

(Re-)Programming of Subtype Specific Cardiomyocytes

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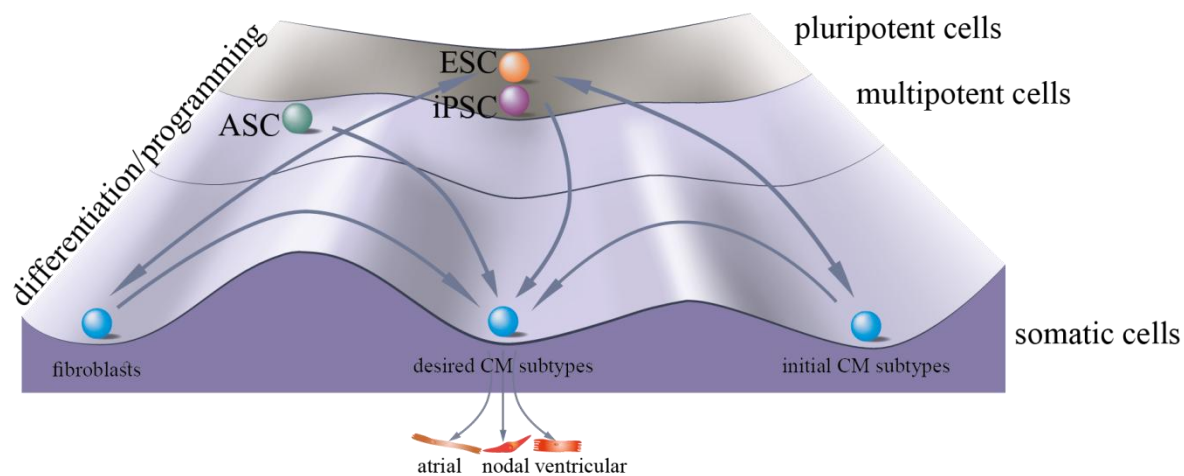
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Abstract

Adult cardiomyocytes (CMs) possess a highly restricted intrinsic regenerative potential –a major barrier to the effective treatment of a range of chronic degenerative cardiac disorders characterised by cellular loss and/or irreversible dysfunction and which underlies the majority of deaths in developed countries. Both stem cell programming and direct cell reprogramming hold promise as novel, potentially curative approaches to address this therapeutic challenge. The advent of induced pluripotent stem cells (iPSCs) has introduced a second pluripotent stem cell source besides embryonic stem cells (ESCs), enabling even autologous cardiomyocyte production. In addition, the recent achievement of directly reprogramming somatic cells into cardiomyocytes is likely to become of great importance. In either case, different clinical scenarios will require the generation of highly pure, specific cardiac cellular-subtypes. In this review, we discuss these themes as related to the cardiovascular stem cell and programming field, including a focus on the emergent topic of pacemaker cell generation for the development of biological pacemakers and *in vitro* drug testing.



Keywords

ESC; iPSC; cardiovascular development; subtype differentiation; system-based data analysis; nodal cells; pacemaker

List of abbreviations

ADSC	adipose tissue-derived mesenchymal stem cell
Alcam	activated leukocyte cell adhesion molecule
AMI	acute myocardial infarction
ANF	natriuretic factor
AP	action potential
ASC	adult stem cell
AV	atrioventricular
AVB	atrioventricular bundle
AVN	atrioventricular node
BB	bundle branch
BCT	bioartificial cardiac tissue
bHLH	basic helix-loop-helix
Bry	Brachyury
CABG	coronary artery bypass graft
Ca_v1.3	calcium voltage-gated channel subunit alpha1 D
Ca_v3.1	calcium voltage-gated channel subunit alpha1 G
CCS	cardiac conduction system
CF	cardiac fibroblast
CHD	congenital heart disease
CM	cardiomyocyte
CMPC	cardiomyocyte progenitor cell
CMVEC	cardiac microvascular endothelial cell
CPC	cardiac progenitor cell
CS	conduction system
CV	cardiovascular
CVD	cardiovascular disease
Cx30.2	connexin30.2
Cx40	connexin40
Cx43	connexin43
Cx45	connexin45
ECG	electrocardiogram
EMILIN2	elastin microfibril interface 2
EPC	endothelial progenitor cell
EPCS	electric-pulse current stimulation
ESC	embryonic stem cell
FDA	Food and Drug Administration
FGF	fibroblast growth factor

