperitoneal injection (ip) of medetomidine (1 mg/kg; Sededorm) and ketamine (75 mg/kg; Clorketam), and its adequacy was monitored by the pedal withdrawal reflex.

Isolation and culture of Sca-1^{+} CPCs

CPCs were isolated as previously described [29]: 6 week male C57BL/6 mice hearts were minced and digested using 0.25% Trypsin/ethylenediaminetetraacetic acid (EDTA) and collagenase II (1500 U; Worthington) in phosphate buffered saline (PBS). Tissue fragments were cultured in Dulbecco's Modified Eagle Medium (DMEM; Lonza) that was supplemented with 10% fetal calf serum (Lonza), 100 IU/mL penicillin and 100 µg/mL streptomycin, insulin-transferrin-selenium 1X, 300 ng/mL retinoic acid, 0.8 µg/mL linoleic acid, 2 mM L-glutamine, 0.1 ng/mL insulin-like growth factor 1, and 0.1 ng/mL endothelial growth factor (complete medium). After 12-15 days, adherent cells migrating out from fragments were harvested and magnetically sorted using anti-hematopoietic lineages antibody cocktail (Miltenyi Biotec). Sca-1^{+} cells were enriched from the Lin^{-} fraction by two sets of magnetic cell sorting protocols (Miltenyi Biotec) and expanded in culture in complete medium before transfection.

Establishment of iCPC^{Sca-1}

Murine TERT coding sequence, obtained from pGRN190 (kind gift from Geron Corporation), was inserted into *Eco*RI restriction site in pCINeo plasmid (Promega). Lin^{-} Sca-1^{+} cells were transfected with pCINeo-TERT or control vector using the Calcium Phosphate Method (Promega), according to the manufacturer's instructions and

incubated in G418-containing medium (Sigma-Aldrich) for at least 3 weeks. For clonal selection, immortalized cells were plated at single-cell density by limiting dilution and cultured in Sca-1^{+}-conditioned medium. Clones derived from a single cell were expanded under G418 selection and characterized for Sca-1 expression by flow-cytometry. Proliferative capacity of the established line was calculated using the formula $\log_{10}$(total no./start no.)/$\log_2$.

In vivo tumorigenicity assay

The tumorigenicity of $iCPC^{Sca\text{-}1}$ was assayed by heterotopic injection into syngeneic animals [30, 31]. Eight-week-old C57BL/6 female mice were subjected to a subcutaneous injection of $1x10^6$ $iCPC^{Sca\text{-}1}$ [in 0.2 mL of 2% fetal bovine serum (FBS) in α-minimal essential medium (α-MEM)] at each shoulder pad. Animals were monitored daily for the appearance of palpable tumors. Mice were necropsied after a 5-month surveillance and the injection region as well as the heart, spleen, lung, and liver were harvested, fixed in 10% formalin neutral buffer (VWR BDH and Prolabo), and paraffin embedded.

Flow cytometry

Before flow-cytometry analysis, single-cell suspensions were labeled with the following antibodies: APC–anti-Pdgfrα (Biolegend), c-Kit and Flk-1 (eBiosciences); PE–anti-CD105 (Biolegend), CD106 (Biolegend), CD44 (Immunotools), CD45 (Immunotools), CD34 (Biolegend), CD31 (BD Pharmingen), CD62L (Immunotools), CD40L (BD Pharmingen), CD90.2 (BD Pharmingen), and Stro-1 (Santa Cruz Biotech); and FITC–anti-Sca-1 (eBiosciences) and CD40 (BD Pharmingen). Samples were acquired on an FACS Canto II and analyzed using FlowJo software.

Gene expression

For the reverse transcription polymerase chain reaction (RT-PCR), total RNA was extracted using Trizol Reagent (Sigma-Aldrich), reverse transcribed according to the manufacturer's instructions (Bioline) and PCR was performed using BIOTAQ DNA polymerase (Bioline) and gene-specific primers. For quantitative RT-PCR (qRT-PCR) analysis, cDNAs were synthesized using PrimeScript™ RT reagent kit following manufacturer's instructions (Takara Bio, Inc.). qRT-PCR was performed using iQ™

Sybr® Green Supermix (Bio-Rad) and gene specific primers. Reactions were carried out in triplicate on the iCycler iQ5 Real-Time PCR system (Bio-Rad). Values were normalized to *glyceraldehyde-3-phosphate dehydrogenase* housekeeping gene. Primer sequences are available on request.

Immunocytochemistry (ICC)

iCPC^{Sca-1} were fixed with 4% paraformaldehyde (PFA) or cold methanol for Gata4 detection, permeabilized with 0.2% (cytoplasmic) or 1% Triton X-100/PBS (nuclear epitopes), and blocked for 1 h with 1% goat serum, 1% bovine serum albumine (BSA), or M.O.M.™ Immunodetection Kit (Vector Lab). Incubation with anti-Nestin (Abcam), anti-Gata4 (Santa Cruz Biotech.), anti-Sarcomeric α-actinin (α-actinin, Sigma-Aldrich), anti-α-Smooth Muscle Actin (α-SMA, Sigma-Aldrich) or anti-von Willebrand Factor (vWF, Sigma-Aldrich) was carried out for 1-2 h at room temperature (RT). Incubation with secondary antibodies was for 1 h at RT, with the exception of M.O.M.™ Biotinylated Anti-Mouse IgG Reagent (Vector Lab) by which the manufacturer's instructions were followed. Slides were mounted in Vectashield with 4,6-diamidino -2-phenylindole (DAPI; Vector Lab) and observed on the inverted fluorescence microscope Axiovert 200 (Zeiss).

iCPC^{Sca-1} differentiation

For cardiomyogenic differentiation, cells were (i) co-cultured with neonatal CMs for 7 days, (ii) cultured in the presence of transforming growth factor-β (TGF-β) for 21 days [32], or (iii) maintained using a commercial cardiomyocyte differentiation medium (Millipore) for 21 days. For the co-culture set-up, CMs were isolated from neonatal (postnatal day 1-3) C57BL/6 as previously described [33]. iCPC^{Sca-1} were pre-stained with viable red fluorescent dye Vybrant^{TM} DiI (Molecular Probes) before seeding onto CMs for 7 days. For endothelial differentiation, iCPC^{Sca-1} were pre-conditioned for 10 days in Endothelial Growth Medium with EGM BulletKit (EGM; Lonza) or in α-MEM (Gibco) with 10% FBS as control. After this period, cells were plated for immunocytochemistry (ICC), processed for CD31 flow cytometry staining, or seeded onto Matrigel Growth Factor Reduced Matrix (BD Biosciences). After overnight culture, cells were fixed and stained for vWF (PBS/0.05% Tween-20/1% BSA for 4 h at RT) or for

calcein AM (BD Biosciences). Briefly, cells were incubated for 30 min at 37ºC in calcein/PBS and imaged using the inverted fluorescence microscope Axiovert 200 (Zeiss). To evaluate tube formation, number of branch points and tube length were assessed using ImageJ1.42. For smooth muscle cell differentiation, iCPC^{Sca-1} were cultured in DMEM high glucose containing 2% FBS and 50 ng/ml of platelet derived growth factor BB (PDGF-BB; R&D Systems) for 10 days, as described earlier [7].

MI, iCPC^{Sca-1} delivery, and echocardiography

MI was induced by permanent ligation of left anterior descending (LAD) coronary artery as described elsewhere [34] with minor alterations. After anesthesia, female C57BL/6 were intubated and ventilated using a small-animal respirator (Minivent 845; Harvard Apparatus). The heart was exposed (Ø 5-7 mm) via left thoracotomy on the third intercostal space, and the pericardial sac was gently disrupted. A non-absorbable 7-0 suture (Silkam®; B. Braun) was used for ligation. Immediately afterward, 5x10^5 iCPC^{Sca-1} (n=8) or vehicle (0.5% BSA/PBS) only (n=9) were delivered by four intramyocardial injections (5 µL each) with a 30-gauge syringe (Hamilton company). Thoracic incision was closed using an absorbable 6-0 suture (Safil®; B. Braun), and surgical staples were used for skin closure. Anesthesia was reverted by atipamezole (ip, 5 mg/kg; Revertor). Analgesia and fluid therapy were performed by ip delivery of butorphanol (1 mg/kg; Butador) and a subcutaneous injection of 5% glucose physiological saline, respectively. This procedure was repeated every 12 h for approximately 72 h post-surgery or until full recovery.

Echocardiographic assessment

Transthoracic echocardiography was performed at 2 weeks after LAD coronary artery ligation using a portable ultrasound apparatus (GE Vivid I; General Electric) that was equipped with a 12 MHz linear probe (GE 12L-RS Linear Array Transducer; General Electric). To evaluate LV structural changes, several parameters from M-mode were measured, that is, the LV internal diameter at diastole (LVIDd) and at systole (LVIDs), the interventricular septum at diastole and at systole, the LV posterior wall at diastole (LVPWd) and at systole (LVPWs), and heart rate. Left ventricular ejection fraction (EF)

and fraction shortening (FS) were calculated as an index of systolic function: FS (%) = [(LVIDd - LVIDs)/LVIDd] x 100 and EF (%) = [(LVIDd^3 – LVIDs^3)/LVIDd^3] x 100.

Histological procedures

Hearts were harvested at 2 weeks post-surgery after an injection with potassium chloride and fixed in 10% Formalin neutral buffer (VWR BDH & Prolabo) for approximately 24 h before paraffin embedding. Representative LV sampling (~ 12 sections at 3 µm) was obtained by transverse sectioning from apex to base with 300 µm intervals. Masson's Trichrome (MT) staining was performed using the Trichrome (Masson) Stain kit (Sigma-Aldrich) with the following modifications: Nuclei were pre-stained with Celestine Blue solution following Gill's Hematoxylin staining, and tissue was incubated for 1 h in Bouin's solution before muscle staining with Biebrich Scarlet-Acid Fuchsin. Sections were photographed with a stereomicroscope (Leica) before morphometric analysis.

MI size calculation and morphometric analysis

Infarct size measurement was based on collagen deposition in the ischemic LV wall 2 weeks post-infarction and calculated by the *area method* using the semi-automated program *MIQuant* [35]. The thickness of LV free wall was measured on the infarction region using ImageJ1.42 on MT-stained sections.

Fluorescence *in situ* hybridization

Sections were subjected to antigen retrieval using 10 mM sodium citrate buffer (pH 6; Sigma-Aldrich) at 98ºC for 10 min followed by 0.01% acid pepsin (Sigma-Aldrich) at 37ºC for 10 min. For fluorescence *in situ* hybridization (FISH), samples were dehydrated and incubated with a specific probe to whole mouse Y-chromosome (Y-Chr; Cambio) for 5 min at 82ºC and overnight at 37ºC. Samples were incubated with Streptavidin (Invitrogen) for 30 min, mounted in Vectashield with DAPI (Vector Lab), and observed on the inverted fluorescence microscope Axiovert 200 (Zeiss). For α-actinin (Sigma-Aldrich) and CD31 (Santa Cruz Biotech.) immunostaining combined with FISH (immuno-FISH), antigen retrieval was achieved by pronase treatment for 30 min at 37ºC or with 10 mM Tris/1 mM EDTA (pH 9.0) at 98ºC for 30 min. Samples were permeabilized with 0.2% Triton X-100 and blocked using 4% FBS and 1% BSA or

M.O.M™ Immunodetection Kit (Vector Lab). Incubation with anti-Connexin43 (Cx43; Abcam), anti-α-SMA (Sigma-Aldrich) and anti-CD31 was performed at RT for 1-2 h and overnight at 4ºC for anti-α-actinin. After a 1 h incubation with the secondary antibody (Invitrogen), slides were rinsed in 50 mM $MgCl_2$/PBS for 5 min and post-fixed with 4% PFA and 50 mM $MgCl_2$/PBS for 10 min. Samples were dehydrated and incubated with Y-Chr probe as described earlier.

Blood vessel density quantification

After antigen retrieval using 10 mM Tris/1 mM EDTA (pH 9.0) at 98ºC for 30 min, samples were permeabilized with 0.2% Triton X-100 and blocked for 1 h with 4% FBS and 1% BSA. Slides were incubated with anti-CD31 for 1-2 hours at RT, 1 h incubated with secondary antibody and mounted in Vectashield with DAPI. Images were captured using MosaiX (AxioVision modules; Carl Zeiss). $CD31^+$ cells were counted in 30 fields *per* heart over 3-5 sections at infarcted and border zone regions along the heart's long axis (n=9 control group, n=8 $iCPC^{Sca\text{-}1}$) using ImageJ1.42. Density was calculated as CD31-positive cells *per* square millimeter (mm^2).

Data and statistical analysis

Statistical significance was evaluated with SPSS v.19.0 using Mann-Whitney or one-way analysis of variance (ANOVA) with post hoc Tukey's test. Values are presented as mean ± standard error of the mean (SEM). $p<0.05$ was considered statistically significant. *p<0.05, **p<0.01, and ***p<0.001.

RESULTS

Generation of an immortalized line representative of $Lin^-Sca\text{-}1^+$ adult CPCs

To immortalize $Lin^-Sca\text{-}1^+$ CPCs by inducing ectopic expression of mTERT, cells expressing Sca-1 were isolated from murine hearts and transfected with pCINeo vector encoding the mTERT.

Further analysis was restricted to one transfected clone of myocardium-resident $Sca\text{-}1^+$ progenitors, that is, the $iCPC^{Sca\text{-}1}$. The remaining G418-resistant isolated clones were subjected to transcriptional profiling (Supplementary Table S1) and cryopreserved for future investigation.

iCPC^{Sca-1} grew to confluence as a monolayer of spindle-shape adherent cells similarly to their native counterparts (Figure 1A) and presented a doubling time of ~21.6 h. iCPC^{Sca-1} were continuously sub-cultured for more than 24 months with no signs of differentiation and maintaining high levels of Sca-1 expression. Conversely, primary (non-transfected) Lin^{-}Sca-1^{+} CPCs decreased Sca-1 expression after 1 month in culture and senesced after 15-20 passages (Supplementary Figure S1).

Genome integration of mTERT was confirmed by Southern blotting using a specific probe for the neomycin phosphotransferase gene (Figure 1B). Chromosome number *per* metaphase plate of iCPC^{Sca-1} was compared with Sca-1^{+} primary cells; the majority of the cells were diploid and displayed a median chromosome number of 44 and 40 in iCPC^{Sca-1} and primary culture, respectively (Figure 1C). In addition, tetraploid cells were present in both conditions. The tumorigenic potential of iCPC^{Sca-1} was evaluated by means of a classical tumorigenesis assay in which syngeneic animals are used as recipients for heterotopic delivery of the cell inocula [30]. Hence, iCPC^{Sca-1} were subcutaneously delivered to the shoulder pads of syngeneic animals that were daily monitored for tumor progression. No local mass formation was observed after a 5-month inspection period and normal histological appearance was verified for the heart, spleen, lungs, and liver (Supplementary Figure S2).

iCPC^{Sca-1} display a typical tri-lineage potential and a stable phenotype after long-term *in vitro* culture

Prospective identification of *bona fide* CPCs based on the unique expression of surface markers is at the present not possible. Hence, it is generally accepted that an accredited cardiac progenitor cell will meet the following criteria: (i) derive from the adult heart; (ii) display either Sca-1 or c-Kit at their surface and lack markers of hematopoietic and endothelial commitment/differentiation; (iii) express cardiac-affiliated transcription factors while lacking mature proteins; and (iv) differentiate both *in vitro* and *in vivo* into CMs, endothelial cells, and smooth muscle cells. iCPC^{Sca-1} were, thus, characterized with regard to the cell surface phenotype, transcriptional profile, and functional properties in order to verify whether this cell line meets the premises mentioned earlier.

Flow cytometry analysis indicated a phenotype consistent with adult $Lin^{-}Sca\text{-}1^{+}$ CPCs, that is, a population constituted of cells with high Sca-1 levels (99.8% ± 0.21%), lacking hematopoietic (Stro-1, CD45, CD34) and endothelial (Flk-1, CD31 and CD34) markers, and expressing mesenchymal-associated proteins (*e.g.* CD29, CD44, CD105, CD106 and Pdgfrα), several of which are critical to cell migration, adhesion, and communication (Figure 1D). Furthermore, the characterized clone (Clone No. 3, Supplementary Table S1) did not express c-Kit, a protein that singly or together with Sca-1 has been used to isolate CPCs. A detailed transcriptional profile was carried out in parallel with primary $Sca\text{-}1^{+}$ CPCs ($CPC^{Sca\text{-}1}$), embryonic stem cells, bone marrow, and heart as controls. The side-by-side transcriptional analysis demonstrated that $iCPC^{Sca\text{-}1}$ and primary $Sca\text{-}1^{+}$ cells consistently expressed stemness-related genes (*Bcrp1, Bmi1,* and *Nestin*) and early cardiac transcription factors (*Gata4, Isl1, Mef2c,* and *Tef1*) while lacking transcripts for mature contractile myofilaments (*α*- and *β-Mhc*), fibroblasts (*Tcf21*) and regulators of pluripotency (*Sox2, Nanog, Oct4*), thus corroborating that $iCPC^{Sca\text{-}1}$ preserves the phenotype of cardiac-affiliated $Sca\text{-}1^{+}$ multipotent progenitors (Figure 1E). It should be noted that both primary and immortalized $CPC^{Sca\text{-}1}$ expressed *c-Myc*, a transcription factor known to block differentiation in distinct model-systems [36] and particularly shown to synergize with Pim-1 to induce the proliferation and survival of CPCs [37]. Overall, the data demonstrated that the immortalization of the cells did not alter the transcriptional profile of $Sca\text{-}1^{+}$ CPCs. The only obvious difference between the established line and the native counterparts is the lack of c-Kit in the immortalized cells, as a result of clone selection. Flow cytometry analysis clearly exposed the enrichment for the Sca-1-expressing fraction in $CPC^{Sca\text{-}1}$ (91.8%) and $iCPC^{Sca\text{-}1}$ (99.8%). Moreover, likely as a result of clonal selection, the $iCPC^{Sca\text{-}1}$ displayed a stronger and less disperse pattern of Sca-1 expression as compared with primary cells. Proper sub-cellular allocation of stemness-associated proteins Gata4 and Nestin was further confirmed by ICC for both primary and immortalized cells (Figure 1F). To test $iCPC^{Sca\text{-}1}$ multipotency, distinct sub-passages were cultured under cell differentiation conditions. Cardiomyocytic differentiation potential was evaluated by co-culture with mouse neonatal CMs. $iCPC^{Sca\text{-}1}$ were pre-stained with viable red fluorescent dye Vybrant DiI to enable detection in the co-culture system.

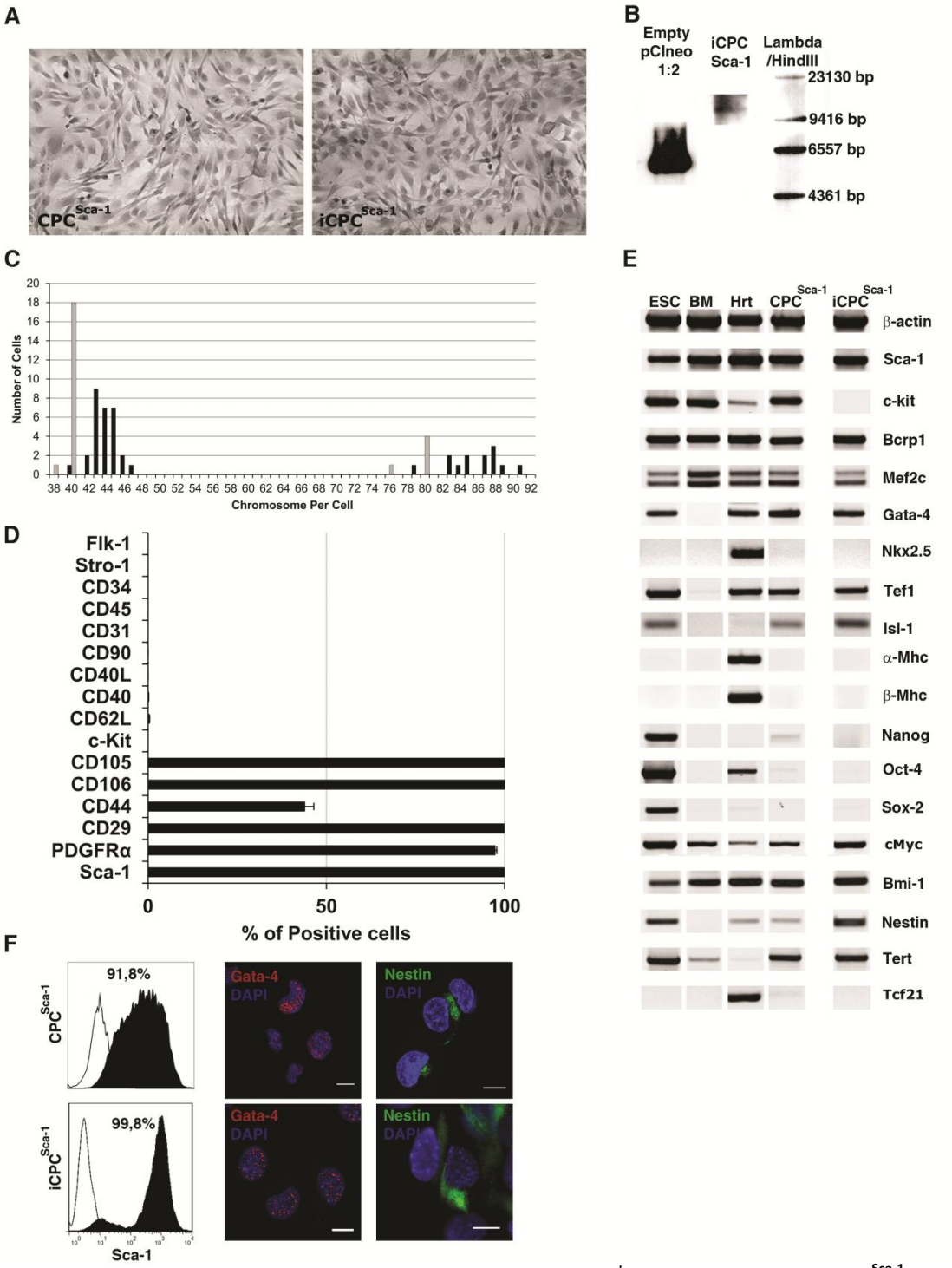
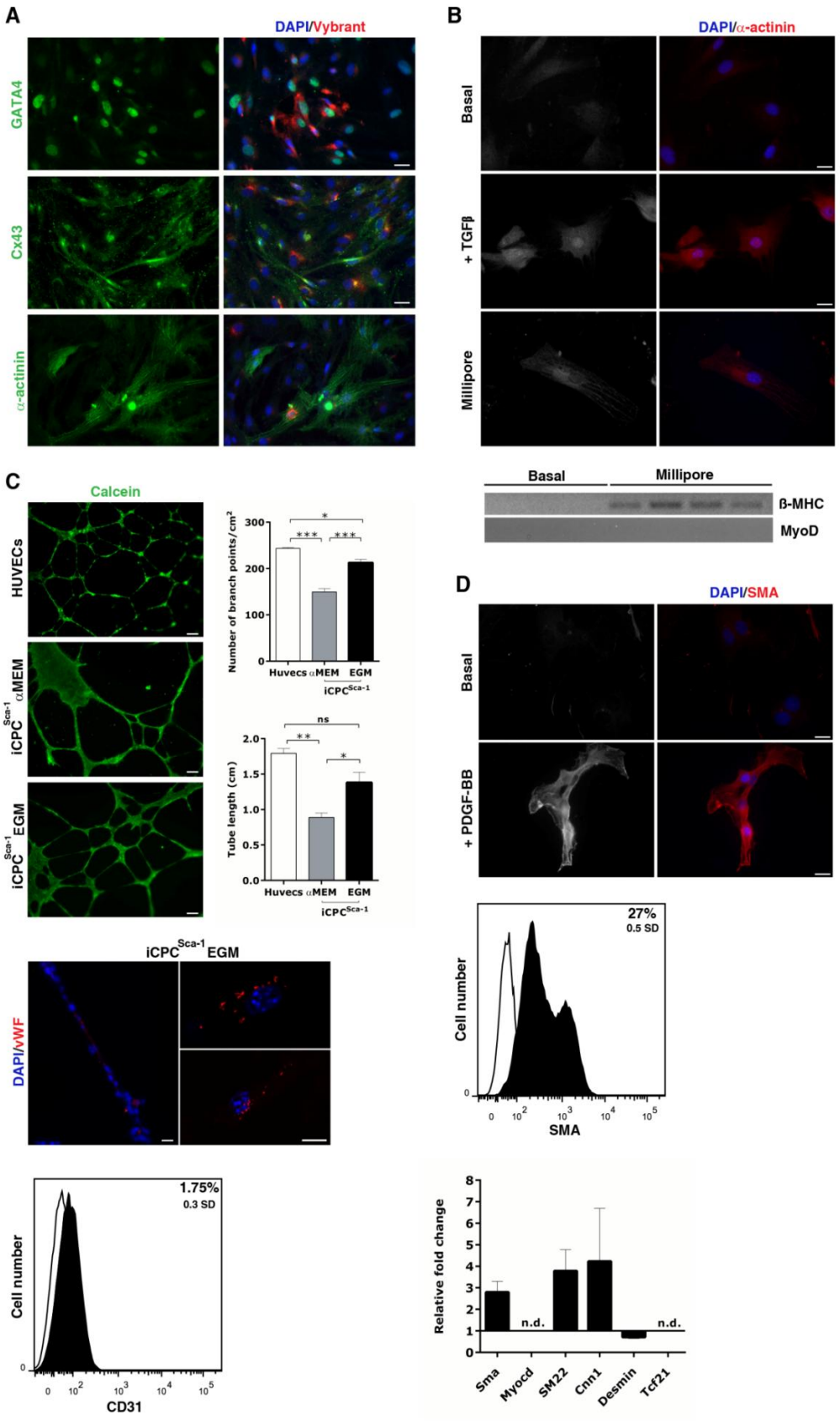


Figure 1. Generation and characterization of immortalized Lin^{-}Sca-1^{+} cardiac progenitors (iCPC^{Sca-1}) cell line. (A) Representative image of primary (CPC^{Sca-1}) and immortalized (iCPC^{Sca-1}) Sca-1^{+} CPCs. (B) Successful integration of pCINeo-murine telomerase catalytic subunit into iCPC^{Sca-1} genome was confirmed by Southern blotting for Neomycin. The empty plasmid was used as positive control. (C) Chromosome counts on (■) iCPC^{Sca-1} metaphase spreads show aneuploidy (n=42) when compared with (▩) Sca-1^{+} counterparts (n=23). (D) Flow cytometry analysis of iCPC^{Sca-1} displays high expression of Sca-1 and mesenchymal-associated antigens (Pdgfrα, CD29, CD44, CD105, and CD106). iCPC^{Sca-1} lack hematopoietic- (CD45, c-Kit) and endothelial-affiliated (Flk-1, CD31, CD34) markers. Bars are represented as mean ± standard error of the mean (SEM).

After a week, iCPC^{Sca-1} upregulated Gata4, displayed a functional pattern of Cx43 and a typical myofibrillar assembly of sarcomeric α-actinin (α-actinin) (Figure 2A). Although the gold-standard protocol to trigger CPCs differentiation is the direct co-culture with neonatal CMs [33], this assay is prone to misleading interpretations due to spontaneous cell fusion events [38, 39]. Hence, we further addressed iCPC^{Sca-1} differentiation in CMs by culturing the cell line for 21 days in cardiomyocytic differentiation medium (Millipore) or in the presence of TGF-β. In this conditions, iCPC^{Sca-1} were also able to upregulate α-actinin expression when compared with cells subjected to basal, that is, cell-maintenance, conditions for the same time period (Figure 2B). Furthermore, when iCPC^{Sca-1} were transduced with a green fluorescent protein (GFP) reporter under the cardiac troponin T (cTnT) promoter and subjected to TGF-β [32] for 45 days, approximately 97% of cells displayed GFP expression (Supplementary Figure S3). Since cTnT and α-actinin are also expressed in the developing skeletal muscle, we evaluated the possibility of iCPC^{Sca-1} having a skeletal origin. The latter was clearly excluded by demonstrating the upregulation of *β-Mhc* expression after differentiation, while no detectable expression of myogenic differentiation 1 (*MyoD*), a marker of skeletal commitment, was found (Figure 2B). Endothelial differentiation potential was assessed by priming iCPC^{Sca-1} with EGM medium for 10 days followed by a classical Matrigel assay to evaluate the capacity of the cells to form tubular-like structures. Primed iCPC^{Sca-1} seeded onto Matrigel exhibited tube-like interconnected structures, similar to the capillary assembly of human umbilical vein endothelial cells (HUVECs) (Figure 2C, n=6), whereas unprimed iCPC^{Sca-1} (iCPC^{Sca-1} pre-cultured in α-MEM) were unable to assemble a fully organized capillary network (n=6). In fact, the tubular complex formed by unprimed cells presented a significantly smaller number of branch points (p<0.001) and tube length (p<0.05 and p<0.01) as compared with that assembled by EGM-primed iCPC^{Sca-1} and

(E) Reverse transcription polymerase chain reaction (RT-PCR) profile of primary CPC^{Sca-1} and iCPC^{Sca-1} reveals the expression of stemness-related genes (*Bcrp1, Bmi1,* and *Nestin*) and early cardiac transcriptional regulators (*Gata4, Isl1, Mef2c,* and *Tef1*). Mature contractile myofilaments [α- and β-cardiac myosin heavy chain (*Mhc*)] and pluripotency (*Sox2, Nanog, and Oct4*) transcripts were not detected. Embryonic stem cells (ESC); bone marrow (BM), and heart (Hrt) were used as controls. (F) Flow cytometry displays the profile of Sca-1 expression in primary CPC^{Sca-1} and iCPC^{Sca-1} and immunocytochemistry (ICC) corroborates the expected sub-cellular location of progenitor-associated proteins Gata4 and Nestin. Scale bar: 10 μm.


A
DAPI/Vybrant
GATA4
Cx43
α-actinin
B
DAPI/α-actinin
Basal
+ TGFβ
Millipore
Basal
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ß-MHC
MyoD
C
Calcein
HUVECs
iCPC^{Sca-1} αMEM
iCPC^{Sca-1} EGM
Number of branch points/cm^2
Huvecs αMEM EGM
iCPC^{Sca-1}
Tube length (cm)
ns
iCPC^{Sca-1} EGM
DAPI/vWF
Cell number
1.75%
0.3 SD
CD31
D
DAPI/SMA
Basal
+ PDGF-BB
Cell number
27%
0.5 SD
SMA
Relative fold change
n.d.
n.d.
Sma
Myocd
SM22
Cnn1
Desmin
Tcf21

HUVECs (Figure 2C). An endothelial phenotype was further addressed by analyzing vWF and CD31 protein expression. vWF was only detected in iCPC^{Sca-1} cells after EGM-priming (Figure 2C). Accordingly, CD31 was increased by 1.75% on primed cells when compared with iCPC^{Sca-1} maintained in α-MEM for the same period (Figure 2C).

The capacity to differentiate into smooth muscle cells was evaluated by subjecting iCPC^{Sca-1} to PDGF-BB for 10 days. After this period, iCPC^{Sca-1} became elongated and displayed α-SMA protein expression, as demonstrated by ICC and flow cytometry (27%) (Figure 2D). In addition, the expression levels of the smooth muscle-affiliated genes, *α-SMA, Myocardin (Myocd), Transgelin (SM22), Calponin 1 (Cnn1),* and *Desmin* were also assessed by qRT-PCR (Figure 2D). After 10 days of PDGF-BB treatment, iCPC^{Sca-1} upregulated *α-SMA, SM22,* and *Cnn1*; while no clear alteration was observed for *Desmin* mRNA levels. In addition, no detectable expression of *Myocd* was found, even on PDGF-BB stimulation. Since some of the genes mentioned earlier are also displayed by fibroblasts/myofibroblats, expression of the fibroblast marker *Tcf21* was evaluated in order to discard a fibroblastic origin of the iCPC^{Sca-1}. Detectable expression of *Tcf21* was not found, neither in basal conditions nor after PDGF-BB treatment (Figures 1E and 2D). Overall, these results demonstrate that iCPC^{Sca-1} were not derived

Figure 2. iCPC^{Sca-1} *in vitro* differentiation potential. (A) Immunostaining demonstrating increase in Gata4 expression and presence of Cx43 and α-actinin in Vybrant-stained iCPC^{Sca-1} after 1 week co-culture with neonatal cardiomyocytes. Scale bar: 20 µm. (B) iCPC^{Sca-1} α-actinin expression is increased after 21 days of culture in cardiomyocytic differentiation conditions: transforming growth factor-β or commercial medium (Millipore). Scale bar: 20 µm. RT-PCR representing the upregulation of *β-Mhc* in iCPC^{Sca-1} cultured in Millipore medium, while no *MyoD* transcript was observed in basal conditions or after differentiation. (C) Calcein staining evidencing the tubular structures formed by human umbilical vein endothelial cells (HUVECs) and iCPC^{Sca-1} [pre-conditioned in either α-minimum essential medium (α-MEM) or endothelial growth medium (EGM)] on Matrigel. Scale bar: 200 µm. The tube length and the number of branch points formed in each culture condition were assessed. Data are represented as mean ± SEM [n=6, ***p<0.001; **p<0.01; *p<0.05 and not significant (ns) by one-way ANOVA with post hoc Tukey's test]. EGM-preconditioned iCPC^{Sca-1} grown on Matrigel exhibit increased von Willebrand Factor (vWF) and CD31 expression as assessed by ICC and flow cytometry, respectively. Scale bars: 20 µm and 5 µm. Data are represented as mean ± standard deviation (SD). (D) α-Smooth muscle actin (α-SMA) expression in control iCPC^{Sca-1} and after 10 days of culture in differentiation medium containing PDGF-BB. Scale bar: 20 µm. Histogram plots show control in *white* and specific staining in *black*. Data are represented as mean ± SD. Quantitative RT-PCR revealed an upregulation in *α-SMA, SM22,* and *Cnn1* mRNA levels; while no dramatic alteration in *Desmin* expression levels was found. No detectable levels of expression were observed for *Myocd* and *Tcf21* (n.d.).Data are represented as a fold increase relative to basal condition (n=3).

from cardiac fibroblasts and that, after PDGF-BB stimulation, cells undergo an incomplete smooth-muscle differentiation program as the levels of *Myocd* and *Desmin* have not changed after PDGF-BB treatment.

Taken together, the data demonstrate that iCPC^{Sca-1} preserved the *in vitro* phenotype and functional ability to moderately differentiate as described by our team [29, 33] and others [5, 12, 13, 16] for the native Lin^{-}Sca-1^{+} counterparts.

iCPC^{Sca-1}-transplanted hearts show improved systolic function and lessened LV remodeling after MI

The beneficial effect of Lin^{-}Sca-1^{+} CPCs administration into the MI heart has been clearly established. Notably, CPCs are able to improve cardiac function and attenuate LV remodeling after MI. However, the *in vivo* differentiation of the CPCs in CMs and endothelial cells is minimal, and the amelioration observed on MI is primarily attributed to paracrine effects [5]. Aiming at demonstrating that iCPC^{Sca-1} display the hallmarks of CPCs in the myocardium in the onset of injury, a proof-of-principle experiment was performed using a commonly reported experimental injury setting [16, 40]. Female C57BL/6 mice subjected to MI via LAD coronary artery ligation were immediately injected on the peri-infarct region with either vehicle medium (n=9) or male-derived iCPC^{Sca-1} (n=8). MI size measurement (based on collagen deposition) at 14 days post-infarction showed that MI extent was comparable between both experimental groups with 52% of the LV wall being affected (Figure 3A). Notwithstanding, echocardiography at 14 days post-surgery in animals injected with iCPC^{Sca-1} showed improved LV function when compared with vehicle controls, as demonstrated by significantly increased (p<0.05) EF and FS in iCPC^{Sca-1}-transplanted hearts (Figure 3B).

Altered cardiac loading after MI due to extensive loss of myocardial cells induces changes in the heart shape, size, and function that are commonly designated as cardiac remodeling. A stereoscopic view of representative cross-sections of infarcted hearts showed that iCPC^{Sca-1} transplantation prevented the major LV wall thinning and LV chamber expansion observed in the vehicle control group (Figure 3C). This was

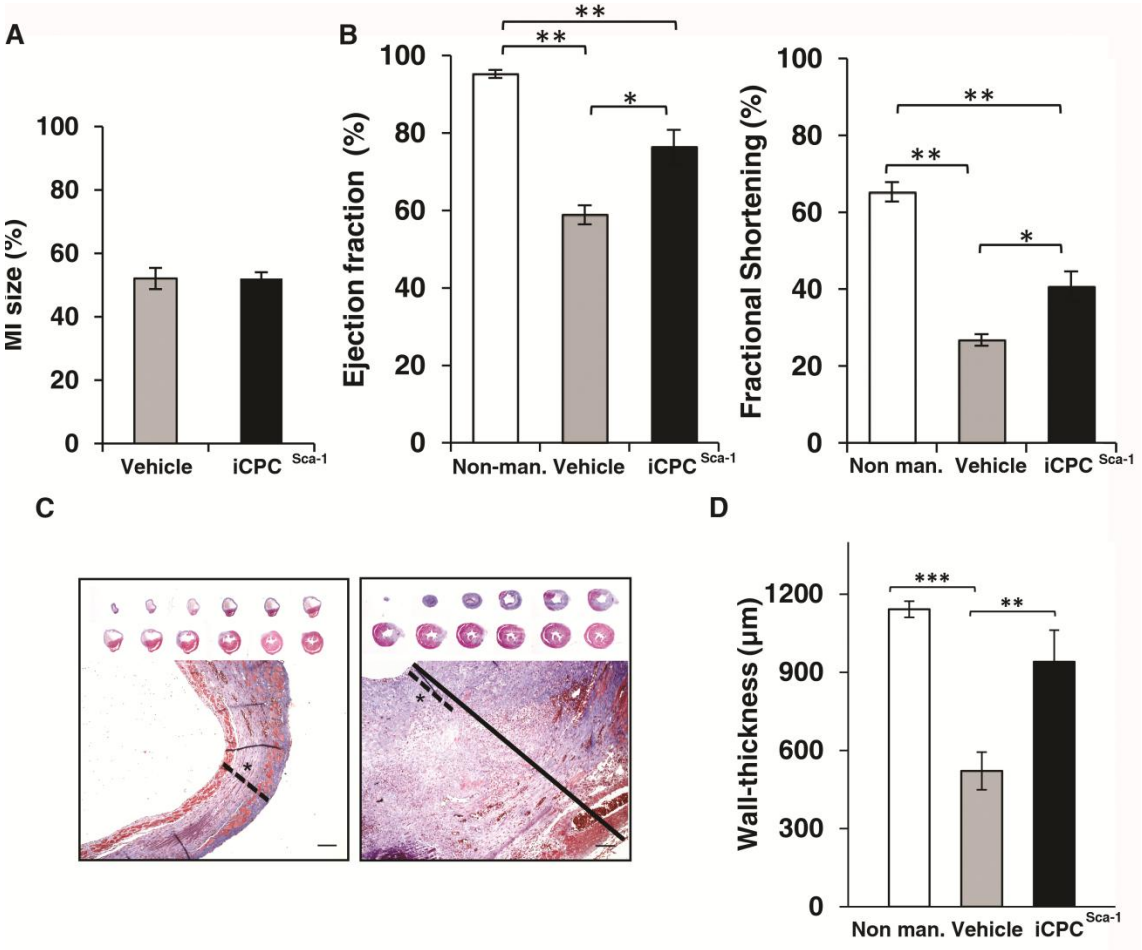

Figure 3. Functional and histological assessment of ischemic hearts transplanted with either iCPC^{Sca-1} or vehicle. (A) Both iCPC^{Sca-1} and vehicle-injected groups display similar myocardial infarction (MI) size. (B) Ejection fraction and fractional shortening measured in non-manipulated animals, vehicle-control and iCPC^{Sca-1}-injected hearts at 2 weeks post-intervention show a significant improvement in left ventricle (LV) contractile capacity in iCPC^{Sca-1}-transplanted hearts. (C) Increased LV wall thickness and decreased LV dilation in iCPC^{Sca-1}-injected animals, demonstrated by Masson's Trichrome staining of LV sections. *Broken* and *continuous lines* highlight the thickness of the infarcted LV wall in vehicle and iCPC^{Sca-1} transplanted animals, respectively. (D) Measurements of LV wall thickness in non-manipulated hearts and in the ischemic region corroborate a significant difference between both experimental groups. Scale bar: 100 µm. Data are represented as mean ± SEM (***p<0.001; **p<0.01, and *p<0.05 by Mann-Whitney test).

corroborated by a morphometric analysis of MT stains, evidencing an increase (p<0.01) in LV wall thickness of iCPC^{Sca-1}-transplanted hearts (Figure 3D) to a value not statistically different from the non-manipulated animals (Figure 3D). However and despite this increase, the LV contractile function of iCPC^{Sca-1}-injected animals was not comparable with that of non-manipulated animals (p<0.01, Figure 3B).

Overall, the data suggest that iCPC^{Sca-1} exerted a cardioprotective effect, translated on a reduction of the deleterious consequences of ischemia and a partial recovery of cardiac functional parameters at 2 weeks post-MI.

Transplanted iCPC^{Sca-1} improve neovascularization of the infarcted myocardium, engraft, and differentiate into CMs-, endothelial-, and smooth muscle-like cells

To address whether cardiac performance amelioration after iCPC^{Sca-1} administration occurred at least partly via stimulation of the *de novo* vessel formation by paracrine mechanisms, the density of small blood vessels in the infarcted myocardium and in the border zone was quantified at 2 weeks post-surgery (Figure 4A). Overall, iCPC^{Sca-1} transplantation resulted in a significant increase in CD31^{+} cells *per* mm^{2} when compared with the vehicle control (709±19 *vs.* 555±24, respectively, $p<0.001$, Figure 4B). iCPC^{Sca-1}, identified by Y-chromosome (Y-Chr) staining were consistently observed in close proximity to vessels, suggesting contribution to either endothelial precursors mobilization/proliferation and/or differentiation into endothelial cells. The latter possibility was evaluated by FISH combined with immunostaining for CD31, and, indeed, double positive events were observed in all analyzed animals although at very low frequencies (4.1±0.7 cells *per* transverse heart section) (Figure 4C and 5I). In fact, the majority of small vessels were not formed by donor cells, hinting that iCPC^{Sca-1} contribution to neovascularization is more likely to occur by stimulating the mobilization and/or proliferation of host endothelial precursors/cells (paracrine action).

We next investigated iCPC^{Sca-1} functional engraftment and contribution to the *de novo* cardiac cells formation. In vehicle-injected female mice, no Y-Chr^{+} cells were detected in the heart (Figure 5A); whereas in transplanted animals, numerous iCPC^{Sca-1} were observed throughout ischemic myocardium and MI border zone at 2 weeks post-infarction. This demonstrates that cells were able to survive and engraft the LV ischemic region (Figure 5B). Moreover, iCPC^{Sca-1} or their progeny were not found in healthy remote areas of the injury site. Ki67 staining was frequently associated with Y-Chr evidencing iCPC^{Sca-1} proliferation within the host myocardium (data not shown). Assembly of the gap junction protein Cx43 at the cellular membrane confirmed that

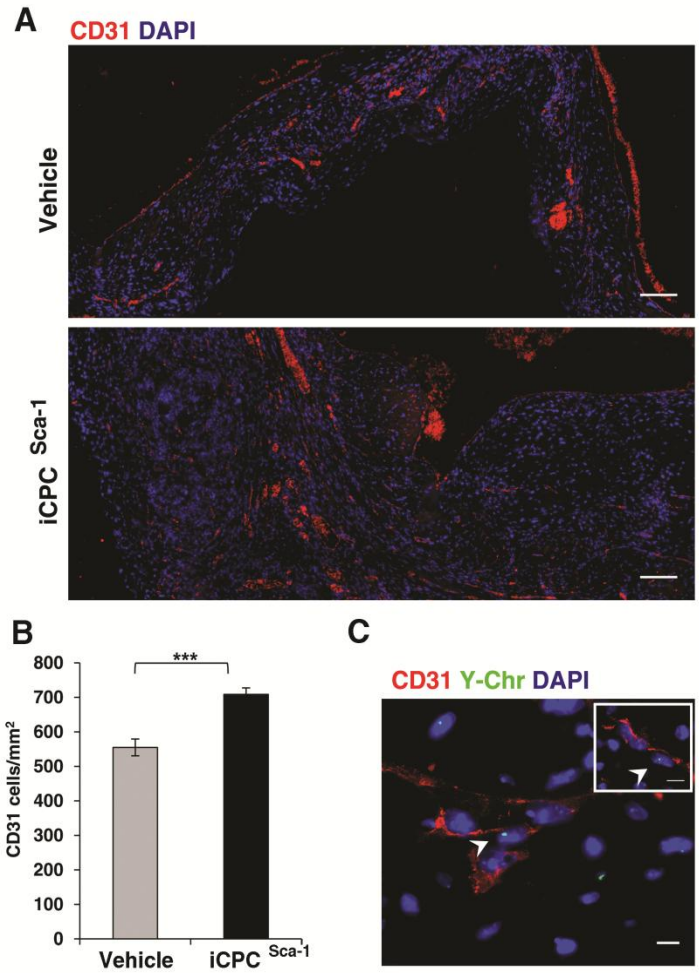

Figure 4. Contribution of iCPC^{Sca-1} to neovascularization post-MI. (A) Immunolocalization of CD31 in the ischemic region of control and iCPC^{Sca-1}-transplanted animals. Scale bar: 100 µm. (B) Quantification of CD31^{+} cells demonstrates a significant increase in small vessels number in iCPC^{Sca-1}-injected animals. (C) Immunostaining combined with fluorescence *in situ* hybridization (immuno-FISH) demonstrating iCPC^{Sca-1} differentiation into CD31^{+} cells (*arrowheads*). Scale bar: 10 µm. Data are represented as mean ± SEM (*** p<0.001 by Mann-Whitney test).

donor cells established functional communication to both donor and to host cells (Figures 5C and 5D), substantiating successful iCPC^{Sca-1} engraftment within the host tissue.