FHF	first (primary) heart field
GF	growth factor
GFP	green fluorescence protein
GO	gene ontology
HCN4	hyperpolarization-activated cyclic nucleotide-gated cation channel 4
hPSCreg	Human Pluripotent Stem Cell registry
HTS	high-throughput sequencing
ICM	induced cardiomyocyte
iPSC	induced pluripotent stem cell
iSAB	induced sino-atrial body
Isl1	ISL LIM homeobox 1
JNK	c-Jun N-terminal kinase
LVEF	left ventricular ejection fraction
MAPK	mitogen-activated protein kinase
MB	molecular beacons
MEA	multi-electrode-array
Mlc2v	myosin, light polypeptide 2, regulatory, cardiac, slow
MSC	mesenchymal stem cell
MYH6	myosin, heavy chain 6, cardiac muscle, alpha
Myh7	myosin, heavy polypeptide 7, cardiac muscle, beta
Na_v1.5	sodium voltage-gated channel alpha subunit 5
Nkx2-5	NK2 homeobox 5
Nppa	natriuretic peptide A
ODE	ordinary differential equation
PA	polyacrylate
PDMS	polydimethylsiloxane
PLGA	polylactide-co-glycolide
PMC	pacemaker cell
PPT	protein-protein interaction
PSC	pluripotent stem cell
Rarg	retinoic acid receptor, gamma
ROCK	rho-associated, coiled-coil containing protein kinase
Rxra	retinoid X receptor, alpha
SA	sino-atrial
SAN	sinoatrial node
SCD	sudden cardiac death
SCN5A	sodium channel, voltage-gated, type V, alpha subunit
SHF	second heart field
Shox2	short stature homeobox 2
SIRPA	signal-reduced protein alpha

SSS	sick sinus syndrome
Tbx18	T-box 18
Tbx3	T-box 3
TF	transcription factor
THF	tertiary heart field
VCAM1	vascular cell adhesion molecule 1
VCS	ventricular conduction system
VEGF	vascular endothelial growth factor
wt	wild-type

1. Introduction

The advent of regenerative medicine has opened up new perspectives for so far insoluble clinical problems. Recent progress in understanding the biology of stem cell pluripotency and endogenous repair mechanisms has fostered a deeper understanding of its remarkable therapeutic potential for tissue repair or replacement. Such novel approaches are urgently required to effectively treat the growing burden of disorders characterized by irreversibly damaged or diseased tissue resulting in loss of organ/tissue function associated with a rapidly ageing population. Furthermore, through the production of autologous pluripotent stem cells, regenerative strategies hold promise in providing truly patient-specific therapies for structural and functional repair in disease.

Cardiovascular disease (CVD) is the leading cause of death worldwide (accounting for 31.3 % in 2015) and is projected to rise further (WHO 2017). CVD encompasses a range of chronic disease states, including ischemic, rheumatic and hypertensive heart disease, in addition to extra-cardiac disorders such as stroke. Heart failure represents the final common phenotype resulting from a diverse range of inherited and acquired cardiac insults and affects ~26 million individuals worldwide [1]. Individuals with severe heart failure have a dismal prognosis with a worse 5-year adjusted mortality than many cancers [2]. To date, allogeneic heart transplantation remains the only available treatment option for patients with end-stage heart failure who are symptomatic despite optimal medical and device (cardiac resynchronization) therapy [3,4]. Despite advances in surgical technique, perioperative management and immunomodulation, a major limitation to its wider application is donor organ scarcity: in Europe in 2015, only 604 donor organs were successfully engrafted, while 1140 patients are on the active Eurotransplant waiting list [5]. An additional 209 recipients died before they could undergo heart transplantation [5]. Even for those transplanted, while symptomatic improvement and survival are in general markedly improved, outcomes (median ~11 year survival) are limited by long-term complications, in part associated with immunosuppression, including malignancy, infection, renal dysfunction and allograft vasculopathy [6]. In view of such limitations, highly innovative approaches are under exploration with the ultimate goal of establishing safe, durable cellular replacement and repair of injured or diseased myocardium, in addition to *in vitro* disease modelling and drug development applications [7–9]. A key requirement for these approaches is to ensure highly reliable and robust generation of fully functional cardiomyocytes with physiological properties as close as possible to their natural counterparts. Partially or terminally differentiated cells offer a relevant alternative to somatic stem cell transplantation, given that the latter are still a matter of controversial debate regarding their moderate therapeutic outcomes [10,11]. Pluripotent stem cells (PSC) and their derivatives offer an attractive source for both cell replacement and studying key cellular and molecular processes involved in cardiovascular disease. Equally, resident cells (e.g. fibroblasts) may also represent a readily accessible source of cells to study cell fate transition not only within, but even across, germ layers.