Although the majority of grafted cells retained an undifferentiated morphology and Sca-1 expression (Supplementary Figure S4), discrete differentiation events were observed. Co-localization of Y-Chr with α-SMA was observed in all analyzed animals (41.2±5.6 cells *per* transverse heart section) (Figure 5I), either integrating small vessels (Figure 5E) or displaying a fibroblast-like morphology (Figure 5F). In fact, in addition to smooth muscle cells, α-SMA is also expressed by myofibroblasts, cells capable of

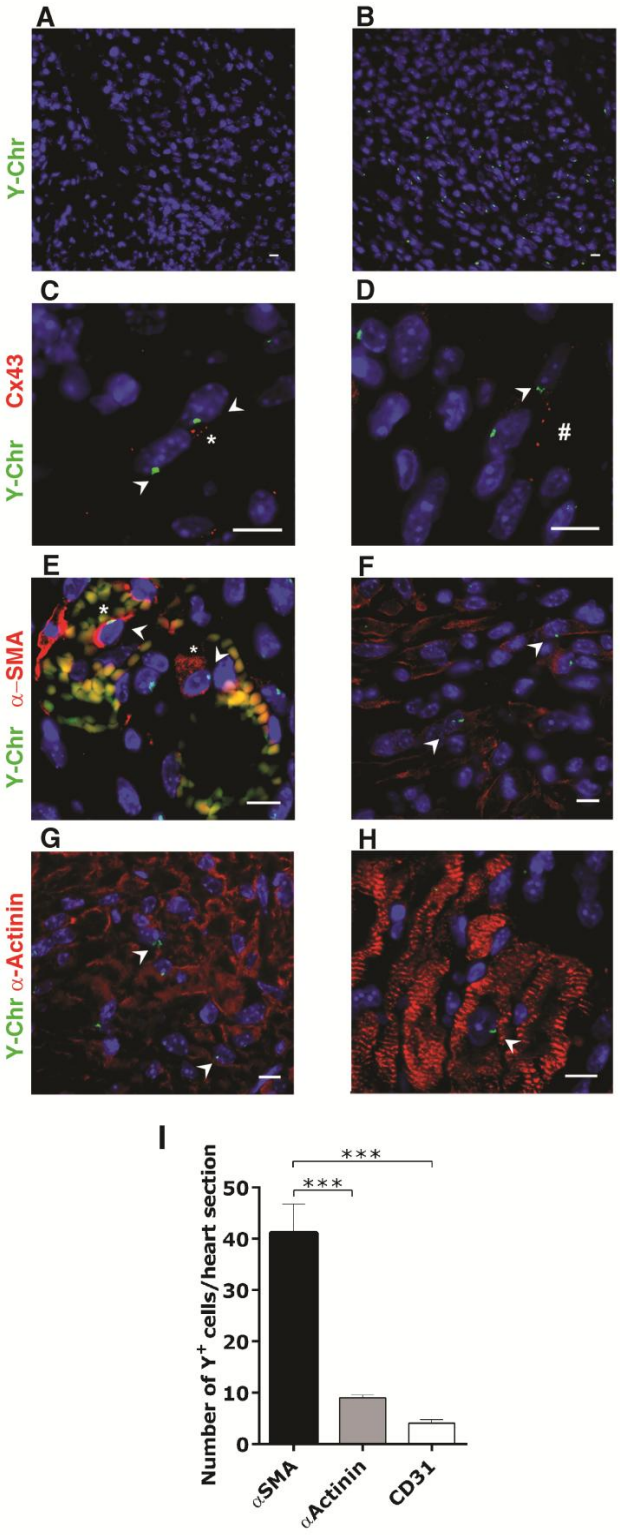

Figure 5. *In vivo* characterization of iCPC^{Sca-1} on transplantation into MI hearts. (A, B) Y-Chromosome detection (FISH) in vehicle (A) and male-derived iCPC^{Sca-1}-injected hearts (B) demonstrates the immortalized cell line successful engraftment. (C-H) Functional communication between donor-donor [*, (C)] and donor-host [#, (D)] were revealed by the assembly of Cx43 at the cellular membrane. iCPC^{Sca-1} expressing α-SMA (*arrowheads*) were found integrating small vessels [*, (E)] and scattered throughout the myocardium displaying a fibroblast-like morphology (F). α-actinin^{+} iCPC^{Sca-1} (*arrowheads*) in the infarcted tissue displayed an immature phenotype (G) and, less-frequently, well-defined cross-striations (H). Scale bar: 10 μm. (I) Graph displaying the number of cells, *per* transverse histological section, bearing the Y-Chr and expressing α-SMA, α-actinin or CD31. Data are represented as mean ± SEM (*** p<0.001 by one-way ANOVA with post hoc Tukey's test).

establishing cell connections through gap junctions, and relevant players in formation of the contractile granulation tissue [41]. However, considering that Tcf21 is a marker of fibroblasts [42] and is also involved in the activation of myofibroblasts [43], and that iCPC^{Sca-1} failed to express this marker even when specifically stimulated (Figures 1E and Figure 2D), there is little support for considering a myofibroblast fate. Furthermore,

iCPC^{Sca-1} contributed to the cardiomyocytic compartment as evidenced by α-actinin expression. The majority of α-actinin^{+} iCPC^{Sca-1} presented an immature phenotype in three out of six analyzed animals (9.0±0.6 cells *per* transverse heart section) (Figure 5I), suggesting an incomplete differentiation. Moreover, α-actinin^{+} iCPC^{Sca-1} were found in close proximity with α-actinin^{+} host cells displaying a similar immature phenotype, suggesting an eventual iCPC^{Sca-1}-mediated recruitment/activation of endogenous precursors (Figure 5G). Donor cells presenting well-defined cross-striations, characteristic of a more mature phenotype, were found in one animal (Figure 5H; Supplementary Figure S5). While we do not rule out the possibility that these scarce events resulted from cell fusion, differentiation into more mature CMs seems a more consistent scenario (Supplementary Figure S5).

Overall, these results are in agreement with the previous observations, that Lin^{-}Sca-1^{+}-mediated beneficial effects after MI are partially mediated by paracrine mechanisms [5, 44] in detriment to a major contribution to cardiac cell types after transplantation.

Discussion

Different progenitor cell subsets have been so far suggested as candidates for the optimal cell population to be implanted in infarcted and/or failing hearts (Figure 6), but a relationship among these populations remains undetermined. The absence of a specific molecular signature as well as the multiplicity of the isolation and characterization protocols do not enable an understanding of whether the described CPCs populations constitute different subsets of a common progenitor or independent cell lineages of distinct ontogeny. Indeed, it has been suggested that the various identified CPCs partake in the same cell lineage, and the different phenotypes are a consequence of the particular culturing protocols used in different laboratories [21]. Importantly, other aspects account for an increased difficulty in the identification of CPCs and to understand their real efficiency. Namely, primary cells can easily change along time in culture as a consequence of genotypic and phenotypic drift and/or senescence [45-47] and, thus, lead to a lack of reproducibility across studies.

Taken together, the experience accumulated in the last decade indicates that only two strategies are used to implement a reliable cell therapy: to try and fail, that is, to

implant whatever progenitor cell phenotype in the patient trusting to identify the perfect treatment, or to systematically dig the complexity of the stem/progenitor cell behavior and, based on novel data and concepts, to formulate a logically stringent procedure to transfer experimental observations to the clinical setting.

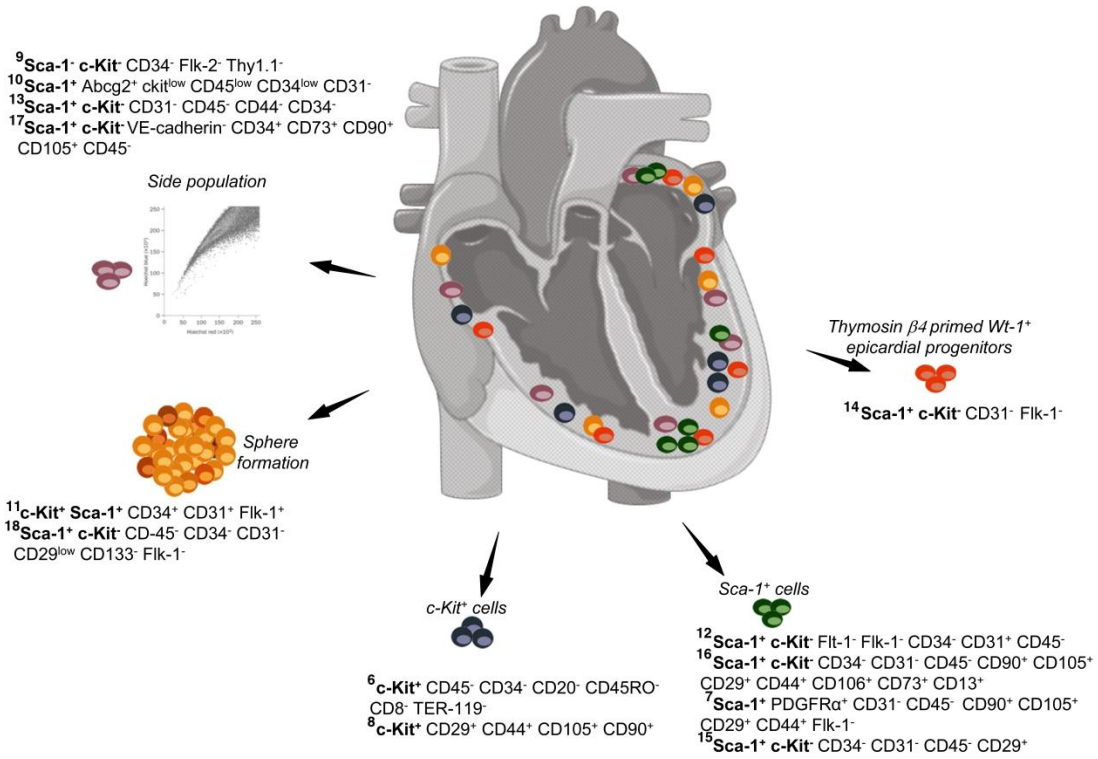

Figure 6. Illustrative scheme of described multipotent progenitor cell populations isolated from the adult heart. Several isolation methods have been applied to separate CPCs based on the expression of specific cell-surface markers (*e.g.* c-Kit^{+} and Sca-1^{+}), *in vitro* functional assays (*e.g.* cardiospheres, side population), or the expression of a specific genetic marker (Wt-1 epicardial progenitors). Regardless of the increasing number of putative CPCs reported, the functional, hierarchical, and anatomical relationship of the illustrated cell progenitor subsets is still elusive.

Novel tools must be developed to help understanding and, thus, governing the CPCs complexity and phenotype variability and to enable the standardization of the experimental sets and the inter-laboratory comparison. Consistently, the present study has been carried out to create an immortalized cardiac progenitors cell line retaining a constant phenotype corresponding to native features. CPCs immortalization has been achieved by inducing TERT overexpression, in order to circumvent the limitations determined by the diminished mitotic capacity of somatic cells [48-52]. The use of

immortalized lines that retain the *in vivo* features of primary cells, by combining the advantages of a high proliferative cell source with a stable phenotype in long-term culture, is a major breakthrough in cell biology. Indeed, we have previously shown that bone marrow-derived mesenchymal stromal/stem cell (MSC) immortalized lines preserve multipotency in standard and three-dimensional conditions *in vitro* [49, 53]. Although telomerase overexpression does not induce oncogenesis, it may provide cells with a greater opportunity to accumulate mutations, predisposing to later malignant transformation [54, 55]. The iCPC^{Sca-1} karyotype analysis showed aneuploidy, characteristic of malignant cells. However, an *in vivo* tumorigenicity assay [30, 31], monitored for 5 months, did not reveal tumor formation either locally (subcutaneously) or in any of the analyzed organs (heart, spleen, lungs, and liver).

Consistently with adult Lin$^-$Sca-1$^+$ CPCs, the iCPC^{Sca-1} phenotype was characterized by the expression of high levels of Sca-1, stemness-related genes (*Bcrp1, Bmi1,* and *Nestin*), mesenchymal-associated proteins (*e.g.* CD29, CD44, CD105, CD106, and Pdgfrα), and early cardiac transcription factors (*Gata4, Isl1, Mef2c,* and *Tef1*). Conversely, immortalized cells did not express c-Kit while also lacking hematopoietic, endothelial, fibroblasts, mature contractile myofilaments (*α-Mhc* and *β-Mhc*), and pluripotency (*Sox2, Nanog,* and *Oct4*) markers.

iCPC^{Sca-1} displayed the capability to generate *in vitro* cardiomyocyte-, smooth muscle-, and endothelial-like cells, with the differentiation toward smooth muscle-like cells being the most efficient. This differential *in vitro* potential of Sca-1$^+$ cells has been previously demonstrated by others [16]. Whether this indicates a higher commitment of CPC^{Sca-1} to smooth muscle-like cells or to the fact that the *in vitro* differentiation conditions are less favorable to activate endothelial and cardiomyocytic pathways, and, thus, not suitable to demonstrate the full potential of these cells, still needs to be determined.

iCPC^{Sca-1} is a phenotypically stable cell line that provides investigators with the same unique cell model to dissect CPCs behavior and differentiation mechanisms. Furthermore, iCPC^{Sca-1} could represent a benchmark to compare with other cells posited to bear stemness characteristics. In fact, the immortalized CPCs retain the

features of their *ex vivo* counterparts, that is, moderate differentiation into cardiomyocyte-, endothelial-, and smooth-muscle-like cells, when subjected to appropriate stimuli *in vitro* as well as *in vivo*, in a myocardial injury setting. Furthermore, the cell line combines the advantages of a high proliferative cell source with a stable phenotype in long-term culture. Indeed, murine cells are prone to undergo spontaneous immortalization after prolonged *in vitro* culture [46, 56, 57], which may result in accumulation of unpredictable cytogenetic modifications and malignant transformations [47]. The use of uncharacterized spontaneously immortalized cell clones as *in vitro* models for their native cell counterparts should be circumvented due to the dissimilar phenotypes generated by unpredictable cytogenetic alterations in overextended sub-culture *in vitro*.

The iCPC^{Sca-1} capability to mimic their native non-immortalized corresponding cells has also been apparent in *in vivo* experiments. In fact, iCPC^{Sca-1} were able to contribute toward repairing the myocardial injuries experimentally provoked by coronary ligation *in vivo, as* shown by minor LV chamber expansion and increased thickness of the LV-free wall. Importantly, echocardiography follow-up revealed improved systolic function after iCPC^{Sca-1} transplantation into MI animals. iCPC^{Sca-1} abundance in the host infarcted and peri-infarcted myocardium was particularly surprising, and cells were able not only to survive but also to engraft. Moreover, engrafted cells established donor-donor and donor-host Cx43-mediated connections. Indeed, Cx43 promotes MSC survival in the ischemic heart [58], and it is critical for protection against ventricular tachycardia, which is a resultant from the transplantation of embryonic CMs in MI [59]. Accordingly, it can be conjectured that Cx43 is involved in iCPC^{Sca-1} resistance to the adverse scenario of myocardial ischemia.

The implantation of immortalized cells into infarcted mouse hearts also induced a significant improvement in the vascularization of the damaged region, very likely, through a paracrine action. Since an improved vascularization is associated with a “pro-regenerative” response, the formation of new vessels in the infarcted tissue was carefully assessed. iCPC^{Sca-1} transplanted hearts exhibited a denser capillary network, when compared with the vehicle control group. In addition, iCPC^{Sca-1} contributed directly to *de novo* vessel formation, as demonstrated by CD31 and α-SMA expression

on Y-Chr bearing cells. However, endothelial differentiation was modest; hence, direct contribution of engrafted cells to new vessel formation is minimal, suggesting that paracrine mechanisms are involved in the recruitment and/or proliferation of endothelial cells and/or precursors.

CPCs differentiation into CMs has been frequently demonstrated by the expression of proteins of the contractile machinery [5, 6, 12, 15]. Indeed, although CPCs appear to grasp a lot of expectations with regard to cardiac cell therapies, most studies still report an immature phenotype for the differentiated cells, to a great extent resembling fetal-neonatal CMs [5, 6, 12, 15]. In our *in vivo* setting, iCPC$^{Sca\text{-}1}$ differentiated into cardiomyocyte-like cells, though the majority displayed a disorganized structure with no detectable sarcomeres, characteristic of an immature phenotype. It should be noted that both primary CPC$^{Sca\text{-}1}$ and iCPC$^{Sca\text{-}1}$ express the transcription factor *c-Myc*, which has been previously shown to play a role in the blockage of cell differentiation [36] and in the survival and proliferation of CPCs [37]. Whether the levels of c-Myc are regulating CPCs cell-fate decision to differentiate at the expenses of cell proliferation remains to be determined and should be subject to further investigation. Since iCPC$^{Sca\text{-}1}$ showed an *in vitro* and *in vivo* differentiation potential similar to their native counterparts, it is our conviction that iCPC$^{Sca\text{-}1}$ constitutes a reliable easy-to-use model to dissect the key molecular mechanisms governing cardiomyocytic differentiation from adult CPCs.

In this work, an immortalized cell line for modeling mouse Lin^{-}Sca-1^{+} CPCs was specifically generated by means of TERT overexpression. The immortalization did not appear to impair any important cellular property, as iCPC$^{Sca\text{-}1}$ were phenotypically and functionally similar to their primary cell counterparts. Overall, the results indicate that iCPC$^{Sca\text{-}1}$ are representative of the native Sca-1^{+} cardiac population, thus constituting a suitable tool for functional and mechanistic studies in need in the field. Hence, with regard to the heterogeneity and low frequency in the adult heart of described CPCs populations (Figure 6), iCPC$^{Sca\text{-}1}$ comes forth as a uniquely validated off-the-shelf line for *in vitro* high-throughput and bioengineering studies. The latter combined with the remarkable capacity of iCPC$^{Sca\text{-}1}$ to engraft and differentiate *in loco* makes this cell line

a valuable model system to investigate *in vivo* the role of the Sca-1^{+} stem/progenitor cells resident in the adult heart.

Last but not the least, the widespread use of this and other similarly generated cardiac progenitor-subset cell lines will enable the reduction of animal usage and will contribute in a properly standardized manner to the definition of benchmark(s) for stem/progenitor cells from distinct organ systems and from different laboratories.

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Author disclosure statement

No competing financial interests exist.

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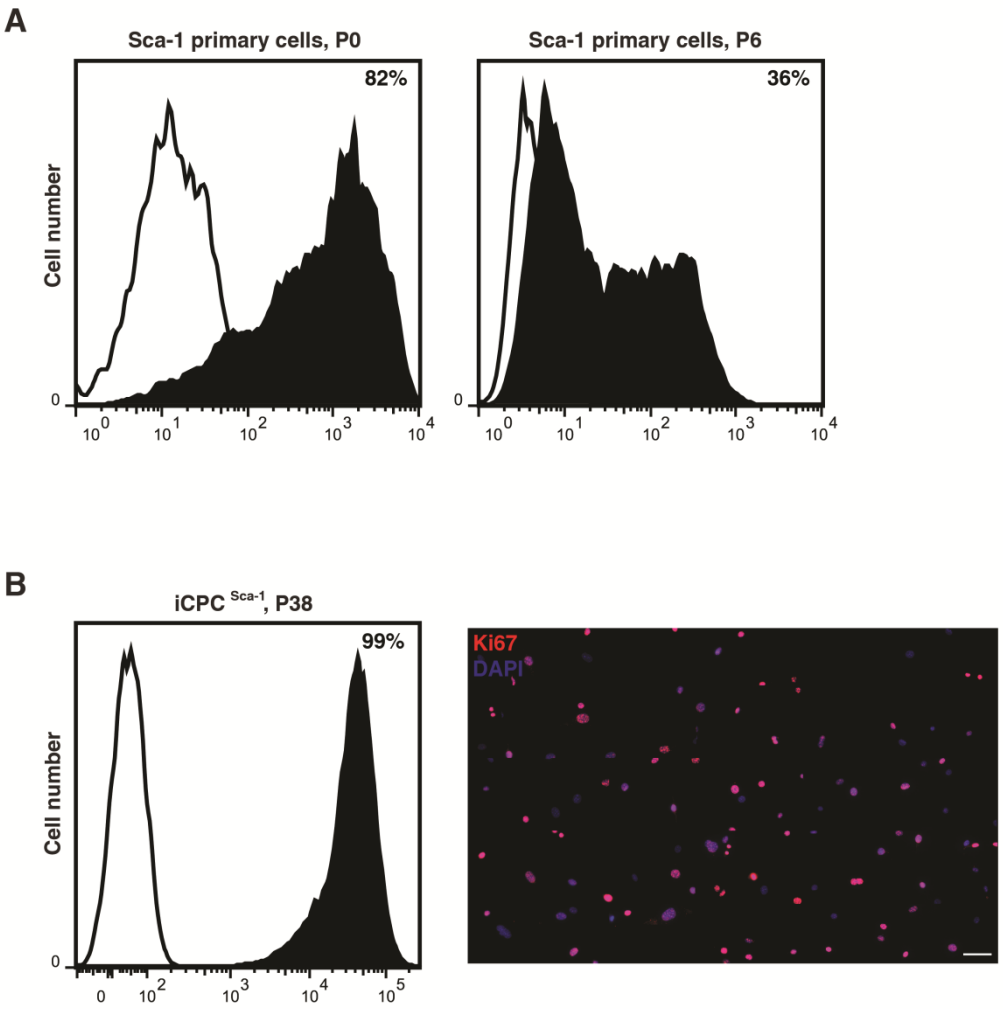
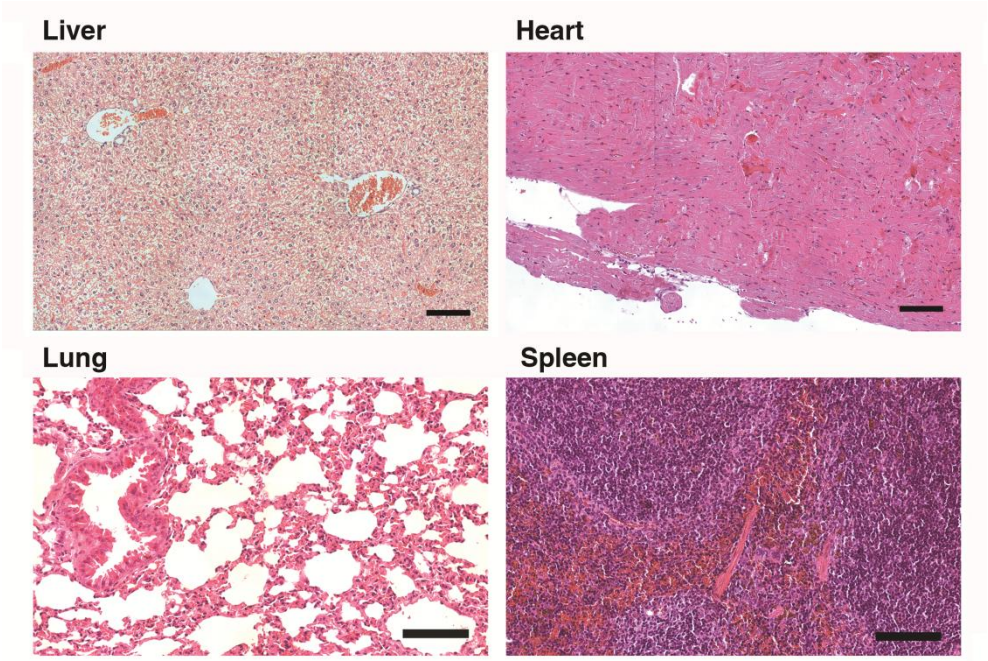
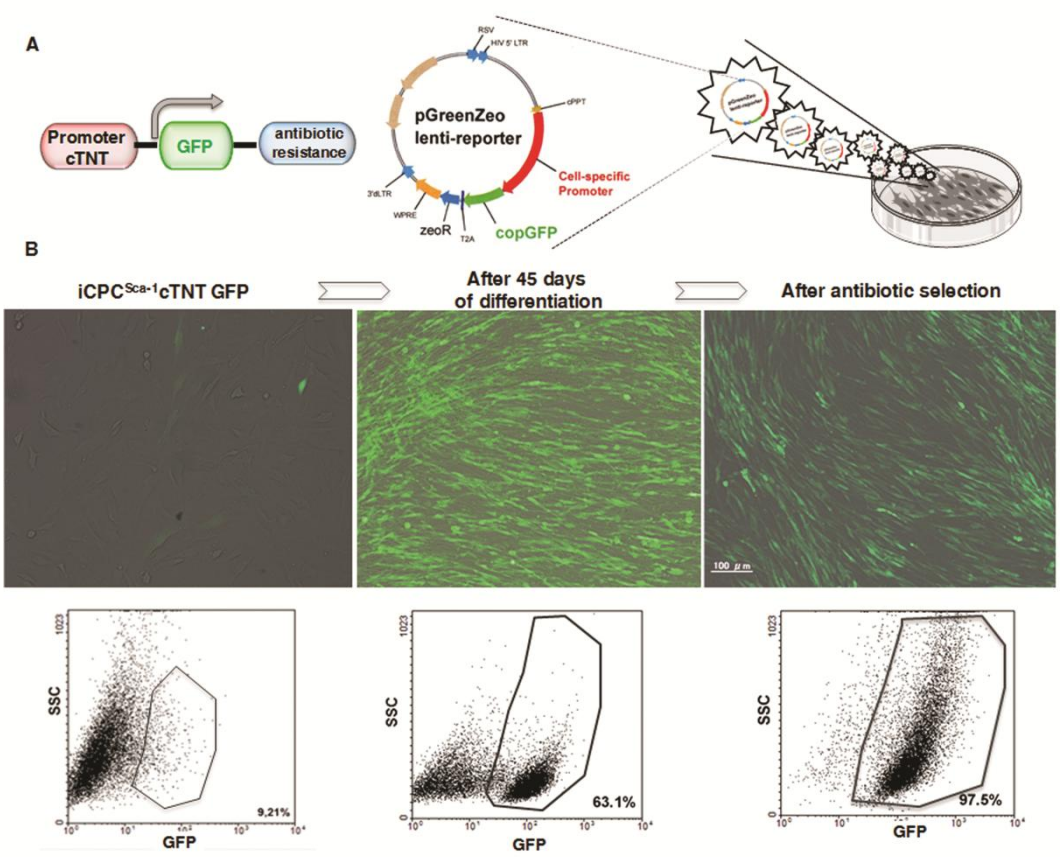
Supplementary information



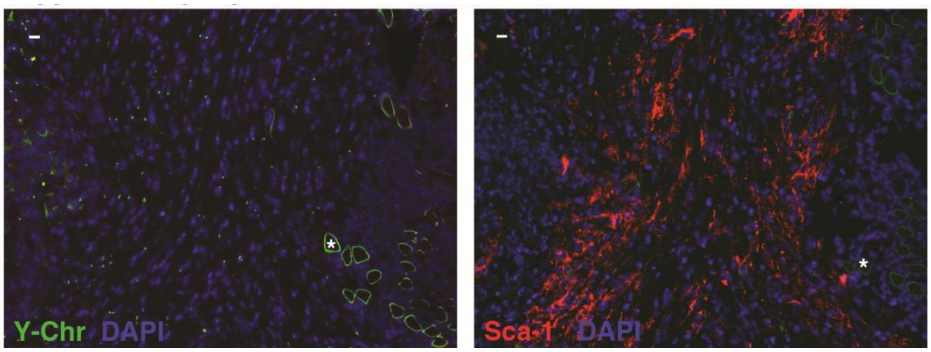
Supplementary Figure S1. iCPC$^{Sca\text{-}1}$ display a stable phenotype in long-term *in vitro culture*. (A) Lin$^-$Sca-1$^+$ primary (non-transfected) CPCs undergo a dramatic decrease in Sca-1 expression following 1 month in culture (P6, 36%) as compared with the high Sca-1 levels after isolation (P0, 82%). (B) iCPC$^{Sca\text{-}1}$ maintain their undifferentiated phenotype even after long-term culture, as evidenced by high Sca-1 expression (P38, 99%) and proliferation rate (P38, Ki67 staining). Scale bar: 20 µm. P, passage number.



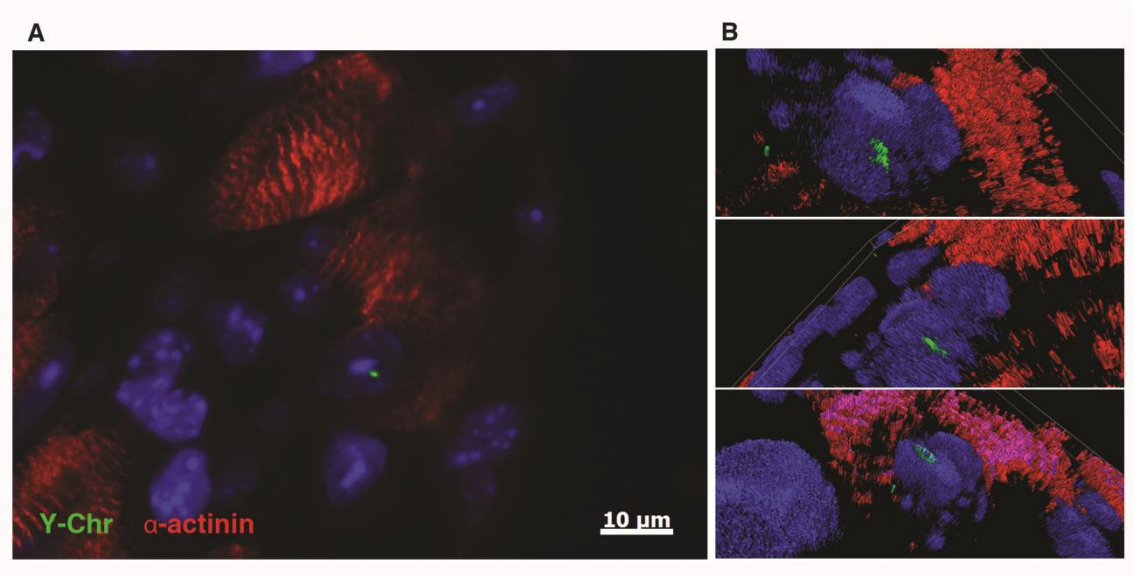
Supplementary Figure S2. iCPC^{Sca-1} tumorigenic behavior was evaluated *in vivo* by injecting subcutaneously 1.0 x 10^6 cells in each flank of the shoulder blades of three C57BL/6 mice. Animals were monitored for 5 months for observation of tumor mass formation in the injected area. After this period, no abnormal tissue was found locally, surrounding the transplanted region, or in the analyzed organs (liver, heart, lung, and spleen). Representative images of hematoxylin and eosin-stained cross-sections of the collected organs demonstrate normal histology. Scale bars: 100 µm.



Supplementary Figure S3. Real-time imaging of GFP under cardiac Troponin promoter indicates that iCPC$^{Sca\text{-}1}$ differentiated into cardiomyocyte-like cells when subjected to the appropriate stimulus. (A) The pGreenZeo lenti-reporter containing the mouse cardiac Troponin (pGreenZeo-cTnT) promoter driving GFP and Zeocin selection (Cambrigde Bioscience) enables the identification and real-time imaging of differentiating cells. (B) iCPC$^{Sca\text{-}1}$ cells transduced with the reporter (iCPC$^{Sca\text{-}1}$ cTnT GFP) were cultured in cardiomyocytic differentiation medium containing TGF-β, and GFP levels were assessed overtime. After 45 days in culture, the expression of cTnT was greatly upregulated, as indicated by the high GFP levels (63%) when compared with the initial culture (9%). Zeocin-selected cTnT-GFP yielded approximately 97% purity. Scale bar: 100 µm.



Supplementary Figure S4. Representative sections of iCPC^{Sca-1} transplanted hearts at 2 weeks after MI were consecutively processed for either FISH for Y-Chr or Sca-1 immunostaining. The majority of grafted cells (Y-Chr^{+}, green) retained an undifferentiated morphology and also Sca-1 (red) expression. The analyzed region is adjacent to the infarcted area, and, thus, remnants of sutures are observed (*). Scale bar: 10 µm.



Supplementary Figure S5. Detailed view of a representative iCPC^{Sca-1} transplanted cell resembling a mature cardiomyocyte at 2 weeks after transplantation. (A) Sarcomeres are highlighted by α-actinin staining (red), and Y-Chr (green) demonstrates the donor cell origin. (B) Three-dimensional rendering of z-stack images further supports the origin of the differentiated cells by showing that the Y-Chr is in the same plane as the cell nuclei. Scale bar: 10 µm.

Supplementary Table S1.

Transcriptional profiling of 20 selected Lin$^-$Sca-1$^+$ Cardiac Progenitor Cell Clones by RT-PCR

Clone	Sca-1	c-kit	Nkx2.5	Mef2c	Isl-1	α-Actinin	Nanog	Oct 3/4
1	+	-	-	+	+	+	+/-	-
2	+	-	-	+	+	-	+/-	-
3	+++	-	-	+/-	+	-/+	-	-
4	-	+	+	+	-	-	+++	-
5	+++	-	-	+	++	+	-	-
6	+++	+	-	+	+++	-	+	-
7	-	+	-	-	-	+	+	-
8	+	-	-	+	+	-	+/-	-
9	+	-	-	+	+	-	+	-
10	-	+	+/-	+	-	-	++	-
11	-	-	+	+	-	-	+	-
12	-	-	-	-	+/-	-	+	-
13	-	+	-	-	+	-	++	-
14	-	+++	+	+	-	-	+/-	-
15	-	-	-	+	-	-	+/-	-
16	-	+	+	-	-	-	-	-
17	-	++	+	+	-	-	++	-
18	++	+/-	+	+	+	-	+	-
19	+	+	+	-	-	+	-	-
20	-	++	+	++	-	+	++	-

According to the densitometry analysis of the gel bands, the expression levels were categorized as follows: (-) not detected, (+/-) low level of expression, (+) moderate level of expression, (++) high level of expression, and (+++) very high level of expression. Clone 3 was selected for a further analysis, while the remaining clones were cryopreserved for future investigation.

Chapter IV

Hes5 specifies cardiac fate in a time-dependent manner

Hes5 specifies cardiac fate in a time-dependent manner

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Summary

Studies in developing embryos and in differentiating mouse embryonic stem cells have unveiled the complex signaling environment that leads to heart formation. Within an interconnected network with other pathways, Notch signaling plays an essential role in specifying cardiac lineages but the exact molecular regulatory mechanisms remain elusive. In this study we identified Hes5 as a mediator of Notch1 in the specification of cardiac lineages. Loss of Hes5 compromised Notch1 activity in cardiogenesis while favoring hematopoietic commitment. Our data demonstrate that Hes5 enhances cardiac fate in a transient temporal window while its sustained activity compromises cardiac maturation. We identified Isl1 and Scl as putative effectors of Hes5 activity in cardiac specification, supporting a tight inter-connection between hematopoietic and cardiac regulatory networks. These findings provide mechanistic insights into how the Notch pathway controls cardiac specification and identify a novel role for Hes5 as a key regulator in the early cardiac molecular program.

Introduction

The heart is the first organ formed in a developing embryo and thus, specification of cardiac lineages occurs very early during embryonic development. Although cardiac morphogenetic events, such as the contribution of first and second heart field progenitors as well as from external sources are well described (Brade et al., 2013), the process of cardiac fate specification is still largely undefined. Cardiac precursors derive from mesodermal cells at the early primitive streak stage and segregate shortly after the migration of prospective hematopoietic progenitors to the yolk sac (Parameswaran and Tam, 1995). Given the ontogenic proximity, it is not surprising that the networks that control heart and vascular/hematopoietic development are tightly inter-connected and mutually antagonistic (Caprioli et al., 2011; Schoenebeck et al., 2007; Simoes et al., 2011).

Differentiating mouse embryonic stem cells (mESCs) recapitulate *in vitro* the molecular events from pre-gastrulation up to cardiomyocyte formation and thus, contribute mechanistic insights into early lineage specification (Doetschman et al., 1985; Kattman et al., 2006; Maltsev et al., 1993; Miller-Hance et al., 1993). Studies in the embryo and in differentiating mESCs have unveiled the complex temporal and spatial crosstalk between Nodal/Activin, Wnt, BMP and Notch signaling pathways. Detailed knowledge of the regulatory signaling network of embryonic heart development will shed light for regenerative strategies, especially, considering that pathways governing heart formation are functional or reactivated in cardiac disease (Freire et al., 2014).

Notch is an evolutionarily conserved signaling pathway involved in a multiplicity of biological processes (*e.g.* cell fate specification, cell differentiation, boundary formation, progenitor cell maintenance, apoptosis and cell proliferation) (Bray, 2006). In mammals, Notch signaling is mediated by the interaction of transmembrane receptors (Notch1-4) and ligands (Jagged1 and 2, and Delta-like1, 3 and 4) expressed on the surface of neighboring cells. The activation of a Notch receptor results in further cleavage of the intracellular domain (NICD) which translocates to the nucleus and binds RBP-J/CSL, resulting in activation of Notch targets, such as members of *Hes* and *Hes-related* (*Hesr*) families (Bray, 2006). *Hes* and *Hesr* genes encode basic helix-loop-

helix (bHLH) transcriptional regulators which mediate Notch functions, such as maintenance of progenitors and binary cell-fate decisions; thereby, controlling the normal timing of differentiation (Kageyama et al., 2007). Notch signaling is crucial for heart morphogenesis (Grego-Bessa et al., 2007; Rones et al., 2000; Rutenberg et al., 2006) and loss-of-function mutations in several Notch members, *e.g. Notch1, Hey1* (also known as *Hesr1*)*, Hey2 (*also known as *Hesr2*)*, Heyl (*also known as *Hesr3*)*, and Hes1* result in severe cardiac phenotypes (Nemir and Pedrazzini, 2008; Rochais et al., 2009). Importantly, the effects of Notch activity are highly dependent on the cellular context. For example, differentiation to cardiomyocytes is enhanced in mESCs deficient in RBP-J (Schroeder et al., 2003) or Notch1 receptor (Nemir et al., 2006) whereas the activation of NICD in mesodermal and hemangioblast populations induces cardiac fate (Chen et al., 2008). Nevertheless, it remains unclear how Notch specifies cardiac fate from mesodermal progenitors, although the mechanism involves in part the regulation of Wnt and BMP signaling (Chen et al., 2008).

In this study we aimed to identify downstream players of the Notch1 receptor involved at the onset of the cardiogenic molecular program. We show that *Hes5*, a member of the *Hes* gene family, is a downstream mediator of Notch1 in the specification of cardiac lineages while repressing the hematopoietic program. Moreover, we unveil the temporal window for cardiac induction in response to Hes5 and identify the putative downstream effectors responsible for its cardiogenic activity. Our results provide mechanistic insights into how the Notch pathway controls cardiac specification and report for the first time a role for Hes5 as a determinant player in the early cardiac molecular network.

Results

Hes5 is a putative Notch1 mediator in cardiac specification

To identify downstream effectors of the Notch1 pathway in cardiac specification we used the AinV/Bry-GFP/NICD1 mES cell line (Cheng et al., 2008) that expresses the Notch1 intracellular domain (NICD1) under the control of a Doxycycline (Dox)-inducible promoter. In addition, this cell line contains the GFP targeted to the *Brachyury* (*Bry*; also known as *T*) *locus*, a pan-mesodermal marker (Showell et al., 2004). The mESCs

were differentiated towards mesoderm as previously described (Kattman et al., 2011) with some modifications (Figure 1A). Within the mesodermal compartment, expression of Flk-1 identifies prospective vascular/hematopoietic and cardiovascular progenitor subsets (Faloon et al., 2000; Kattman et al., 2006). Bry-GFP^{+}Flk-1^{+} mesodermal cells were isolated at day (D) 3.75 of differentiation and NICD1 expression was induced by the addition of Dox. Consistent with the earlier findings (Chen et al., 2008), we observed enhanced cardiac output, as indicated by an increased frequency of cells expressing the contractile filament, cardiac Troponin T (cTnT) (26.9% to 46.3%) upon NICD1 induction (Figure 1B).

Next, as a preliminary screen for Notch effectors involved in cardiac specification, the published dataset (Chen et al., 2008) was organized to identify genes upregulated at 12 hours (h) after NICD4 activation in Bry-GFP^{+}/Flk-1^{+} cells. Among the top ranked genes, *Hes5* and *Heyl,* encoding members of the *Hes* family were identified (Figure 1C). We asked whether these genes would be similarly affected upon Notch1 activation, given that NICD1 overexpression was also shown to induce cardiac fate (Chen et al., 2008).

Assuming that *Hes* and *Hesr* genes are generally responsible for Notch functions, we evaluated the expression of *Hes5, Hes1, Heyl ,Hey1* and *Hey2* at 0, 4, 12, 24, 48 and 96 h after NICD1 induction in D3.75 mesodermal progenitors. As result of NICD1 activation, *Hes5, Heyl* and *Hey2* were upregulated, *Hey1* mRNA levels were moderately increased and *Hes1* expression was not altered (Figure 1D). As previously reported for Notch4 (Chen et al., 2008) our results show particular *Hes5* and *Heyl* upregulation, suggesting that Notch1 and Notch4 play similar regulatory functions. However, while *Heyl* showed sustained upregulation after NICD1 induction, *Hes5* levels were highly increased within 24 h followed by a dramatic decrease (Figure 1D). This gene expression pattern suggests a temporal role for Hes5 as a mediator of Notch1 in the onset of the cardiac molecular program.

Hes5 depletion favors the hematopoietic program

To evaluate whether loss of Hes5 would have an effect on the differentiation of cardiac lineages, its mRNA expression was downregulated in AinV/Bry-GFP/NICD1 mESCs using 2 different short hairpin (sh) RNAs targeting *Hes5* coding sequences (sh1_*Hes5* and

sh2_*Hes5*). NICD1 was induced at D3.75 to promote *Hes5* upregulation and the mRNA levels were analyzed after 24 h to determine the knockdown (KD) efficiency for each shRNA. Transduction with sh1_*Hes5* and sh2_*Hes5* resulted in 70% and 80% of *Hes5* downregulation, respectively when compared to cells transduced with control shRNA targeting *Luciferase* (sh_*Luc*) (Figure 1E).

Cells infected with sh2_*Hes5* were differentiated towards mesoderm-derived lineages to evaluate the effect of *Hes5* downregulation in the emergence of contracting cardiomyocytes. Interestingly, rare contracting areas were observed in *Hes5*-KD cultures at D8 of differentiation. Nevertheless, the emergence of hematopoietic-like cells was enhanced, as demonstrated by morphology and the accumulation of brown-reddish pigmentation indicative of hemoglobinization (Figure 1F and Movie S1). In contrast, control cultures were characterized by a mixture of contracting cardiac cells and hematopoietic cells (Figure 1F and Movie S2). These results suggest that Hes5 acts as a repressor of the hematopoietic program.

Following these observations, mRNA expression levels of cardiac genes (*Tbx5, Gata4, Isl1* and *Myh6*) and hematopoietic regulators (*Scl* (also known as *Tal1*) and *Gata1*) were analyzed at D4.75 and at D8 of differentiation (Figure 1G). *Tbx5, Gata4* and *Isl1* are transcriptional regulators mainly expressed in cardiac progenitors, while *Myh6*, encodes the alpha heavy chain subunit of cardiac myosin that comprises the contractile machinery, indicative of differentiation into cardiomyocytes. *Scl* is essential for specification of the hemogenic program from mesoderm (Endoh et al., 2002; Schlaeger et al., 2005) and *Gata1* expression is required for embryonic and definitive terminal erythroid maturation (Fujiwara et al., 1996). *Hes5*-KD cells had markedly decreased levels (varying from 2.0 up to 12.5-fold differences) of cardiac gene expression, especially *Gata4* at D4.75 and *Isl1* at D8 of differentiation (Figure 1G). In addition, *Scl* mRNA levels were similar to control levels; whereas, *Gata1* expression was significantly increased at D4.75 (4.8-fold increase) and comparable to control levels at D8 (Figure 1G).

These results show that loss of Hes5 favors commitment to the hematopoietic program. Furthermore, the decreased cardiac output, likely the consequence of

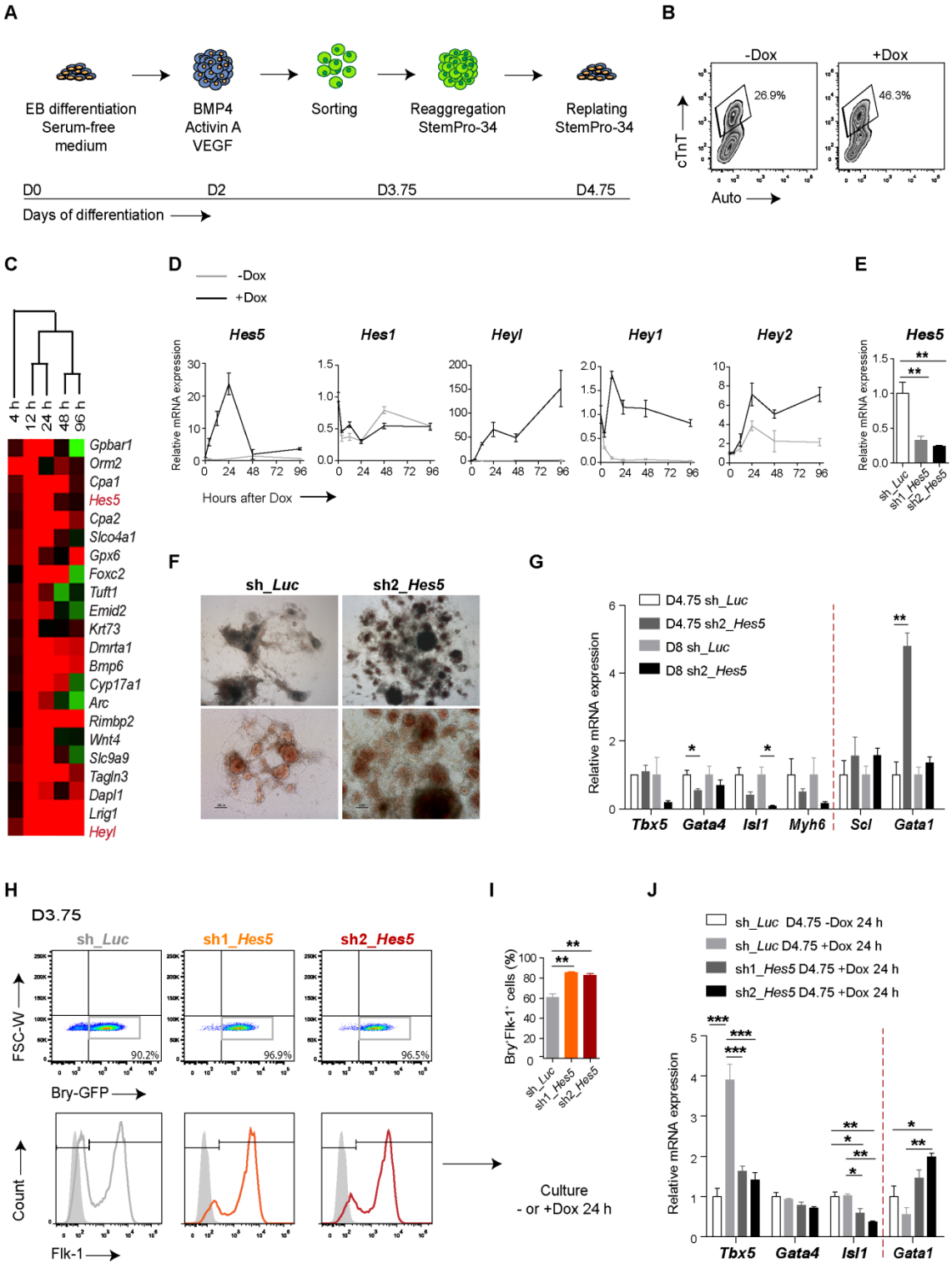


Figure 1. Hes5 is a mediator of Notch1 in mesoderm specification into cardiac and hematopoietic derivatives. (A) Experimental procedure for mesoderm differentiation. Generation of EBs was promoted in serum-free medium. After 48 h cells were reaggregated in the presence of Activin A, BMP4 and VEGF. At D3.75 mesodermal progenitors were sorted, allowed to form aggregates for 24 h and plated in gelatin-coated plates. (B) Flow cytometry analysis shows increase in cTnT^{+} cell percentage after NICD1 activation (+Dox). Auto, autofluorescence.

perturbing the cardiac and hematopoietic regulatory networks, hints a function for Hes5 in cardiogenesis. Taken together, these observations suggest a role for Hes5 as a mediator of Notch1 in the specification of mesodermal progenitors into cardiac and hematopoietic derivatives.