2. Tissue regeneration and repair for cardiovascular disease

Normal cardiac function and physiological homeostasis is achieved through the complex interaction of a diverse range of cell types broadly constituting myocyte, vascular and stromal compartments. Even amongst specific cell types, such as cardiomyocytes (CM), there exist different phenotypes (e.g. sinoatrial, atrial, nodal, Purkinje and ventricular). Disease processes do not affect all these cell types uniformly, with relatively greater impact on specific tissue components such as fibrosis or vascular insufficiency.

The human heart does exhibit some regenerative potential, albeit very low, with an annual cardiomyocyte turnover rate of 1 % at age 25 years, reducing further to 0.45 % by 75 years [12]. As a corollary, adult human cardiomyocytes are long-lived cells, such that less than 50 % will be replaced over a life-span of 75 years. In contrast, the proportion of CM situated in mitosis and cytokinesis is highest in infancy and contributes to developmental growth, suggesting significant cardiac regenerative potential in children and adolescents [13]. Other studies, including data from animal models, have highlighted that CMs, upon transition from the mononucleate to a mature binucleate state, exit the cell cycle and lose their proliferative potential during a short postnatal period [14–16]. In the setting of common CVD such as acute myocardial infarction (MI), leading to the abrupt loss of up to ~1 billion CM, this intrinsic regeneration potential is vastly inadequate, resulting in structural (i.e. scar) rather than functional (i.e. contractile) repair, and potentially to progressive deleterious ventricular remodeling and post-MI heart failure. However, the identification of adult CM repopulation raises the possibility that either normally resident cell populations such as cardiac progenitor cells (CPCs), or pre-existing CM may represent sources for myocardial repair post-injury [13,17,18].

Accordingly, development of experimental protocols to robustly generate distinct cardiac cell types and define their specific clinical/preclinical applications is required. We will address the progress made recently with attempts at stem cell and somatic cell-based programming, detailing their therapeutic potential and current stage of development. A major contribution to these has been provided by applying insights gained from the study of cardiovascular developmental biology to which we turn our attention next.

3. Cardiogenesis during development and its regulation

Cardiac development occurs during the early stages of the embryonic phase, and is crucial to ensure adequate nutrient and oxygen supply to, as well as removal of waste from, the growing organism. The mature mammalian heart is highly complex in structure, divided macroscopically into four chambers macroscopically and constituting specific muscle and non-muscle cell types, including left and right atrial CM, left and right ventricular CM, and cells forming the conduction system, sinoatrial pacemaker, vascular smooth muscle, endo- and epicardium [19–23]. The generation of such developmentally diverse cell fates are achieved via spatiotemporally stringent molecular regulation, with clear evidence that myocardial cells derive from Brachyury⁺ (Bry⁺) mesodermal progenitor cells of the primitive streak during gastrulation through the impact of Wnt signaling [24–26].

Thereafter, two crucial transcription factors (TF) are regarded as cardiovascular fate-determining factors: the bHLH TF MesP1 (mesoderm posterior 1) [27–30] and the surface molecule Flk1 (also known as VEGFR2: vascular endothelial growth factor receptor 2) [31,32]. Further development is achieved from multipotent cardiac progenitor cells [33] and can be distinguished mainly in two origins: i) the first (primary) heart field (FHF) demarcating an Nkx2.5⁺/Hcn4⁺ cell population which forms the cardiac crescent [34–41], and ii) the second heart field (SHF) demarcating a Nkx2.5⁺/Isl1⁺ cell population derived from the pharyngeal mesoderm and lying medially and posterior to the FHF [41–46]. In avians, a decisive role for the tertiary heart field (THF) in pacemaker development of the sino-atrial (SA) node has also been reported [47,48]. Primary heart field progenitor cells will yield the myocardium of the left ventricle as well as a limited portion of the right ventricle, the right and left atria and large parts of the conduction system (CS), such as the atrioventricular (AV) node and the ventricular CS [20,42]. Multipotent progenitor cells of the SHF will yield myocardium of the right and left atria, the right ventricle and the outflow tract, as well as cardiac vascular smooth muscle and the endocardium [31,46,49]. In addition to these, epicardial progenitor cells give rise to cardiac fibroblasts, vascular smooth muscle, atrial and venous endothelial cells [20,50–52]. Moreover, pro-cardiogenic factors and signaling pathways play a decisive role during development and are distributed from the surrounding endoderm and mesoderm. These include bone morphogenetic proteins [53–57], notch [58], nodal and fibroblast growth factors [59–61], in addition to canonical and non-canonical Wnt/JNK [62–66].

A highly coordinated signaling network determines early cardiac progenitor as well as late specific cell fates, whose disruption can lead to abnormal embryonic development and congenital heart disease (CHD) characterized by malformation of specific cardiac structures [67]. CHD is the most common major congenital defect worldwide with a birth prevalence of between 0.58 – 0.9 % [68,69]. Thus, dysregulation of TFs (e.g. Nkx2.5 [70–78], Gata4 [77,79–82] or members of the forkhead family [83]) is associated with various abnormalities including atrioventricular block, septal defects or pulmonary stenosis [84–86]. Exemplifying this, smoking-associated cardiac defects have been linked to promotor DNA hypermethylation of Tbx5 and Gata4 caused by maternal nicotine exposure [87]. In contrast, mutations in genes encoding cardiac ion channels are largely associated with phenotypes associated with sudden cardiac death (SCD) resulting from lethal arrhythmias [88].

4. Programming strategies for cardiovascular lineages

Insights into cardiac development and the potential serious sequelae arising from its disruption are a critical prerequisite for furthering disease modelling, drug development and cell replacement strategies.

In this chapter, we address approaches to enhance cardiovascular cell differentiation from adult stem cells (ASCs) (“directed differentiation”; 4.1) and from ESCs and iPSCs (“forward programming”; 4.2), in addition to discussing attempts to convert terminally differentiated somatic cell types into cardiac cells (“direct reprogramming”; 4.3) (Figure 1).

The overriding aim of all programming strategies should be the generation of cells as physiologically close as possible to their natural counterparts. Moreover, to enable technology transfer from bench to bedside, procedures will have to be xeno-, serum-, feeder- and DNA-free. Accordingly, the approaches described below address potential options to overcome hurdles associated with the purity, yield and safety of physiologically functional CM subtypes (Figure 1). As an exemplar of this, we describe approaches aimed at the generation of biological pacemaker cells for both therapeutic replacement and *in vitro* drug testing (subsection 4.4), including our own recent progress in global transcriptome network analysis of “induced sino-atrial bodies (iSABs)” [89–91].