Loss of Hes5 compromises Notch1 effects on preferential cardiac commitment

We next investigated whether loss of Hes5 compromises early events downstream Notch1 activation in Bry$^+$Flk-1$^+$ mesodermal cells. Cells transduced with sh1_*Hes5* and sh2_*Hes5* were differentiated towards mesoderm and the percentage of Bry$^+$ and Flk-1$^+$ cells was analyzed at D3.75 (Figures 1H and 1I). Loss of Hes5 did not affect mesodermal commitment, assessed by the appearance of Bry-GFP-expressing cells (>90%) (Figure 1H), while generation of Flk-1$^+$ mesoderm was enhanced, as shown by the higher frequencies of Bry$^+$Flk-1$^+$ cells (61% to 83-85%) (Figures 1H and 1I).

NICD1 expression was induced (+Dox) in D3.75 Bry$^+$Flk-1$^+$ cells and mRNA levels of *Tbx5*, *Gata4, Isl1* and *Gata1* were analyzed after 24 h (D4.75). After NICD1 induction, control cells (sh_*Luc,* +Dox) showed upregulated *Tbx5* expression, while no changes were observed in *Gata4* and *Isl1* levels when compared to non-induced (sh_*Luc,* -Dox) cells (Figure 1J). In contrast, as result of NICD1 activation *Gata1* was downregulated (Figure 1J). When compared to induced control (sh_*Luc,* +Dox), induced *Hes5*-KD cells (sh1_*Hes5* and sh2_*Hes5,* +Dox) demonstrated significant downregulation of *Tbx5* (2.4

(C) Heat map depicting the top-ranked genes at 12 h after NICD4 activation from the published data set (GSE12425). The data was analyzed by Cluster 3.0 and displayed by TreeView. *Hes5* and *Heyl* (highlighted in red) are members of the Notch pathway. Red and green colors represent increased and decreased expression relatively to -Dox, respectively. (D) Relative mRNA expression of Notch targets (*Hes5, Hes1, Heyl, Hey1* and *Hey2*) at 0, 4, 12, 24, 48 and 96 h after NICD1 activation (+Dox). Expression is normalized to 0h. (E) *Hes5* mRNA levels in cells infected either with sh_*Luc*, sh1_*Hes5* or sh2_*Hes5* at 24 h after NICD1 activation (n=3). Expression is normalized to sh_*Luc*. (F) Brightfield images show preponderance of hematopoietic-like colonies when *Hes5* is depleted (sh2_*Hes5*) relatively to the control (sh_*Luc*). Top: lower and bottom: higher magnification. Scale bar: 50 µm. (G) qRT-PCR results show downregulation of cardiac-related genes (*Tbx5, Gata4, Isl1* and *Myh6*) at D4.75 and D8 and upregulated *Gata1* expression at D4.75 in *Hes5*-KD cells (sh2_*Hes5*), while *Scl* mRNA levels are similar to control (n=3). Expression is normalized to sh_*Luc* at each time point. (H and I) Flow cytometry profile (H) and quantification (I) of D3.75 Bry$^+$Flk-1$^+$ cells demonstrate increased percentage in *Hes5*-KD cultures (sh1_*Hes5* and sh2_*Hes5*) (n=3). (J) qRT-PCR data demonstrate significant *Tbx5* and *Isl1* downregulation and *Gata1* upregulation in *Hes5*-KD cells at 24 h after NICD1 induction (+Dox) (n=3). Expression is normalized to sh_*Luc* -Dox. Data are represented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001. See also Movies S1 and S2 (https://www.dropbox.com/sh/9220b3egbpkhkk4/AADNg0mDURnjgVmpy_50qI4Ka?dl=0).

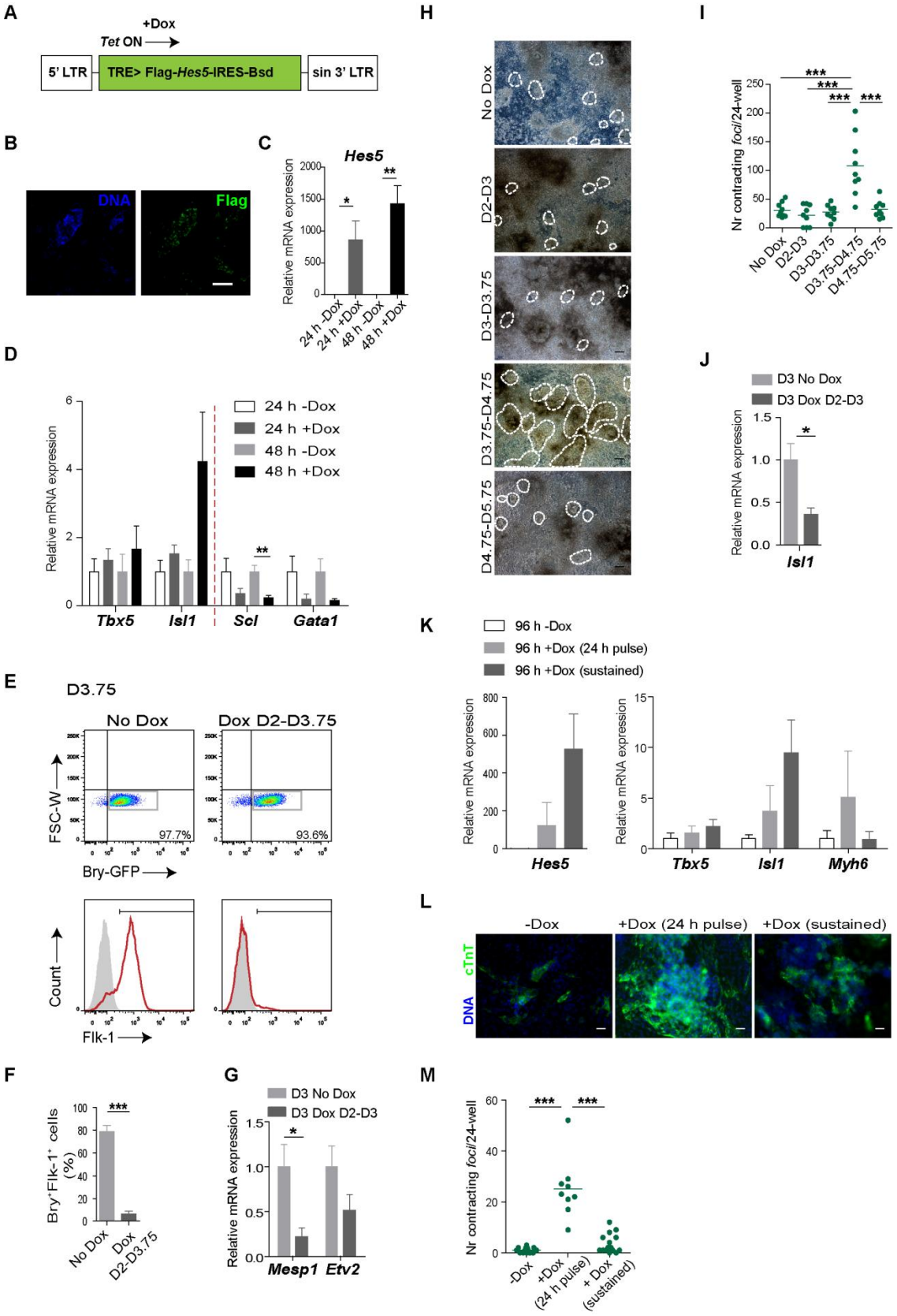
and 2.8-fold) and *Isl1* (1.7 and 2.7-fold) and a moderate decrease of *Gata4* (Figure 1J). As predicted, *Gata1* expression significantly increased compared to sh_*Luc* +Dox condition (2.7 and 3.6-fold), achieving levels superior to the non-induced control cells (sh_*Luc,* -Dox) (Figure 1J). Taken together, our observations indicate that Hes5 mediates Notch1 activity by preferentially directing mesodermal cells towards a cardiac fate, while repressing the hematopoietic program.

Temporal mapping discloses a confined transient window for cardiac specification in response to *Hes5* activation

To dissect the role of Hes5 in the specification of cardiac lineages over hematopoietic fate we specifically induced *Hes5* overexpression in mesodermal progenitors using a Dox-inducible gain-of-function expression system. A lentiviral cassette containing Flag-tagged *Hes5* cDNA driven by a tetracycline response element (TRE) promoter was transduced into reverse tetracycline transactivator (rtTA)-expressing AinV/Bry-GFP mESCs (Figure 2A). In this system, exogenous *Hes5* is expressed only in the presence of Dox. To select Flag-tagged *Hes5* overexpressing clones, transduced mESCs were single-cell sorted and screened for Flag epitope expression (Figure 2B).

Sorted D3.75 Bry^+ mesodermal progenitors were cultured in the presence or absence of Dox. Analyses of mRNA levels after 24 h and 48 h demonstrated effective *Hes5* induction by Dox (Figure 2C). *Hes5* activation promoted upregulation (4.2-fold) of the cardiac gene *Isl1* measured 48 h after induction; however, no substantial changes were observed in the mRNA levels of *Tbx5*. *Scl* and *Gata1* hematopoietic regulators were downregulated (2.8 and 5.0-fold, respectively) 24 h after *Hes5* induction and further decreased (4.2 and 6.7-fold, respectively) at 48 h (Figure 2D). These results confirm that Hes5 regulates cardiac and hematopoietic outputs from mesodermal progenitors.

Given the temporal and context-dependent nature of *Hes* genes (Kageyama et al., 2007), we conducted a temporal mapping to identify the time-window permissive to cardiac induction in response to Hes5 activity. Our *Hes5*-KD studies suggested an effect on the generation of $Bry^+Flk\text{-}1^+$ progenitors; therefore, we investigated whether early induction of *Hes5* would impair the onset of $Flk\text{-}1^+$ mesoderm. To that end, we induced exogenous *Hes5* expression at D2 of differentiation, concomitant with the addition of


A
+Dox
Tet ON
5' LTR
TRE> Flag-Hes5-IRES-Bsd
sin 3' LTR
B
DNA
Flag
C
Hes5
Relative mRNA expression
24 h -Dox
24 h +Dox
48 h -Dox
48 h +Dox
D
Relative mRNA expression
24 h -Dox
24 h +Dox
48 h -Dox
48 h +Dox
Tbx5
Isl1
Scl
Gata1
E
D3.75
No Dox
Dox D2-D3.75
FSC-W
Bry-GFP
97.7%
93.6%
Count
Flk-1
F
Bry+Flk-1+ cells (%)
No Dox
Dox D2-D3.75
G
D3 No Dox
D3 Dox D2-D3
Relative mRNA expression
Mesp1
Etv2
H
No Dox
D2-D3
D3-D3.75
D3.75-D4.75
D4.75-D5.75
I
Nr contracting foci/24-well
No Dox
D2-D3
D3-D3.75
D3.75-D4.75
D4.75-D5.75
J
D3 No Dox
D3 Dox D2-D3
Relative mRNA expression
Isl1
K
96 h -Dox
96 h +Dox (24 h pulse)
96 h +Dox (sustained)
Relative mRNA expression
Hes5
Tbx5
Isl1
Myh6
L
-Dox
+Dox (24 h pulse)
+Dox (sustained)
cTnT
DNA
M
Nr contracting foci/24-well
-Dox
+Dox (24 h pulse)
+ Dox (sustained)

Nodal and BMP signals, and analyzed the effects at D3.75 of differentiation. *Hes5* induction from D2 to D3.75 of differentiation had no effect on the development of Bry^+ cells but significantly compromised the emergence of $Bry^+Flk\text{-}1^+$ progenitors (79% to 6%) (Figures 2E and 2F). Given the role of Mesp1 (Lindsley et al., 2008) and Etv2 (Lee et al., 2008) as inducers of $Flk\text{-}1^+$ mesoderm, their mRNA levels were analyzed at D3 following 24 h of *Hes5* induction (Dox D2-D3). In addition, Mesp1, although firstly described as a master cardiac regulator (Lindsley et al., 2008; Saga et al., 2000), was recently demonstrated to define mesodermal patterning into cardiac, hematopoietic or skeletal myogenic progenitors in a context-dependent manner (Chan et al., 2013). Etv2 promotes commitment towards hemogenic mesoderm (Kataoka et al., 2011; Liu et al., 2012). Early *Hes5* activation significantly compromised the expression of *Mesp1* (4.5-fold) while *Etv2* was slightly decreased (2.0-fold) (Figure 2G); possibly, explaining the diminished frequency of $Bry^+Flk\text{-}1^+$ cells.

Figure 2. A short-pulse of *Hes5* is required for cardiac specification and maturation in a confined temporal window. (A) AinV/Bry-GFP mESCs were transduced with a cassette containing a tetracycline response element (TRE) promoter driving the expression of exogenous Flag-tagged *Hes5* and blasticidin (Bsd) via an IRES element in the presence of Dox. (B) Immunofluorescence demonstrating expression of Flag in mESCs derived from a blasticidin-resistant clone. Scale bar: 50 µm. (C) Efficient *Hes5* overexpression after addition of Dox to D3.75 Bry^+ cells (n=3-4). Expression is normalized to -Dox at each time point. (D) qRT-PCR data show *Isl1* upregulation and *Scl* and *Gata1* downregulation after *Hes5* activation in D3.75 Bry^+ cells, while no substantial changes are detected in *Tbx5* mRNA levels (n=3-4). Expression is normalized to -Dox at each time point. (E and F) Flow cytometry profile (E) and quantification (F) of D3.75 $Bry^+Flk\text{-}1^+$ cells after *Hes5* activation from D2 (Dox D2-D3.75) (n=3). (G) Decreased *Mesp1* and *Etv2* mRNA expression at D3 after *Hes5* induction from D2-D3 (n=4). Expression is normalized to No Dox. (H and I) Non-induced control (No Dox) and cultures treated with Dox at different stages of differentiation (D2-D3, D3-D3.75, D3.75-D4.75 or D4.75-D5.75). Dashed white lines delimiting contracting *foci* (H) and quantification *per* well (3 wells *per* biological triplicate) (I) show higher number of contracting *foci* as result of *Hes5* activation from D3.75-D4.75. Scale bar: 100 µm. (J) qRT-PCR analysis at D3 demonstrates *Isl1* downregulation as result of *Hes5* induction from D2-D3 (n=4). Expression is normalized to No Dox. (K) Relative mRNA quantification shows lower *Hes5* levels after a 24 h pulse (+Dox, 24 h pulse) compared to sustained addition of Dox (+Dox, sustained) at 96 h after induction. *Tbx5* and, particularly, *Isl1* expression levels increase with longer exposure to Dox, while *Myh6* levels upregulate only in 24 h pulse induced cultures (n=3). Expression is normalized to -Dox. (L) Immunofluorescence detecting cTnT protein indicates increased expression in 24 h pulse induced cultures (+Dox, 24 h pulse) when compared to cultures that were not induced (-Dox) or continuously induced (+Dox, sustained). Scale bar: 50 µm. (M) Quantification of the number of contracting *foci per* well (3 wells *per* biological triplicate) demonstrates enhanced cardiomyocytic output as result of a 24 h pulse (+Dox, 24 h pulse) when compared to sustained (+Dox, sustained) or non-exogenous *Hes5* activation (-Dox). Data are represented as mean ± SEM. **p*<0.05; ***p*<0.01; ****p*<0.001.

To dissect the temporal effect of Hes5 on cardiac differentiation, pulses of *Hes5* expression were applied over a time-course of embryoid body (EB) differentiation (D2-D3, D3-D3.75, D3.75-D4.75 and D4.75-D5.75). Interestingly, only an activation pulse between D3.75-D4.75 resulted in a significantly higher cardiac output, as reflected by the number of contracting *foci per* well; whereas, in the other stages of differentiation this number was comparable amongst groups (Figures 2H and 2I). Given that Isl1 cooperates with Mesp1 in mESCs to promote cardiac differentiation (Bondue et al., 2011), its expression was analyzed at D3 of differentiation 24 h after Dox addition (Dox D2-D3). Levels of *Isl1* which positively responded to *Hes5* induction at D3.75 (Figure 2D), decreased significantly as a consequence of early *Hes5* activation (Figure 2J). These observations indicate a confined temporal window permissive for efficient cardiac induction in response to Hes5.

Sustained *Hes5* induction impairs cardiac maturation

Our results demonstrate that NICD1 activation at D3.75 promotes transient *Hes5* upregulation (Figure 1D) and that induction of *Hes5* at this time results in increased cardiac output (Figures 2D, 2H and 2I). These observations indicate a confined temporal window for Hes5 activity in cardiac specification. We then asked whether cardiac specification mediated by Hes5 requires a short pulse-activation, implying that continuous *Hes5* induction would impair cardiac maturation. A 24 h pulse or sustained expression was induced at D3.75 and the mRNA levels of cardiac progenitor genes (*Tbx5, Isl1*) and cardiomyocytes (*Myh6*) were analyzed 96 h after induction. We observed that when *Hes5* was induced (+Dox), either as a 24 h pulse or sustained, *Tbx5* (1.6 and 2.2-fold, respectively) and *Isl1* (3.7 and 9.5-fold, respectively) mRNA levels increased; whereas, *Myh6* was only upregulated when a 24 h pulse was applied (5.1-fold) (Figure 2K). In addition, expression of cTnT protein was more prominent in cells induced for 24 h (+Dox, 24 h pulse) than in sustained (+Dox, sustained) or non-induced conditions (-Dox) (Figure 2L). These results were further supported by a significantly higher number of contracting *foci* after a pulse of *Hes5* (Figure 2M). Taken together, our observations demonstrate that Hes5 activity is required as a transient pulse to permit the initiation and further progression of the cardiac program.

The genetic program regulated by Hes5 is implicated in cardiogenic and hematopoietic processes

To investigate the underlying mechanism by which Hes5 promotes cardiac fate, chromatin immunoprecipitation combined with DNA sequencing (ChIP-Seq) was performed at 48 h after *Hes5* activation in D3.75 Bry^+ cells. Hes5 was recruited to more than 6500 sites, the majority of them located within 1 kb from the transcription start site (TSS) (Figure 3A and Table S1). Most of the target genes were implicated in protein-binding and transcriptional regulation (Figure 3B).

Functional annotation analyses using the Panther classification system revealed that the targets of Hes5 were significantly enriched in cardiovascular and vascular/hematopoietic developmental processes (Figure 3C and Table S2). Of interest, genes related to the Wnt pathway were also present in the significantly enriched categories (Figure 3C and Table S2). The Notch pathway has been demonstrated to specify cardiac fate in part by inhibiting canonical Wnt and activating BMP pathways (Chen et al., 2008). These results suggest that regulation of the Wnt pathway might be, at least partially, mediated by Hes5. We next performed a survey using the Mouse Genome Informatics (MGI) online resource to identify mammalian abnormal phenotypes caused by mutations in genes regulated by Hes5. These analyses showed significant enrichment for genes associated with embryonic (p=3.17x10^{-49}) and extraembryonic (p=1.59x10^{-33}) tissue development, as well as, heart morphology and cardiovascular development (p=1.57x10^{-31} and p=1.01x10^{-29}) (Figure 3D and Table S3). Furthermore, using the Online Mendelian Inheritance in Man (OMIM) disease database we identified genes associated with leukemia at the top of the ranked list (p=1.12x10^{-06}) (Figure 3E and Table S4). Examples of Hes5 targets include: *Scl* (binding site at 3.8 kb from the TSS) and *Gata2* (binding sites at the promoter-TSS and at 1.8 kb from the TSS), both crucial for early hematopoiesis (Pimanda et al., 2007), and *Isl1* and *Gata4* (binding sites at the promoter-TSS), which are essential for cardiac development (Dodou et al., 2004) (Figure 3F).