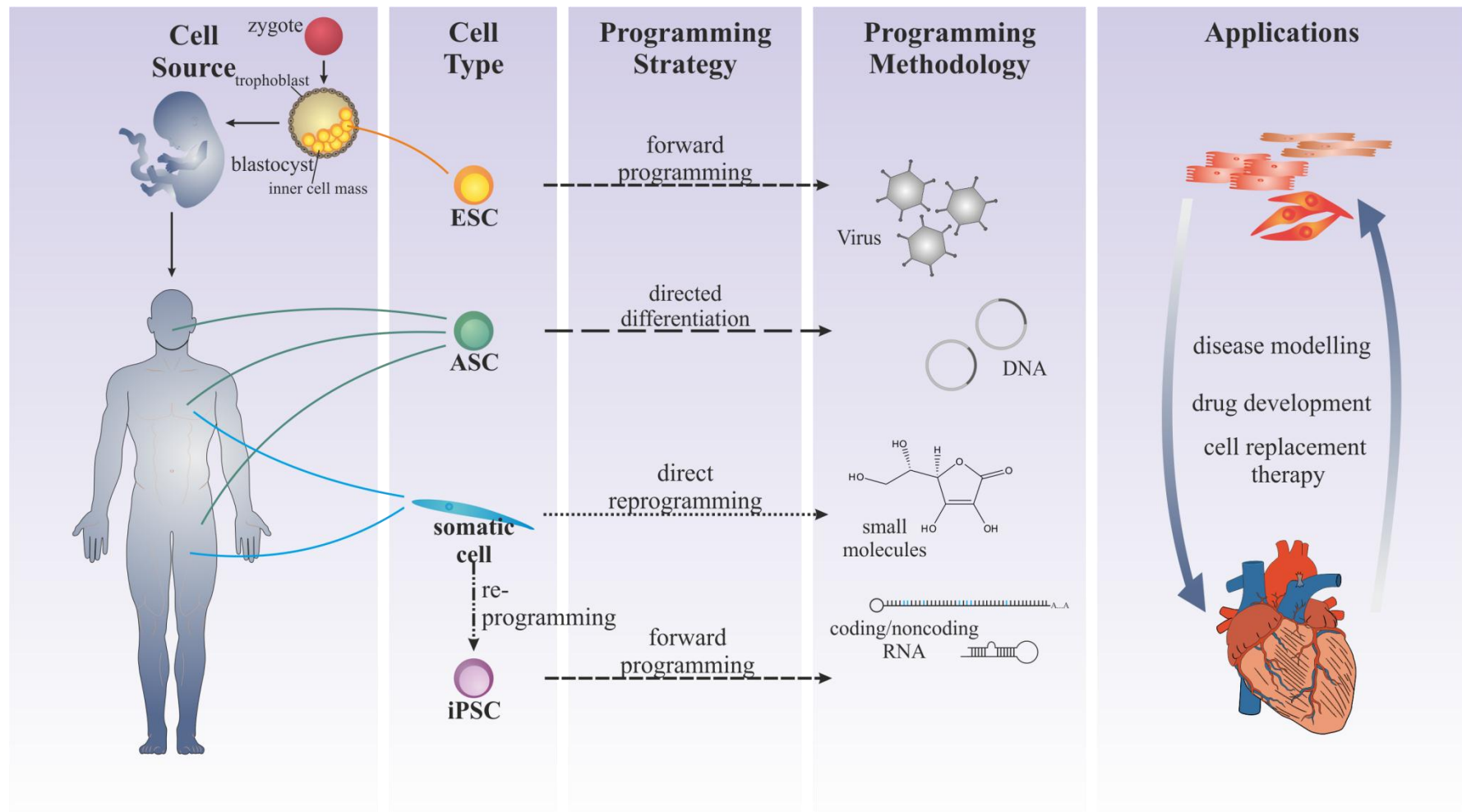


Figure 1: Common programming strategies for cardiovascular lineages.

4.1. Forward programming of multipotent stem cells

4.1.1. Cardiac programming of adult stem cells

Early attempts at introducing adult (marrow) stem cells for transplantation therapy were performed in 1957 by E. Donnall Thomas between identical twins, with the recipient suffering from leukemia. This ushered in a phase of experimental work and clinical trials in haematopoietic transplantation which ultimately led to the award of Nobel Prize in Physiology or Medicine in 1990, for Thomas together with Joseph Murray “for their discoveries concerning organ and cell transplantation in the treatment of human disease” (http://www.nobelprize.org/nobel_prizes/medicine/laureates/1990/).

While the use of stem cell transplantation for cardiovascular disorders is a promising concept approaching clinical translation [10,92,93], based on neovascularization and improved endothelial function [94], its clinical efficacy has been the subject of repeated debate in view of the modest outcomes [10]. A small study of intramyocardial delivery of purified CD133+ bone marrow SC demonstrated an encouraging but marginal improvement in left ventricular ejection fraction (LVEF) by ~ 6% after coronary artery bypass graft (CABG) surgery: CABG-only - preoperative 37.9 ± 10.3 % to 41.3 ± 9.1 % after 6 months; CABG with CD133+ cell injection: preoperative 37.4% +/- 8.4% to 47.1% +/- 8.3% after 6 months [95]. Comparable results have been reported in studies of patients following acute myocardial infarction (AMI). Given the challenges in patient recruitment to cardiac regenerative trials [10], meta-analysis has been used to better discern the size of the potential therapeutic effect. A recent systematic review of 8 prospective randomized clinical trials containing 449 participants found no overall significant improvement in LVEF (1.47 %, CI -4.5 – 7.45) in the setting of AMI following mesenchymal SC (MSC) transplantation [96]. However, exploratory subgroup analysis revealed a significant improvement in LVEF in those transplanted in the first week and also dependent upon cell dose administered (up to 3.3%).

(Re-)programming of ASCs may enable greater benefit from these multipotent cells. The sources of adult SC for potential cell fate alteration include MSC from bone marrow [97–104], adipose-tissue [105–110] or dental follicles [111], in addition to endothelial progenitor cells (EPCs) isolated from peripheral blood of patients with AMI or umbilical cord blood [112]. At least partial cardiogenic differentiation of these can be induced using a variety of exogenous manipulation strategies (Table1) including: i) treatment with methylation inhibitors, such as 5-azacytidine and histone deacetylase inhibitors, such as trichostatin A [97,99,100,103–106,109,112]; ii) co-culture with isolated neonatal cardiomyocytes [98,101,109]; iii) forced exogenous overexpression of either TFs, such as Shox2 [101] and Gata4, Nkx2.5 [98], or use of a TF-cocktail [106], or non-coding RNAs, including miRNAs [102] and lncRNAs [104]; or iv) stimulation via media supplemented with various growth factors [100,107,108] or small molecules such as ascorbic acid [108] or suberoylanilide hydroxamic acid [111].