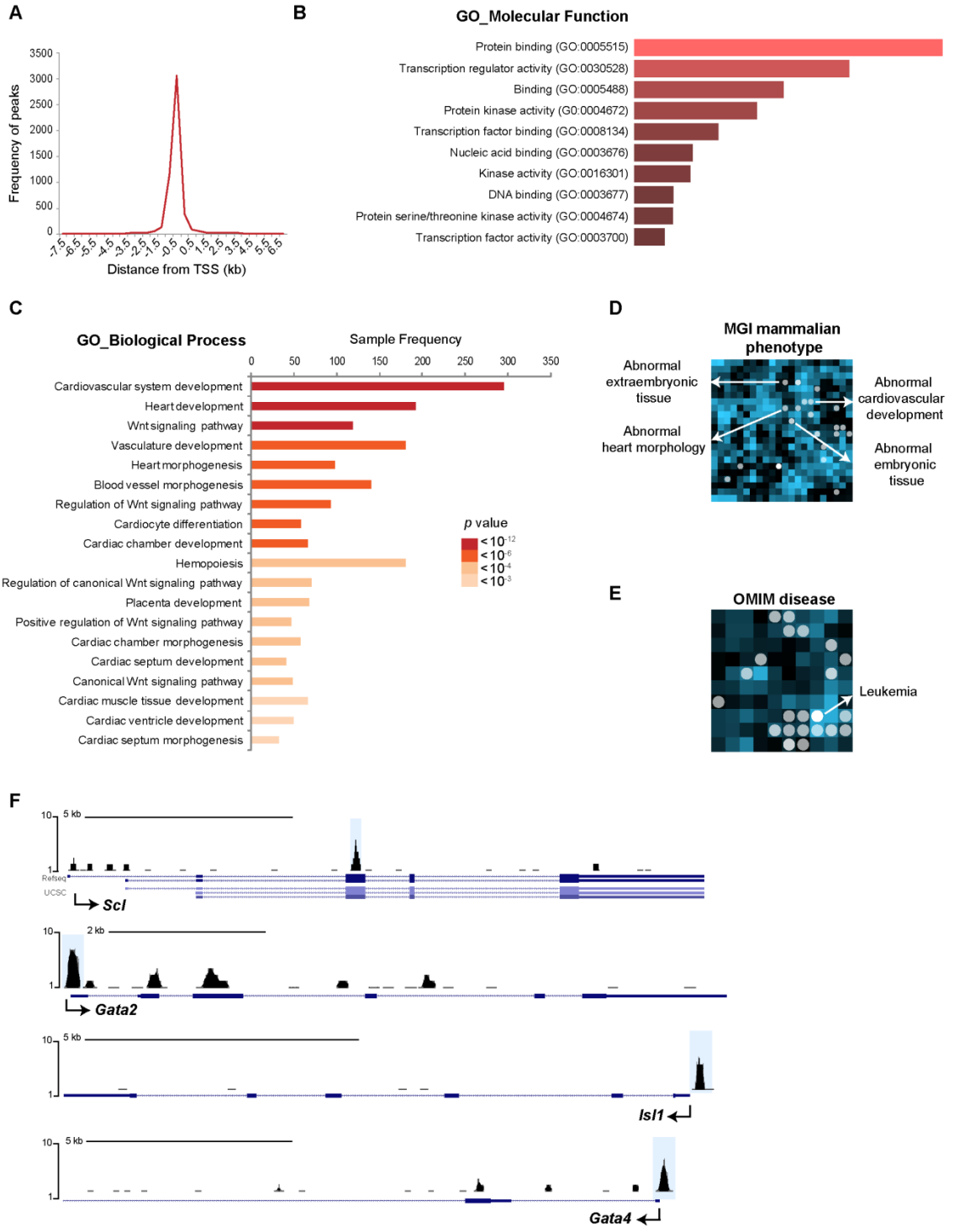


Figure 3. The genetic program regulated by Hes5. (A) Frequency of peaks by ChIP-Seq demonstrates that Hes5 binds within 1 kb of the TSS in the majority of targets. (B) Gene ontology (GO) molecular function analysis of Hes5 target genes using the Enrichr gene list enrichment analysis tool (http://amp.pharm.mssm.edu/Enrichr/index.html) (Chen et al., 2013). (C) Gene ontology (GO) biological process analysis of Hes5 target genes using the Panther classification system database (http://www.geneontology.org/) demonstrates significant enrichment in cardiovascular and vascular/hematopoietic developmental processes.

Hes5 promotes cardiac specification from mesoderm by regulating Isl1 and Scl

In primitive-streak stage embryos Flk-1 expression overlaps with Pdgfrα (also known as CD140a) expression in some cells from the nascent mesoderm. However, in contrast to Flk-1, Pdgfrα is not detected in the blood islands (Kataoka et al., 1997). Similarly, in the mESCs differentiation system, subpopulations based on the expression of these receptors emerge at different times and hold distinct potentials (Sakurai et al., 2006). Presumptive primitive/unpatterned mesodermal progenitors co-express both receptors whereas progenitors that downregulate Pdgfrα are putatively committed to vascular/hematopoietic lineages (Kataoka et al., 2011; Liu et al., 2012). At D3.75 of differentiation the majority of Bry^+ cells co-express both receptors (67%) (Figure 4A).

To understand whether Hes5 plays an instructive role in mesodermal specification, progenitors committed to a hemogenic fate were excluded based on the lack of Pdgfrα expression within the Flk-1^+ compartment. A 24 h pulse of Dox was then given to the population enriched for putative unpatterned mesodermal progenitors (Bry^+Flk-1^+Pdgfrα^+) (Figure 4A). Owing to *Hes5* pulse-activation, cardiac commitment was highly favored, as shown by enhanced emergence of contracting *foci* (Figure 4B and Movies S3 and S4). To address whether Hes5 could play a selective rather than an instructive role, proliferation and apoptosis were assessed after *Hes5* induction. The former was analyzed 24 h after Dox addition with a 5 h pulse of bromodeoxyuridine (BrdU). Apoptosis was measured by combined Annexin V and 7-aminoactinomycin D (7-AAD) staining which allows discrimination of apoptotic, dead and living cells. Aggregates were allowed to adhere 24 h after Dox addition and apoptosis was assessed after 12 h. Non-induced and induced cultures contained similar percentages of BrdU-labeled (9% and 7%, respectively) (Figure 4C) and apoptotic cells (16%) (Figure 4D). These data suggest that Hes5 does not promote the selection of particular subsets of progenitors, but rather acts instructively on unpatterned mesodermal cells.

(D and E) Canvas created by Network2Canvas (http://maayanlab.net/N2C/) representing gene list enrichment analysis using the Mouse Genome Informatics (MGI) (D) and Online Mendelian Inheritance in Man (OMIM) disease (E) online resources. White circles highlight enriched categories. Relevant terms are indicated. (F) Representation of Hes5 binding sites at *Scl*, *Gata2*, *Isl1* and *Gata4 loci*. The blue highlighted boxes indicate the statistically significant Hes5 binding peaks. See also Tables S1, S2, S3 and S4 (https://www.dropbox.com/sh/9220b3egbpkhkk4/AADNg0mDURnjgVmpy_50qI4Ka?dl=0).

Hes and Hesr proteins repress transcription by DNA binding-dependent and independent mechanisms, the latter includes prevention of DNA binding by lineage-specific bHLH activators, such as MyoD or Mash1 (Fischer and Gessler, 2007). Therefore, we asked if Hes5 regulates Scl in a similar manner as Hey1 regulates MyoD, by counteracting the formation of the MyoD/E47 heterodimer (Sun et al., 2001). Our results do not demonstrate protein-protein interactions between Hes5 and Scl (Figure 4E), indicating that Hes5 and Scl are not likely to form an inactive heterodimeric complex with impaired DNA-binding abilities.

We reasoned that the Hes5 targets *Gata4, Isl1, Scl* and *Gata2* would be good candidates to validate in further studies. The quantification of mRNA levels 24 h after *Hes5* induction in the $Bry^{+}Flk\text{-}1^{+}Pdgfr\alpha^{+}$ population showed a significant increase in *Isl1* expression levels (3.9-fold) and a decrease in *Scl* levels (4.3-fold); whereas, no significant differences were detected in *Gata4* and *Gata2* levels (Figure 4F). The alignment of DNA motifs found in the ChIP-Seq peak binding regions allowed us to identify the putative sites at the *Isl1* and *Scl loci* (Figure 4G). Regulation of Isl1 and Scl levels was further confirmed at the protein level with an increased percentage of Isl1-expressing cells (5% to 22%) at 48 h after Dox addition (Figure 4H), while the opposite effect was observed for Scl (16% to 4%) (Figure 4I). Taken together, these findings indicate that Hes5 directly regulates *Isl1* and *Scl* by a DNA binding-dependent mechanism and suggest a role for these genes, as downstream mediators of Hes5 in cardiac specification.

Discussion

Pluripotent stem cells offer the unique advantage of allowing mechanistic studies aimed at the dissection of early lineage specification. Here we identify Hes5 as a novel regulator, downstream of the Notch1 pathway, in the specification of cardiac lineages from mesodermal progenitors. *Hes5* is a member of the *Hes* gene family, which encodes bHLH transcriptional regulators. Hes proteins regulate the maintenance of progenitors and binary cell fate decisions; thereby, controlling the normal timing of differentiation (Kageyama et al., 2007).

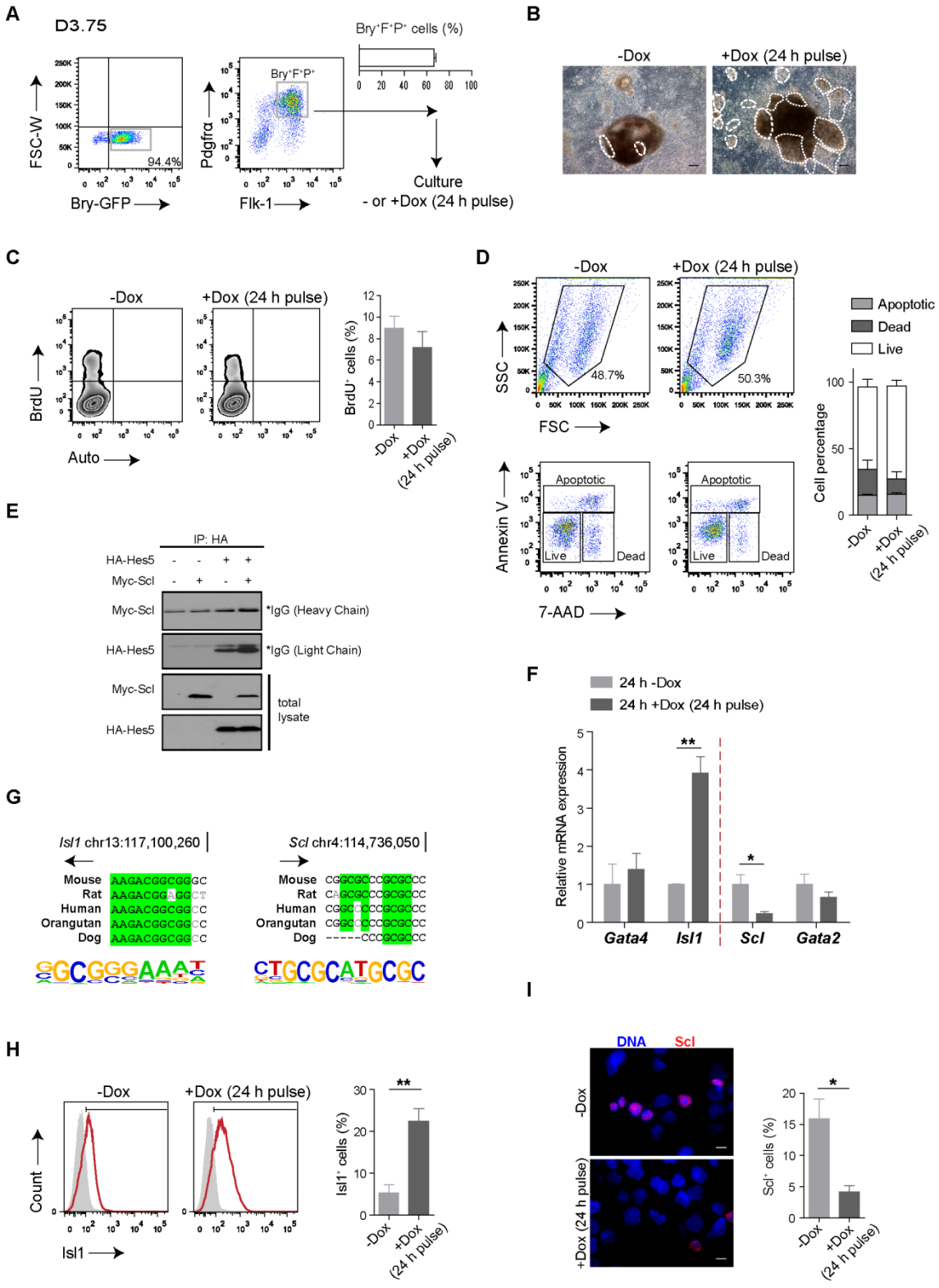


Figure 4. Hes5 regulates Isl1 and Scl levels in primitive mesodermal progenitors. (A) Flow cytometry profile and quantification of Bry$^+$Flk-1$^+$Pdgfrα$^+$ (Bry$^+$F$^+$P$^+$) cells at D3.75 (n=8). (B) Morphological evaluation shows distinct lineage outcomes as result of *Hes5* 24 h pulse induction. Dashed white lines indicate contracting *foci*. Scale bar: 100 µm.

Hes5-null mouse embryos; although, with apparently normal morphology, show premature neuronal differentiation at E10.5 with a more severe phenotype when combined with a *Hes1* mutation (Ohtsuka et al., 1999). Hes5 regulates the specification of glial cell fate at the expense of neurons in the retina (Hojo et al., 2000) and, in combination with Hes1, modulates arterial cell fate of endothelial cells during brain vascular development (Kitagawa et al., 2013). Yet, to date, Hes5 participation in cardiac development has neither been demonstrated nor suggested.

Our results indicate that Hes5 is a mediator of the Notch1 pathway in cardiogenesis, as its loss compromised the effect of Notch1 on preferential cardiac commitment. *Hes5*-null embryos have no apparent cardiovascular phenotype, but to our knowledge, no studies specifically aimed to address heart malformations in *Hes5*-null embryos have been performed. Nevertheless, is also possible that another bHLH protein(s) compensates for the cardiogenic activity of Hes5. Given the *Hes1* expression profile upon NICD1 induction we suggest that Hes5 function in cardiogenesis is likely not compensated by Hes1, as often demonstrated in other organ systems (Kitagawa et al., 2013; Ohtsuka et al., 1999). However, future investigation is needed to confirm this hypothesis.

We demonstrate that *Hes5*-KD favored hematopoietic commitment while cardiac genes were downregulated, supporting previous findings that the onset of cardiogenesis is concomitant with the repression of the hemogenic program. Our results also corroborate published reports demonstrating that inhibition of the Notch

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(C) Flow cytometry profile and quantification of $BrdU^+$ cells (5 h BrdU pulse) demonstrate no differences in cell proliferation after *Hes5* pulse (n=3). Auto, autofluorescence. (D) Flow cytometry profile and quantification of Annexin V/7-AAD stained cells show no differences in cell apoptosis after *Hes5* pulse (n=3). (E) Immunoprecipitation for HA-tagged Hes5 followed by immunoblotting for HA and Myc detection in lysates from HEK-293T cells co-transfected with HA-tagged *Hes5* and Myc-tagged *Scl*. Results show no protein-protein interaction. (F) Quantification of *Gata4, Isl1, Scl* and *Gata2* mRNA levels shows significant *Isl1* upregulation and *Scl* downregulation as result of *Hes5* pulse (n=3). Expression is normalized to -Dox. (G) Putative Hes5 binding sites at *Isl1* and *Scl loci* identified by alignment of DNA motifs found in the ChIP-Seq peak binding regions. Sequences were analyzed and aligned using VectorNTI and ClustalW softwares. (H) Flow cytometry profile and quantification of Isl1-expressing cells demonstrate increased percentage after *Hes5* pulse (n=3). (I) Immunofluorescence and quantification of Scl^+ cells show decreased percentage after *Hes5* pulse (>20 fields *per* biological triplicate). Scale bar: 10 μm. Data are represented as mean ± SEM. **p*<0.05; ***p*<0.01. See also Movies S3 and S4 (https://www.dropbox.com/sh/9220b3egbpkhkk4/AADNg0mDURnjgVmpy_50qI4Ka?dl=0).

pathway is required for primitive erythropoietic specification (Cheng et al., 2008). Interestingly, when *Hes5* levels were downregulated, the emergence of Flk-1^{+} progenitors was enhanced. We cannot precisely determine whether this increase represents a higher contribution to the hematopoietic reservoir or a disruption in the normal timing of differentiation. However, given the role of Hes proteins in repressing premature differentiation, we hypothesize that *Hes5*-KD cells engaged in premature hematopoietic commitment.

We further show that Hes5 enhances cardiac output in a confined transient temporal window. Activation of the gene at early stages; although, not having an impact in Bry expression greatly compromised the emergence of Flk-1^{+} mesodermal derivatives. Likewise, expression of *Mesp1* and *Etv2*, inducers of Flk-1^{+} mesodermal development (Lee et al., 2008; Lindsley et al., 2008), was also compromised as result of early *Hes5* activation. This is in accordance with previous reports that Notch activation in mESCs inhibits the generation of Flk-1^{+} mesodermal progenitors (Schroeder et al., 2006). Interestingly, a specific temporal window (D3.75-D4.75) was permissive for enhanced cardiac induction, implying an instructive role for Hes5 in primitive mesoderm.

Our data show that Hes5 regulates pivotal target genes for normal vascular/hematopoietic and heart development. In addition, the enrichment for genes related to the Wnt signaling suggests that Hes5 might mediate part of the crosstalk between Notch and Wnt pathways, previously shown to control cardiac and primitive erythropoietic specification (Chen et al., 2008; Cheng et al., 2008).

Furthermore, our observations strongly implicate Isl1 and Scl as the potential downstream mediators of Hes5 action. The crucial roles for these genes in cardiac and hematopoietic development have been demonstrated. Homozygous *Isl1*-null mice have several abnormalities in heart development with lethality at E10.5 (Cai et al., 2003), while *Scl* deficient embryos die at E9.5 with a complete absence of yolk sac hematopoiesis (Robb et al., 1995; Shivdasani et al., 1995). Moreover, *Scl*-null embryos show ectopic cardiogenesis in the yolk sac (Van Handel et al., 2012). Hes proteins repress transcription by DNA binding-dependent and independent mechanisms. DNA binding-dependent mechanisms usually involve recruitment of co-factors, such as

Groucho/TLE co-repressors, and independent mechanisms include prevention of DNA binding by lineage-specific bHLH activators, such as MyoD or Mash1 (Fischer and Gessler, 2007). Hes5 does not seem to form an inactive heterodimer complex with Scl but is rather likely to directly regulate its expression. Although Hes proteins usually act as transcriptional repressors, there is evidence for a switch in the function of Hes1, from a repressor to an activator, by dismissal of the Groucho/TLE complex and occupancy by co-activators at *Mash1* promoter during neuronal differentiation (Ju et al., 2004). Normal organ development relies on the precise switching between gene repression and activation. We show that Hes5 tightly regulates *Isl1* in a temporal manner, as *Hes5* activation in early stage of differentiation (D2-D3) downregulated *Isl1* expression whereas induction at D3.75 had a positive effect on *Isl1* levels. Whether this temporal regulation requires recruitment and further dismissal of the Groucho/TLE complex from *Isl1* promoter needs further investigation.

In addition, our results show that cardiac maturation requires *Hes5* withdrawal. These observations are in agreement with reports demonstrating that Hes1-Hes5 maintain neural stem cells in the embryo telencephalon in an undifferentiated state (Ohtsuka et al., 2001) while in cardiac development, Notch activation in a specific cellular-context impairs normal cardiomyocytic maturation (Chau et al., 2006; Chen et al., 2008; Rutenberg et al., 2006; Watanabe et al., 2006). Our results point that one possible explanation for the blockage of maturation is the continuous promotion of high *Isl1* levels by Hes5. Isl1 is expressed transiently by cardiogenic precursors, suggesting that its function might be detrimental for cardiomyocytic differentiation (Cai et al., 2003).

Collectively, the data we present unveil Hes5 as a novel player in the determination of cardiac lineages. Importantly, the onset of cardiogenesis depends on temporal Hes5 regulation in order to trigger the cardiac program and proceed in maturation. Moreover, our data support the existing knowledge of a tight inter-correlation between hematopoietic and cardiac regulatory networks, where Hes5 balances *Isl1* and *Scl* levels to determine cardiac fate (Figure 5). Our results provide mechanistic insights into how the Notch pathway controls cardiac specification and report for the first time a role for Hes5 as a determinant player in the early cardiac molecular network.

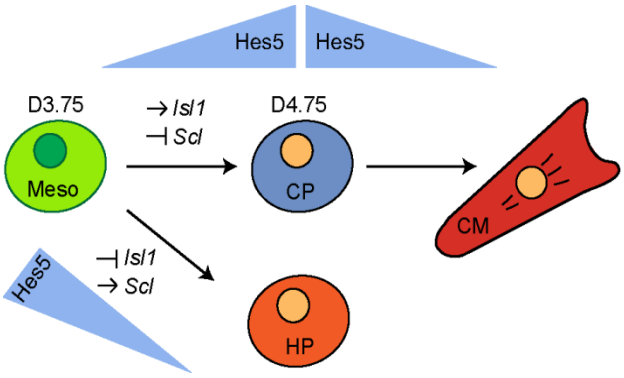

Figure 5. Model portraying the role of Hes5 in cardiac specification. Proposed model for the mechanism underlying cardiac induction mediated by Hes5. At D3.75 of *in vitro* differentiation Hes5 regulates Isl1 and Scl levels in primitive mesodermal progenitors determining cardiac *versus* hematopoietic outcomes. Cardiac induction occurs in a confined temporal window that requires a short pulse activation of *Hes5* to allow cardiac maturation after commitment to the cardiac program.
Meso, Mesodermal progenitor; CP, Cardiac progenitor; HP, Hematopoietic progenitor; CM, Cardiomyocyte; → Activation; ⊣ Repression.

Experimental Procedures

Detailed experimental procedures can be found in the Supplemental Experimental Procedures section.

mESC lines

The AinV/Bry-GFP/NICD1 and AinV/Bry-GFP mESC lines (Cheng et al., 2008) were a kind gift from Dr Gordon M. Keller and Dr Valerie Gouon-Evans.

Cell differentiation

mESCs were differentiated as previously described (Kattman et al., 2011) with some modifications.

shRNA design

shRNA sequences were obtained from the TRC library database (http://www.broadinstitute.org/genome_bio/trc/publicSearchForHairpinsForm.php) and are listed in Table S5.

Generation of *Hes5* overexpressing clones

AinV/Bry-GFP mESCs were infected with a lentiviral pTRE-IRES-Bsd^{R} vector containing Flag-tagged *Hes5* cDNA driven by a tetracycline response element (TRE) promoter.

Cells were cultured under blasticidin selection, single-cell sorted and screened for Flag expression.

Gene expression analysis

RNA was isolated using Trizol reagent and reverse transcribed using PrimeScript RT reagent kit (Takara Bio, Inc.). Gene-specific primers are listed in Table S6.

ChIP-Seq

ChIP-Seq was performed as previously described (Ang et al., 2011) with few modifications.

Data and statistical analysis

Data are represented as mean ± SEM. Statistical significance was determined by unpaired t test and ANOVA as appropriate. $p<0.05$ was considered statistically significant.

ACCESSION NUMBERS

ChIP-Seq data are deposited in NCBI-GEO database under accession number GSE64540.

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Inventory of Supplemental Information

Supplemental Movies and Tables

- **Movie S1, related to Figure 1.** *Hes5*-KD cultures at D8 of differentiation
- **Movie S2, related to Figure 1.** Control cultures at D8 of differentiation
- **Movie S3, related to Figure 4.** Cultures derived from D3.75 $Bry^{+}Flk\text{-}1^{+}Pdgfr\alpha^{+}$ mesodermal progenitors
- **Movie S4, related to Figure 4.** Cultures derived from D3.75 $Bry^{+}Flk\text{-}1^{+}Pdgfr\alpha^{+}$ mesodermal progenitors after *Hes5* pulse
- **Table S1, related to Figure 3.** Targets of Hes5 assessed by ChIP-Seq
- **Table S2, related to Figure 3.** Gene ontology (GO) biological process analysis of Hes5 targets
- **Table S3, related to Figure 3.** Analysis of Hes5 targets using MGI database
- **Table S4, related to Figure 3.** Analysis of Hes5 targets using OMIM disease database

  Supplemental Movies S1-S4 and Tables S1-S4 can be assessed using the link:

  https://www.dropbox.com/sh/9220b3egbpkhkk4/AADNg0mDURnjgVmpy_50qI4Ka?dl=0
- **Table S5, related to Experimental Procedures.** shRNA sequences used for knockdown studies
- **Table S6, related to Experimental Procedures.** Primers used for qRT-PCR

Supplemental Experimental Procedures

Supplemental References

Supplemental Movies and Tables

Movie S1, related to Figure 1. *Hes5*-KD cultures at D8 of differentiation

Absence of contracting *foci* in *Hes5*-KD (sh2_*Hes5*) cultures at D8 of *in vitro* differentiation.

Movie S2, related to Figure 1. Control cultures at D8 of differentiation

Presence of contracting *foci* in control (sh_*Luc*) cultures at D8 of *in vitro* differentiation.

Movie S3, related to Figure 4. Cultures derived from D3.75 Bry$^+$Flk-1$^+$Pdgfrα$^+$ mesodermal progenitors

Presence of rare contracting *foci* in cultures derived from sorted D3.75 Bry$^+$Flk-1$^+$Pdgfrα$^+$ cells that were cultured in the absence of Dox for 1 week.

Movie S4, related to Figure 4. Cultures derived from D3.75 Bry$^+$Flk-1$^+$Pdgfrα$^+$ mesodermal progenitors after *Hes5* pulse

Presence of a high number of contracting *foci* in 24 h pulse induced cultures at 1 week after sorting of D3.75 Bry$^+$Flk-1$^+$Pdgfrα$^+$ cells.

Table S5, related to Experimental Procedures. shRNA sequences used for knockdown studies

shRNA	shRNA sequence
sh_*Luc*	CTTACGCTGAGTACTTCGA
sh1_*Hes5*	CCGTCAGCTACCTGAAACACA
sh2_*Hes5*	GATGCTCAGTCCCAAGGAGAA

Table S6, related to Experimental Procedures. Primers used for qRT-PCR

Gene	Forward	Reverse
Etv2	AGGACTGGGAGCGGAATTTG	TCTTCGTGAGGTAAAGCGGG
Gapdh	CGTCCCGTAGACAAAATGGT	TTGATGGCAACAATCTCCAC
Gata1	TGGGGACCTCAGAACCCTTG	GGCTGCATTTGGGGAAGTG
Gata2	CAGACGACAACCACCACCTTA	CAGTGGCCTGTTAACATTGTGC
Gata4	CCCTACCCAGCCTACATGG	ACATATCGAGATTGGGGTGTCT
Hes1	TCATGGAGAAGAGGCGAAGGGCA	GAGCGCGGCGGTCATCTGC
Hes5	GGAGAAAAACCGACTGCGGA	TGTTTCAGGTAGCTGACGGC
Hey1	GAGAAGCGCCGACGAGACCG	GGCGTGCGCGTCAAAATAACCTTT
Hey2	TGCGTTCCGCTAGGCGACAG	TGAGCTTGTAGCGTGCCCAGG
Heyl	CAGCCCTTCGCAGATGCAA	CCAATCGTCGCAATTCAGAAAG
Isl1	ATGATGGTGGTTTACAGGCTAAC	TCGATGCTACTTCACTGCCAG
Mesp1	GCAGTCGCAGTCGCTCGGTC	CGCTGCTGAAGAGCGGAGATGA
Myh6	GCCCAGTACCTCCGAAAGTC	GCCTTAACATACTCCTCCTTGTC
Scl	CACTAGGCAGTGGGTTCTTTG	GGTGTGAGGACCATCAGAAATCT
Tbx5	GGAGCCTGATTCCAAAGACA	TTCAGCCACAGTTCACGTTC

Supplemental Experimental Procedures

Cell culture and cell differentiation

mESCs 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, 100 U/mL penicillin/streptomycin (Gibco, Life Technologies), 1 mM sodium pyruvate, 2 mM L-glutamine (Gibco, Life Technologies), 0.1 mM β-mercaptoethanol and 1000 U/ml Leukemia inhibitory factor (LIF) (Esgro, Millipore). Mesoderm differentiation was induced as previously described (Gadue et al., 2006; Kattman et al.,2011) with few modifications. Briefly, at day (D) 0 of differentiation mESCs were dissociated with TrypLE Express (Gibco, Life Technologies) and cultured at 1-2x10^5 cell/ml in serum-free differentiation medium that consisted of 75% Iscove's Modified Dulbecco's Medium (IMDM) and 25% Ham's F12 medium (HyClone) supplemented with 0.5x of both N-2 and B-27 (Gibco, Life Technologies), 0.05% bovine serum albumin (BSA), penicillin/streptomycin, 2 mM L-glutamine, 4.5×10^{-4} M 1-thioglycerol (Sigma) and 50 µg/mL ascorbic acid in ultra-low attachment 6-well plates (Corning) for 48 h to allow formation of embryoid bodies (EBs). After this period EBs were collected by centrifugation, dissociated by TrypLE Express and plated at 1.5-4x10^5 cells/ml in the previous medium 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). At D3.75 of differentiation 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), 4.5x10^{-4} M 1-thioglycerol and 0.5 mM ascorbic acid, at 2x10^5 cells/ml in ultra-low-attachment 24-well plates (Corning), as previously described (Kattman et al., 2006). After 24 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. Doxycycline (Dox) (1 µg/mL; Sigma) was added to the medium at indicated time points. Cultures were monitored for cell contraction under the light microscope and photographs and movies were acquired using Leica DMI4000 (Leica) or Axiovert 200 (Zeiss) microscopes.

Flow activated cell sorting

D3.75 cells were dissociated with TrypLE Express and directly sorted based on the expression of GFP-Bry, or incubated with phycoerythrin (PE)-conjugated anti-Flk-1 (Avas 12α1, BD Pharmingen) alone or in combination with allophycocyanin (APC)-conjugated Pdgfrα/CD140a (APA5, BioLegend) at 1:100 dilution for 20 minutes (min)

on ice. Cells were washed twice and resuspended in phosphate buffered saline (PBS) containing 2% FBS, 25 mM Hepes, 1 mM EDTA and sorted using Mo-Flo (DakoCytomation) or FACS Aria (BD Biosciences) cell sorters.

shRNA design

shRNA sequences were obtained from the TRC library database (http://www.broadinstitute.org/genome_bio/trc/publicSearchForHairpinsForm.php). shRNA oligos were synthesized by Integrated DNA Technologies, annealed, and cloned into AgeI/EcoRI sites of the lentiviral-based shRNA expression vector pLKO.1 Blasticidin (Bsd^R) (Addgene) (Moffat et al., 2006) according to the supplier's protocol. All shRNA constructs were confirmed by sequencing. Sequences are listed in Table S5.

Lentivirus production and mESCs transduction

Lentiviral-based shRNA vectors and pCMV-dR8.2 (packaging) and pCMV-VSVG (envelope) plasmids were co-transfected into HEK-293T cells using the calcium phosphate method (Chen and Okayama, 1987). In summary, HEK-293T cells were grown to 70-80% confluency in DMEM with 10% FBS. DNA was mixed with $CaCl_2$ and slowly added dropwise to 2x BES buffered saline (Sigma), while subjected to bubble air caused by continuous pipetting, and incubated for 15 min. The calcium phosphate-DNA complexes were added dropwise onto cells that were kept at 37ºC overnight and then transferred into a 32.5ºC incubator. Viral supernatants were harvested after 36, 48 and 72 h, filtered (0.45 μm) and concentrated with Amicon ultra centrifugal filter units (Millipore). mESCs were incubated overnight with virus in medium supplemented with polybrene (8 μg/ml, Sigma) and cultured in fresh medium for 4 days. After this period, cells were cultured in medium supplemented with 5 μg/mL blasticidin for additional 4 days.

Gene expression analysis

RNA was extracted using Trizol reagent and reverse transcribed using PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions. Quantitative RT-PCR was performed using iQ Sybr Green Supermix (Bio-Rad) and gene-specific primers. Reactions were carried out in triplicate on the iCycler iQ5 Real-Time PCR system (Bio-Rad). Relative gene expression was normalized according to

glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression. Gene-specific primers are listed in Table S6.

Flow cytometry

For cardiac Troponin T (cTnT) staining, cells were dissociated with TrypLE Express and fixed with 4% paraformaldehyde (PFA) for 30 min and then permeabilized with PBS with 10% fetal calf serum and 0.1% saponin for 10 min. After two washes, cells were incubated with unconjugated mouse anti-cardiac Troponin T (13-11, NeoMarkers/Thermo Scientific) at 1:100 dilution for 30 min at room temperature (RT). Cells were then washed and stained with PE-conjugated goat anti-mouse secondary antibody (Invitrogen) at 1:200 dilution for 30 min at RT. Lastly, cells were washed twice with saponin containing buffer and once in PBS with 3% FBS. For Isl1 detection, cells were dissociated with TrypLE Express and stained using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Briefly, cells were fixed and permeabilized in the Fixation/Permeabilization solution, prepared according to manufacturer's instructions, for 45 min at RT in the dark. Cells were then washed in permeabilization buffer and incubated with PE-conjugated anti-Isl1 (Q11-465, BD Pharmingen) at 1:100 dilution at RT for 60 min. Cells were washed once in permeabilization buffer and then once in PBS with 3% FBS. Flow cytometry acquisition was performed on a 5-laser LSRII or FACS Canto II (BD Biosciences) and analyzed using FlowJo software.

Immunofluorescence

For Flag and cTnT detection, cells were fixed with 4% PFA for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Blocking for unspecific staining was achieved using PBS containing 4% FBS and 1% BSA for 60 min and followed by incubation with mouse anti-Flag (M2, Sigma) at 1:100 dilution or mouse anti-cardiac Troponin T (13-11, NeoMarkers/Thermo Scientific) at 1:500 dilution for 120 min. Cells were then incubated with anti-mouse 488 for 60 min. For Scl staining, cells were dissociated, cytospun into slides and fixed with cold methanol for 2 min. After permeabilization with 1% Triton X-100 in PBS for 5 min, cells were blocked for 60 min with PBS buffer containing 4% FBS and 1% BSA. Incubation with goat anti-Scl (C21, Santa Cruz (sc-12984X)) at 1:10000 dilution was carried out for 90 min and followed by incubation

with secondary antibody donkey anti-goat 568 for 45 min. For nuclei staining, samples were incubated with 4,6-diamidino-2-phenylindole (DAPI) at 0.5 µg/ml or mounted in Vectashield with DAPI (Vector Lab). Images were acquired using a Leica DMI4000 (Leica) or a Axiovert 200 (Zeiss) microscope.

ChIP-Seq sample preparation and analysis

Sample preparation for ChIP-Seq was performed as previously described (Ang et al., 2011) with few modifications. Briefly, 48 h after Dox treatment $10x10^6$ cells were dissociated with TrypLE Express and fixed with 11% formaldehyde in medium for 20 min with rotation. The fixation reaction was stopped by adding 125 mM glycine solution and rotating the mixture for 5 min at RT. Cells were then quick frozen and stored at -80°C. Samples were thawed and lysed first in lysis buffer I and then in lysis buffer II. The resulting nuclear pellets were resuspended in lysis buffer III. Sonication was performed using the Covaris sonication instrument using the following settings: Time: 10 min, Duty cycle: 5%, Intensity: 4, Cycles *per* burst: 200, Temperature: 4°C. ChIP pull down was performed using Protein G Dynabeads conjugated to anti-Flag antibody (M2, Sigma) overnight at 4°C. Cells without Dox were used as negative control. DNA library preparation was done using the NEB ChIP-Seq sample preparation kit. The libraries were run on HiSeq 2000 machines at the genomics shared resource facility at Icahn School of Medicine at Mount Sinai. ChIP-Seq analysis was done using the raw fastq files. The raw reads were mapped using bowtie software (v1.0). To determine significant binding peaks MACS (v1.4) analysis software was used. The gene annotation information was derived from the peaks files using the Homer software. The visualization of the ChIP-Seq data was done using UCSC genome browser.

Cell proliferation and cell apoptosis analyses

Cell proliferation was assessed by bromodeoxyuridine (BrdU) incorporation. Cells were incubated with 10 µM BrdU for 5 h and then dissociated with TrypLE Express and fixed with 1% PFA for 20 min at 4ºC. Next, samples were permeabilized with 0.2% Triton X-100 in PBS for 10 min at 4ºC. Washes were carried out in PBS buffer with 2% FBS and 0.1% saponin and followed by incubation in 2M HCl for 20 min. After washing, unspecific staining was blocked with PBS containing 5% normal goat serum (NGS) and

0.1% saponin for 20 min. V450-conjugated BrdU antibody (BD Biosciences) was added for 30 min at RT diluted 1:25 in PBS containing 1% NGS and 0.1% saponin. Apoptosis was assessed using the Annexin V detection kit (eBioscience) according to the manufacturer's protocol. In summary, cells were gently dissociated with StemPro Accutase Cell Dissociation Reagent (Gibco) for 5 min, washed in PBS and then in Binding Buffer provided by the kit. V450-conjugated Annexin V was incubated at 1:20 dilution for 15 min at RT. After washes in Binding Buffer, samples were incubated with 7-Aminoactinomycin D (7-AAD) viability staining solution and acquired in the FACS Canto II (BD Biosciences). Analyses were performed using FlowJo software.

Immunoprecipitation and immunoblotting

HEK-293T cells were cultured in DMEM supplemented with 10% FBS. Cells were transfected with pSIN-EF2-Myc-*Scl* and pSIN-EF2-HA-*Hes5* vectors using the Lipofectamine 2000 transfection reagent (Invitrogen). Immunoprecipitation and immunoblotting were performed as described (Hu et al., 2004; Lee et al., 2007; Lee et al., 2009). Briefly, cells were lysed 48 h after transfection in RIPA-B buffer (20 mM Na_2HPO_4 [pH 7.4], 150 mM NaCl, 1% Triton X-100) in the presence of protease inhibitors (3 µg/ml aprotinin, 750 µg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF and 2 mM sodium orthovanadate) and incubated on ice for 1 h followed by centrifugation to remove cell debris. Samples were incubated with anti-HA antibody (12CA5, Roche) overnight at 4ºC followed by incubation with protein G agarose (Roche) for 2-3 h at 4ºC. After five-time washes in ice-cold RIPA-B buffer with protease inhibitors, the immunoprecipitated complexes were resolved by SDS-PAGE and transferred to PVDF membranes (Bio-Rad) for immunoblotting. Membranes were blocked with TBST buffer (10 mM Tris-HCl (pH 7.9), 150 mM NaCl, and 0.05% Tween 20) containing 5% skim milk for 1 h and incubated with anti-Myc (9E10, Roche) or anti-HA (12CA5, Roche) antibodies at 1:3000 dilution overnight at 4ºC. After 3 washes with TBST, samples were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, washed three times and detected either by ECL or ECL Plus (GE Healthcare).

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Chapter V

A role for Hes5 in adult CPCs

A role for Hes5 in adult CPCs

Short Report

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Abstract

Adult cardiac progenitor cells (CPCs) hold promise as candidates for cell-based therapies aiming at improvement of heart function. However, to date, the mechanisms that trigger the activation of these cells in cardiac homeostasis and disease are still elusive. Knowledge gathered from the embryonic development provides valuable information on the regulators that determine early cell-fate decision. These factors may constitute key candidates for the modulation of cardiogenic processes in endogenous CPCs. We have recently demonstrated a function for Hes5 in promoting cardiac fate from embryonic stem cells (ESCs)-derived mesodermal progenitors in a discrete time window. However a role in the regulation of cardiogenic processes in adult CPCs remains unexplored. In the herein work we show that *Hes5* overexpression in the iCPC$^{Sca\text{-}1}$ cell-line, a representative model system of endogenous CPCs, promoted the upregulation of cardiomyocytic structural genes, likely by inducing transient *Isl1* downregulation. Our data supports a role for Hes5 as a key candidate to enhance cardiomyocytic differentiation in endogenous adult CPCs.

Introduction

In the last years cardiovascular research has been stricken by the identification of a number of heart resident adult CPCs reported to give rise to cardiomyocytes (CMs) and vasculature forming-cells [1-12]. However, the molecular signature and developmental origin of CPCs as well as the role in aging and response to injury are still largely elusive. We have recently been able to generate a cell line of heart-derived $Lin^{-}Sca\text{-}1^{+}$ progenitors specifically immortalized by overexpression of the murine telomerase catalytic subunit (mTERT), and therefore named $iCPC^{Sca\text{-}1}$ [13]. $iCPC^{Sca\text{-}1}$ cell line preserves the hallmarks of the primary cell counterparts while maintaining a stable phenotype in long-term culture, thus representing a unique *in vitro* tool to study biological relevant features related to $Lin^{-}Sca\text{-}1^{+}$ adult CPCs. In resemblance to its freshly isolated counterparts this cell line demonstrates limited ability to differentiate into cardiomyocyte-like cells both *in vitro* and *in vivo*. Thus, the identification of cardiogenic master regulators that can enhance cardiomyogenic differentiation from these cells is of great interest.

We have identified a novel function for Hes5 in the specification of cardiac fate from embryonic stem cells (ESCs)-derived mesodermal progenitors (Freire, AG et al, under peer review). Yet, the role of Hes5 in the regulation of cardiogenic processes in the adult heart, and namely in adult CPCs, has not been unveiled. *Hes* genes encode basic helix-loop-helix (bHLH) transcriptional regulators which are commonly effectors of the Notch signaling [14]. The expression of several Notch pathway members is crucial for heart morphogenesis [15-17] and decreases during postnatal life [18]. Interestingly, Notch pathway reactivation has been demonstrated in response to cardiac stress in association to pro-survival and repair processes [19], including prevention of apoptosis in cardiomyocytes and promotion of neovascularization [18, 20, 21]. Importantly, activated Notch promotes expansion of $Sca\text{-}1^{+}$ CPCs [22] and induces myocytic differentiation from $c\text{-}Kit^{+}$ CPCs [23]. Given Hes5 participation in cardiogenesis and the need to drive efficient cardiomyocytic differentiation in adult CPCs, we proposed to validate Hes5 as a factor to enhance cardiomyocytic differentiation using the $iCPC^{Sca\text{-}1}$ cell line as a model. Data on the role of Hes5 in the regulation of proliferation and

differentiation in iCPC^{Sca-1} is shortly reported thereby opening the way towards elucidation of the role of Hes5 in the adult CPCs context.

Results and Discussion

To examine the role of Hes5 in adult cardiac progenitors, *Hes5* overexpression was promoted in the cell line we have previously validated as a model of Lin$^-$Sca-1$^+$ heart-resident progenitors (iCPC^{Sca-1}). *Hes5* upregulation was observed as early as 24 hours (h) after transfection (Figure 1Ai). After 10 days *Hes5* expression was maintained in stable transfected cells following blasticidin selection (Figure 1Aii). Conversely, control iCPC^{Sca-1} cells transfected with the empty vector (Mock) did not express *Hes5* (Figure 1Ai and 1Aii).

Activation of Notch induces proliferation in immature cardiomyocytes [24] and cell cycle reentry of quiescent cardiomyocytes [25]. Moreover, Hes5 and Hes1 were shown to maintain proliferating neural stem cells in the embryo telencephalon [26]. Therefore, we evaluated whether *Hes5* overexpression induces proliferation in CPCs by assessing the percentage of Ki67-expressing cells at 48 h after transient transfection. The percentage of Ki67$^+$ cells was similar in Mock and *Hes5*-transfected cells (72.2% and 68.6%, respectively) (Figure 1B), suggesting that *Hes5* overexpression has no effect in adult CPCs proliferation.

To assess if *Hes5* overexpression promotes cardiomyocytic differentiation in adult CPCs, the mRNA levels of *Gata4, Isl1, Myh6* and *Myh7* were analyzed at 10 days after transfection in blasticidin-resistant cells growing in basal conditions. Isl1 and Gata4 are early transcriptional regulators in the cardiac program, whereas the mature contractile myofilaments Myh6 (also known as α-Mhc) and Myh7 (also known as β-Mhc) associate with the differentiation into cardiomyocytes. We had previously shown that although in basal conditions iCPC^{Sca-1} expressed *Isl1* and *Gata4* and lacked transcripts for the contractile myofilaments, the cells were capable to upregulate *Myh7* when stimulated in cardiomyocytic differentiation medium [13]. Remarkably, in this work we observed that while *Gata4* and *Isl1* mRNA levels were comparable to control levels at 10 days after transfection, *Myh6* (2-fold) and, significantly, *Myh7* (12-fold) were upregulated following *Hes5* overexpression (Figure 1C). These data indicate that Hes5 is capable of

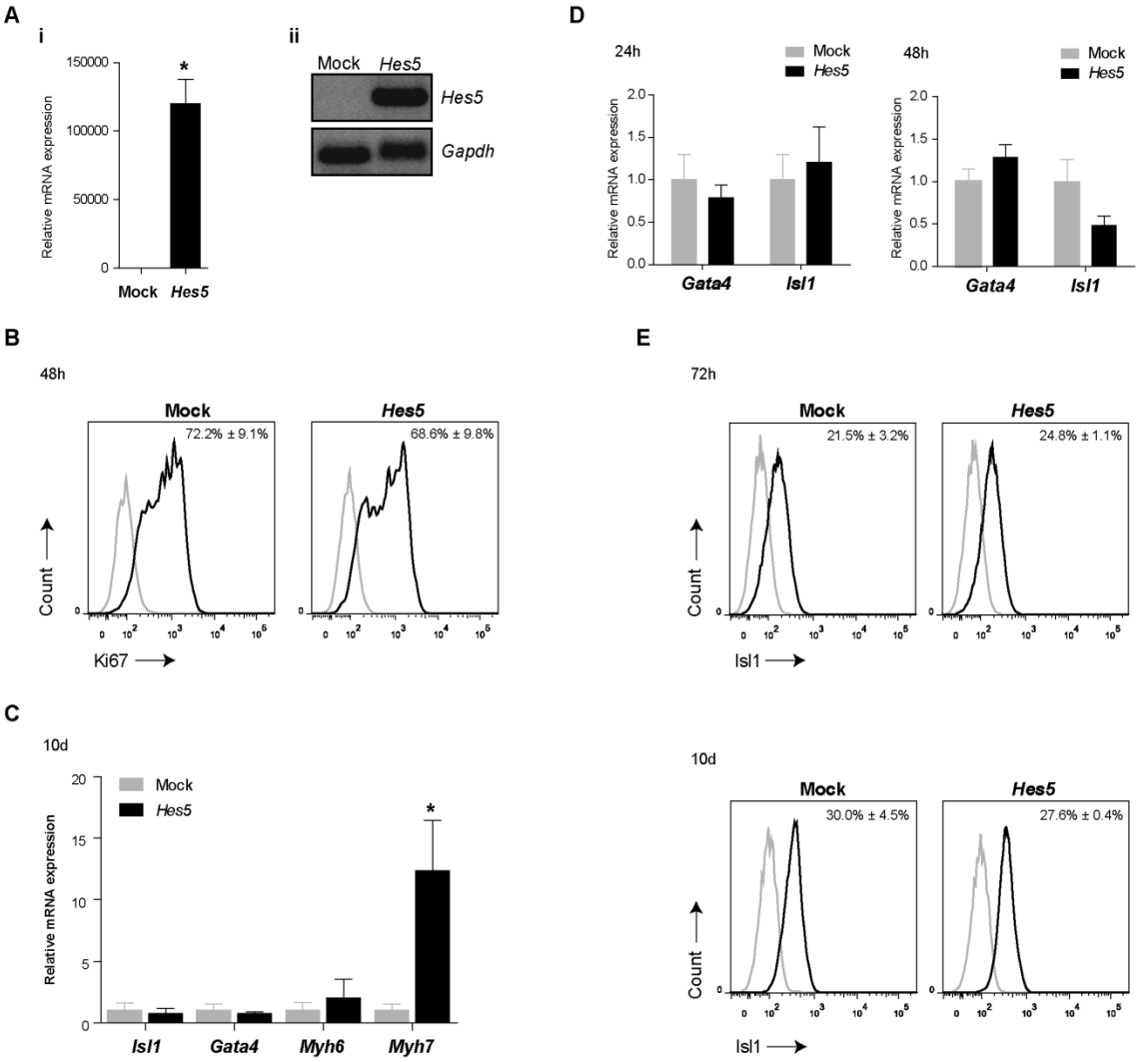

Figure 1. *Hes5*-transfected iCPC$^{Sca\text{-}1}$ engage cardiomyocytic differentiation likely in response to transient *Isl1* downregulation. (A) Quantitative RT-PCR results show efficient *Hes5* overexpression at 24 hours (h) after transfection. Gene expression is normalized to Mock (i). After selection for 4 days in blasticidin-supplemented medium, stable *Hes5*-transfected cells were obtained. Cells transfected with empty vector (Mock) do not express Hes5 (ii). (B) Flow cytometry analysis demonstrates similar percentage of Ki67-expressing cells at 48 h after transfection, indicating no alteration in iCPC$^{Sca\text{-}1}$ proliferation following *Hes5* overexpression (n=2). (C) Relative mRNA expression demonstrates modest and significant upregulation of *Myh6* and *Myh7*, respectively, in blasticidin-resistant cells expressing *Hes5*, while *Gata4* and *Isl1* levels are similar to control levels (Mock) at 10 days (d) after transfection (n=3). Gene expression is normalized to Mock. (D) Quantitative RT-PCR data demonstrate *Gata4* and *Isl1* mRNA levels at 24 and 48 h after transfection. Results show decreased *Isl1* mRNA levels at 48 h after transfection with exogenous *Hes5* (n=3-6). Gene expression is normalized to Mock. (E) Flow cytometry analysis at 72 h (n=3) and 10 d (n=2) after transfection shows similar Isl1^{+} cell percentage in Mock and *Hes5*-transfected iCPC$^{Sca\text{-}1}$ populations. Data are represented as mean ± SEM. **p*<0.05.

triggering the cardiomyocytic program in iCPC$^{Sca\text{-}1}$, and importantly, in the absence of additional stimulation from culture medium components.

Next, we aimed to understand the early events after *Hes5* overexpression that could account for the effect on iCPC$^{Sca\text{-}1}$ differentiation. Our previous work identified *Isl1* and *Gata4* as genes bound and putatively regulated by Hes5 in ESCs-derived mesodermal progenitors. Moreover, the results indicated that Hes5 tightly regulates *Isl1* expression, promoting its significant downregulation or upregulation in a time-dependent manner, suggesting Isl1 as a downstream effector of Hes5 during specification of cardiac fate (Freire, AG et al, under peer review). In light of these results, we questioned whether Hes5 regulates the same genes also in the adult CPCs context. *Isl1* and *Gata4* mRNA expression was evaluated 24 and 48 h after transfection. No considerable differences were observed for both genes after 24 h. Interestingly, *Isl1* mRNA levels were decreased 48 h upon *Hes5* overexpression (2-fold), while *Gata4* levels were similar to control levels (Figure 1D). It is worth noting these results represent gene expression alterations early after transient transfection and prior to blasticidin selection. More dramatic differences would be expected if only transfected cells were analyzed. These data suggest that Hes5 may also bind and regulate *Isl1* in the context of adult CPCs. Although we have previously verified *Isl1* expression at the transcript level in iCPC$^{Sca\text{-}1}$ [13], the frequency of cells expressing the protein among iCPC$^{Sca\text{-}1}$ population had not been evaluated. Therefore, we evaluated Isl1 protein expression in iCPC$^{Sca\text{-}1}$ by flow cytometry, and assessed whether Hes5 expression has an impact on the frequency of cells expressing Isl1 in the iCPC$^{Sca\text{-}1}$ population. At 72 h after transfection similar cell percentages were observed for Mock and *Hes5*-transfected iCPC$^{Sca\text{-}1}$ (21.5% and 24.8%, respectively) (Figure 1E). This observation was also verified at 10 days after transfection following blasticidin selection for transfected cells, where Mock and *Hes5*-transfected iCPC$^{Sca\text{-}1}$ presented similar cell percentages of Isl1-expressing cells (30.0% and 27.6%, respectively) (Figure 1E). In summary, these results show *Is1* downregulation at 48 h but not at 10 days after transfection, and no alterations in the frequency of cells expressing Isl1 among iCPC$^{Sca\text{-}1}$ population after *Hes5* overexpression. These observations suggest that an Isl1^{+} cell-subset within the iCPC$^{Sca\text{-}1}$ population transiently downregulates Isl1 in response to Hes5 expression.

During embryonic development, Isl1 is expressed in cardiac progenitors and downregulates as they differentiate into cardiomyocytes [27], suggesting that Isl1 may be detrimental for the progression of cardiac differentiation. Moreover, this indicates Isl1 may maintain an undifferentiated state. $Isl1^+$ cells have been isolated from postnatal [28, 29] and adult hearts [29, 30], and although postnatal $Isl1^+$ cardioblasts did not show Sca-1 expression [28], adult $Sca\text{-}1^+$ CPCs isolated by us and others [13, 31, 32] express Isl1, suggesting that the $Sca\text{-}1^+$ CPCs reservoir includes a subset of $Isl1^+$ progenitor cells in the adult heart.

The evidence that Isl1 dowregulation is required for cardiomyocyte differentiation [27, 28, 33] supports our previous findings indicating that, after cardiac induction, sustained *Hes5* expression impairs cardiac maturation, likely due to continuous promotion of high Isl1 levels (Freire, AG et al, under peer review). Thus, in light of these observations, we hypothesize that Isl1 levels in $iCPC^{Sca\text{-}1}$ are downregulated early after *Hes5* overexpression triggering the expression of cardiomyocytic markers. Further investigation is warranted to dissect the mechanism of Isl1 regulation mediated by Hes5 and to elucidate whether Hes5 will drive more robust cardiomyocytic differentiation from endogenous CPCs.

Materials and Methods

$iCPC^{Sca\text{-}1}$ cell culture

$iCPC^{Sca\text{-}1}$ were maintained in 70% high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies) and 30% Claycomb medium (Sigma-Aldrich), supplemented with 10% Fetal Bovine Serum (FBS) (Lonza), 100 U/mL penicillin/streptomycin (Gibco, Life Technologies) and 2 mM L-glutamine (Gibco, Life Technologies).

$iCPC^{Sca\text{-}1}$ transfection

For transient transfection Lipofectamine 2000 reagent was used according to the manufacturer's protocol. Briefly, $iCPC^{Sca\text{-}1}$ were plated at $1x10^5$ cells/24-well at the day before transfection to achieve 90% confluency at the time of transfection. 0.8 µg of DNA *per* 24-well were complexed with lipofectamine for 30 minutes (min) at room

temperature (RT). Complexes were added to cells and medium was changed 24 h after transfection. For stable transfection, the calcium phosphate method was followed as previously described [34]. In summary, cells were grown to 70-80% confluency in DMEM with 10% FBS. 10 µg of DNA *per* 6-well was mixed with $CaCl_2$ and slowly added dropwise to 2x BES buffered saline (Sigma), while subjected to bubble air caused by continuous pipetting, and incubated for 15 min. The calcium phosphate-DNA complexes were added dropwise onto cells that were kept at 37ºC overnight. Medium was replaced after 24 h. Four days after transfection cells were cultured in medium supplemented with 5 µg/mL blasticidin and medium was maintained for additional four days.

Gene expression analysis

RNA was extracted using Trizol reagent and reverse transcribed using PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions. RT-PCR and quantitative RT-PCR (qRT-PCR) were performed using BIOTAQ DNA polymerase (Bioline) and iQ Sybr Green Supermix (Bio-Rad), respectively, and gene-specific primers. qRT-PCR reactions were carried out in triplicate on the iCycler iQ5 Real-Time PCR system (Bio-Rad). Relative gene expression was normalized according to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression.

Flow cytometry

For Isl1 and Ki67 detection, cells were dissociated with trypsin and stained using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Briefly, cells were fixed and permeabilized using the Fixation/Permeabilization solution, prepared according to manufacturer's instructions, for 45 min at RT in the dark. Cells were then washed in permeabilization buffer and incubated with PE-conjugated anti-Isl1 (Q11-465, BD Pharmingen) at 1:100 dilution or FITC-conjugated anti-Ki67 (556026, BD Pharmingen) at 1:20 dilution for 30 min on ice. Cells were washed once in permeabilization buffer and then once in phosphate buffer saline (PBS) with 3% FBS. Flow cytometry acquisition was performed on a FACS Canto II (BD Biosciences) and analyzed using FlowJo software.

Data and statistical analysis

Data are represented as mean ± SEM. Statistical significance was determined by unpaired t test. $p<0.05$ was considered statistically significant. *$p<0.05$.

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Chapter VI

Concluding remarks and future perspectives

Concluding remarks and future perspectives

Originally, the proposal for the herein Doctoral dissertation had as driving forces the scarcity of fundamental knowledge on the biology and on the role in heart homeostasis and disease of putative adult cardiac progenitors. In this context we were further challenged by a general lack of standardization on the methods applied in what was an emerging area. Hence, we aimed at engendering *in vitro* systems in which the initiation and further progression of the cardiac molecular program could be robustly investigated. Our objectives have been delineated in order (i) to create and validate a benchmark to be used as a reference for $Lin^{-}Sca\text{-}1^{+}$ adult CPCs, (ii) to identify effectors downstream of Notch in the onset of cardiogenesis using the well-established ESCs *in vitro* model system for cardiac differentiation and, (iii) to validate these factors as key regulators for cardiogenic processes in adult CPCs.

Within this conceptual framework, our work has materialized into tools and knowledge that fulfill some of the needs in the cardiovascular field. A cell line of $Sca\text{-}1^{+}$ CPCs specifically immortalized by overexpression of mTERT ($iCPC^{Sca\text{-}1}$) has been established for the first time. An extensive *in vitro* and *in vivo* characterization has validated the newly generated line as a model system representative of the native cellular counterparts, while preserving a high mitotic capacity and a stable phenotype in long-term culture. Importantly, after immortalization, cells preserved the phenotypic features described for endogenous CPCs expressing Sca-1. $iCPC^{Sca\text{-}1}$ cell line display early cardiac transcription factors and features typical of mesenchymal affiliation, while lacking transcripts for cardiac structural genes and markers associated to hematopoietic/endothelial cells, pluripotent cells and fibroblasts. Importantly, these cells survived and engrafted the injured myocardium while contributing to repair processes, as demonstrated by a decreased LV remodeling and improved cardiac function. Moreover, an increase in neovascularization was observed and has been attributed mostly to the secretion of paracrine factors inducers of mobilization and/or activation of resident progenitors and/or other relevant cells. Indeed, secretion of paracrine factors appear to account for the beneficial effects observed upon cell transplantation in a manner rather independent of the cellular source [1]. $iCPC^{Sca\text{-}1}$, similarly to what is recognized for their native counterparts, exhibited modest

differentiation into cardiomyocyte-, smooth muscle- and endothelial-like cells *in vitro* and after transplanted into infarcted murine hearts, although differentiation into the smooth muscle lineage appeared preponderant. In fact, the multipotency of CPCs has been questioned, as lineage tracing studies tracking Sca-1-derived cells have suggested a restricted lineage potential [2]. Moreover, protocols for *in vitro* differentiation of these cells involve DNA demethylation by 5-aza [3] and/or co-culture with cardiomyocytes [3, 4], rather unusual requirements for *bona fide* stem cells. However, the prospect that CPCs are innately endowed to originate all cardiac lineages cannot be excluded, as the appropriate signals for the unfolding of the full differentiation potential could be absent. Hence, although this particular study did not contribute new knowledge on the biology of CPCs and on their role in cardiac homeostasis and repair, it made available to the scientific community a reliable *in vitro* tool which will ease functional and mechanistic studies on the fundamental biological aspects regarding endogenous Sca-1^{+} CPCs. In light of this, parallel studies from our laboratory and collaborators have been addressing biological features of adult CPCs using this cell line as a model system. As examples, the role of mechano-transduced signals in the regulation of CPCs differentiation [5] and the ability of CPCs to colonize ontogenic stage-specific decellularized ECM (Silva, AC et al.; unpublished data) have been investigated using the $iCPC^{Sca\text{-}1}$ cell line.

Also in the frame of this Thesis we proposed to identify early regulators at the onset of cardiogenesis. The rational for this derived from the recognition that developmental factors are putative regulators in the adult molecular regulatory circuitry and candidates for *in situ* activation of adult CPCs. In this sense, pluripotent stem cells have been proficuous as a model system for “eyeglassing” the molecular events at the onset of lineage determination. Hence, ESCs constitute a unique platform for the identification of master regulators of cardiogenic processes, presently a rather more reliable application than their clinical use, due to the reasons discussed earlier in this dissertation, *e.g.* of immunological, oncological or safety nature.

Apparently at odds with previous studies in embryos and differentiating ESCs, demonstrating an inhibitory role for Notch in cardiac differentiation [6-9], Chen et al, have demonstrated that transient NICD activation directs ESCs-derived mesodermal

progenitors and hemangioblasts towards a cardiac fate [10]. These findings highlight the context-dependent nature of Notch pathway and suggest that the dual effects as repressor or inducer of cardiogenic differentiation are much likely associated to the stage during differentiation the signal is being activated.

Thus, we proposed to identify the downstream mediators of Notch in the determination of cardiac fate using the robustness of the ESCs system for cardiac differentiation. This work demonstrates for the first time a role for Hes5 as an early regulator at the onset of cardiogenesis. In fact, to the best of our knowledge, this is the first collection of experimental data reporting Hes5 participation in cardiogenic processes whatsoever. *Hes5* is a member of the *Hes* gene family of well-known Notch targets and has been described as a mediator of Notch effects in non-cardiac systems [11, 12]. *Hes* genes encode bHLH transcriptional regulators which mediate the maintenance of progenitors and binary cell fate decisions, thereby controlling the normal timing of differentiation [13]. We were able to show that Hes5 mediates, at least in part, the effects of NICD1 in the determination of cardiac fate, contributing mechanistic insights into how Notch pathway determines cardiac fate from mesodermal progenitors. Moreover, the results strongly indicate that Hes5 specifies cardiac over hematopoietic cell-fate choice in mesodermal progenitors, and fit into the current understanding that networks controlling heart and vascular/hematopoietic development are tightly inter-connected and mutually antagonistic [14-16]. In resemblance to what has been documented for activated Notch a time-dependent function and a dual effect on cardiogenic differentiation was observed for Hes5. Loss and gain-of-function of Hes5 early during *in vitro* differentiation affected the Flk-1^+ mesodermal reservoir, resulting in increased and decreased cell percentage, respectively. Due to these observations and the described role for Hes factors in controlling the normal timing of cell differentiation [13], we speculate Hes5 regulates the timing of determination of Flk-1^+ hematopoietic and cardiac mesodermal derivatives.

Furthermore, in ESCs-derived mesodermal progenitors Hes5 binds and putatively regulates pivotal target genes for normal vascular/hematopoietic, *e.g. Scl*, and heart development, *e.g. Isl1*. Isl1 is indispensable for heart development [21], while Scl

prevents ectopic cardiogenesis in prospective hemogenic tissue [22] by occupying primed enhancers used by cardiac regulators for gene activation [23]. Thus, our results suggest that, in mesodermal progenitors, Hes5 directs cell-fate decision towards cardiac over hematopoietic lineages through Isl1 and Scl effectors. Moreover, a dual role for Hes5 as a repressor or an activator of Isl1 at specific stages of ESCs differentiation is hinted. This switch of function, from a repressor to an activator, has been described for Hes1 at *Mash1* promoter during neuronal differentiation [24]. Indeed, normal organ development relies on the precise switching between gene repression and activation. This work contributes a preliminary insight in the mechanism of action downstream of Hes5 in cardiogenesis, and demands the confirmation of effective regulation and sequence-specific binding at *Isl1* and *Scl loci* by endogenous Hes5. Moreover, those studies should contemplate whether tight temporal regulation of *Isl1* expression involves the recruitment and further dismissal of the Groucho/TLE co-repressor complex from *Isl1* promoter.

Notably, we were able to identify the appropriate temporal window for cardiac induction *in vitro* mediated by transient high levels of Hes5. Evidence piles up, including our own, reporting an inhibitory effect for Notch pathway in cardiac maturation, which appears to correlate to an activation at later-stages in cardiac differentiation, after cardiac induction have already occurred [8-10, 18-20]. It is our conviction these data fit like a glove within the prior knowledge, at first glance contradictory, documenting a dual role for the Notch signaling as an inducer or suppressor of cardiogenesis. Moreover, our work highlights the context-specific and time-dependent nature of Hes proteins. Importantly, not only the timing of induction, but also the duration of the signal has impact on cell-fate determination and lineage differentiation. Thus, the inhibitory effect on cardiogenesis seems to be at the level of development and maturation rather than at the time of cardiac specification, as long as inducted at the exact timing.

Worth noting, the use of a genetically-modified ES cell line enabled us to capture a flash-moment which is technically challenging to visualize in the developing embryo. Moreover, a transient temporal window for Hes5 function in cardiogenesis has been identified that otherwise would stay unnoticed.

Although these findings are novel and of great importance, they represent nonetheless effects of loss and gain-of-function mutations. Thus, the validation of the role of Hes5 in specifying cardiac fate in the most close to physiological conditions is undoubtedly required. In our laboratory investigation has been started to address whether endogenous Hes5 is expressed in prospective cardiomyogenic precursors in a cell-autonomous fashion during normal ESCs differentiation and in early/mid-streak stage embryos (prior to E7.0). Notch1 expression is documented in nascent mesodermal cells in the developing embryo [25], correlating with the stage we have identified Hes5 as a downstream effector of NICD1 *in vitro*. Thus, it will be worthwhile to investigate in the embryo whether Hes5 is expressed as a downstream target of Notch1 in nascent mesodermal cells primed to become cardiac progenitors. *Hes5*-null embryos have no apparent cardiac phenotype though, to the best of our knowledge, no studies were conducted to specifically address cardiac malformations. However, the possibility that another bHLH protein could compensate for Hes5 function in cardiogenesis is high. Indeed, compensation between Hes/Hesr factors is suggested in non-cardiac systems [11, 12] and in later stages in cardiac development, as for example Hey1 and HeyL are important in heart morphogenesis but only the double mutant display cardiac abnormalities [26]. Thus, it will be interesting to assess the compensatory function of other Hes/Hesr factors after *Hes5* depletion, and whether these proteins share binding site specificity at the same target genes.

Determinant players in the onset of cardiogenesis during development are putative regulators of the cardiac molecular circuitry in adult CPCs and candidates for *in situ* activation of these cells. Our premise was inspired by the key role developmental transcription factors display in triggering a cardiogenic program in non-cardiac cells, such as cardiac fibroblasts [27-29] or hemangioblasts [10], or favoring cardiac cell-fate determination from mesoderm [30]. In fact, boosted by Yamanaka's findings, the seeking for key factor combinations to reprogram to a given lineage has widespread in multiple fields of research. Complementarily, *in vivo* reprogramming and gene delivery, in many ways still in their infancy, may hold the key for multiple therapeutic applications. The discovery of key factors that can be delivered to particular cell-types,

in which a biological process will be specifically triggered, is a major goal for translation to the clinic.

Hence, in a proof-of-concept approach the role of Hes5 in the regulation of proliferation and differentiation in adult CPCs was evaluated using the iCPC^{Sca-1} cell line as a model of endogenous CPCs. *Hes5* expression in iCPC^{Sca-1} had no effect in cell proliferation while remarkably promoting *Myh6* and*,* particularly, *Myh7* upregulation in basal culture conditions. Interestingly, *Hes5*-transfected iCPC^{Sca-1} showed *Isl1* downregulation, similarly to the regulation we observed when *Hes5* was activated during mesodermal induction from ESCs. Considering that Isl1 expression downregulates once cardiac progenitors differentiate [21, 31, 32], we hypothesize that after *Hes5* transfection the transiently decreased levels of *Isl1* in iCPC^{Sca-1} trigger the progression in the cardiomyocytic program, visible by the upregulation of transcripts for contractile myofilaments. Although a very preliminary assumption, these observations constitute a first glance at the potential Hes5 may hold as a key regulator for enhancing cardiomyogenic differentiation in adult CPCs. Future work is required to demonstrate binding and regulation of *Isl1* promoter by Hes5, although it is tempting to speculate the consistent timely-dependent regulation of Isl1 at different stages of cardiac differentiation may in part explain the effect of *Hes5* in cardiac maturation.

Yet, several questions arise from these findings, as for instance, what is the role of Hes5 in adult heart homeostasis and cardiac repair, and in particular, in the regulation of endogenous CPCs? To the best of our knowledge there are no studies specifically targeted to investigate the role of this gene in the regulation of adult CPCs, not even roughly addressing a participation in cardiac repair processes. Notch signaling however, has been demonstrated to play a cardioprotective role in the damaged myocardium [33, 34]. Importantly, activated Notch promotes expansion of Sca-1^{+} CPCs [35] and induces myocytic differentiation from c-Kit^{+} CPCs [34]. In light of our original findings, Hes5 participation as a mediator in some of the described effects for the Notch pathway, and particularly in the regulation of CPCs differentiation, should be investigated.

Thus, the present Thesis introduces another piece in the complex puzzle that is the interconnected network of combinatorial codes to be perceived by the cells. The decision to engage a determined lineage is far to be a single variable process. Indeed, the particular molecular milieu will define how that specific signal will be decoded by the cell in a given time and space. That said, the context-specific effects of Hes5 in cardiogenesis result from the interactions played by itself, as well as by its targets, with a plethora of transcriptional regulators and co-factors within a particular epigenetic status. Likewise, the genes bound and regulated by Hes5 in a different cellular context are not necessarily the same. Therefore, it is highly unlikely that Hes5 could drive cardiac fate in cells that are not primed to originate that lineage. It would be like planting in an infertile soil. In this sense, Hes5 does not determine cardiac fate or differentiation by itself, but instead through the particular environment it encounters.

In this work we gave the first steps in the translation of Hes5 function to the adult CPCs regulatory network. It will be very interesting to validate whether Hes5 is a key factor to enhance cardiogenic differentiation potential in endogenous CPCs. Importantly, this work hints that the mechanisms of regulation in embryonic and adult systems may overlap, reinforcing the importance of understanding the embryonic development while seeking for novel therapeutic targets.

Ideal regenerative procedures may involve the *in situ* activation of cardiogenic processes. Speculating that Hes5 plays a role in CPCs, one can foresee the design of strategies for targeted Hes5 delivery into CPCs, as for instance by particles functionalized with specific antibodies for the recognition of a particular surface marker. However, given the lack of a specific marker for CPCs, the choice of c-Kit or Sca-1 will not be ideal given the overlap with other cell types. Another issue is the control of the timing and duration of activation. The effects of Hes5 are highly time-dependent, therefore the designed strategy must contemplate the inducible expression of the gene. As alternative, cardiomyogenic differentiation from CPCs can be driven *ex vivo* followed by the transplantation of the differentiated cells. However, one can wonder whether Hes5 plays a different role in CPCs partaking distinct cell-subsets. Assuming that the different subsets correspond to distinct developmental stages from the same progenitor, it will be interesting to address if the effects of Hes5

in different CPCs will recapitulate the set of events occurring along the progression of the embryonic cardiomyocytic program.

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