Recently published reports on cardiomyogenic differentiation suggest that cardiac marker expression of MSC- and CD34⁺ progenitor cell-derivatives are actually based on fusion with endogenous cardiomyocytes of the recipient rather than on trans-differentiation *in vivo* [113,114]. To clarify the relative roles of secreted factors versus direct cell-cell contact, indirect-co-culture using cell culture inserts has been tested, identifying that direct cell-cell contact improves results and can even lead to human adipose tissue-derived mesenchymal stem cell (ADSC)-originating spontaneously beating CM-like

cells [109]. Future studies should define the cardiogenic differentiation potential of ASC by entirely excluding cell fusion as a mechanism, e.g. via specific labeling of the neonatal CM used, or employment of different species. However, if cell fusion is consistently demonstrated to exert a positive influence on myogenic and functional regeneration of the affected tissue, this approach should not be abandoned. The large number of studies using the epigenetic modifier 5-azacytidine highlights the potential for harnessing epigenetic modulation to modify cell fate decisions to drive regeneration. While the outcomes reported are highly variable, expression of specific cardiac markers such as desmin, cardiac actin and Troponin has been demonstrated, in association with Notch signaling. The benefits of MSC preconditioning modification such as improved cell survival and proliferation, stimulation of paracrine factor secretion and increased angiogenesis - thereby promoting cardiac repair - have been described in detail elsewhere [115].

4.1.2. Forward programming of CM progenitor cells

Another promising approach is the support of pre-existing precursor cells: cardiomyocyte progenitor cells (CMPC) can be efficiently isolated from fetal hearts, as either c-kit⁺ [116] or Sca-1⁺ [117,118] cell populations. Overexpression of miR-1 and miR-499 in such cells has been shown to enhance their differentiation into cardiomyocytes via repression of histone deacetylase 4 and Sox6 [118], confirmed with overexpression of miR-499 in another CMPC population resulting in similar effects (repression of Sox6 and Rod1) by Hosoda and colleagues [116]. The cardiogenic potential of CMPC can also be efficiently enhanced using the methylation inhibitor 5-azacytidine in combination with TGF- β [117].

Table 1: Overview of recently published programming strategies of ASCs toward diverse cardiovascular subtypes

Cell origin	Host	Delivery system	<i>in vivo</i> / <i>in vitro</i>	Factor / Substances	Target cell type	Special features	Literature
CMPC	human	synthetic nucleoside protein	<i>in vitro</i>	5 $\mu\text{mol/L}$ 5-AZA following 1 ng/mL TGF- β 1	CM-like	spontaneously beating myocytes; gap-junctional communication and action potentials of maturing cardiomyocytes	Goumans <i>et al.</i> , 2007 [117]
ADSC	rat	protein	<i>in vitro</i>	10 ng/mL TGF- β 1	CM-like	Actin, cMhc	Gwak <i>et al.</i> , 2009 [107]
ADSC	human	synthetic nucleoside	<i>in vitro</i>	10 $\mu\text{mol/L}$ 5-AZA or 100 ng/mL TSA or Co-culture with RNCM or modified cardiomyogenic medium	CM-like	Highest expression in direct contact co-culture with RNCM: Actin, Gata4, Nkx2.5, cTnT spontaneous contractions synchronous Ca^{2+} transient	Choi <i>et al.</i> , 2010 [109]
CMPC	human	miRNA	<i>in vitro</i>	miR-1 and miR-499	CM-like	Repression of HDAC4 and Sox6 Enhanced cardiomyogenesis	Sluijter <i>et al.</i> , 2010 [118]
CMPC	human	miRNA	<i>in vitro</i> / <i>in vivo</i>	miR-499	CM-like	Repression of Sox6 and Rod1 Enhanced cardiomyogenesis	Hosoda <i>et al.</i> , 2011 [116]
ADSC	rat	synthetic nucleoside	<i>in vitro</i>	Planat-Bérnard or 5 $\mu\text{mol/L}$ 5-AZA	CM-like	no spontaneous contraction Actn2, Cx-43	Carvalho <i>et al.</i> , 2012 [105]
BMSC	mouse	miRNA (Lentiviral)	<i>in vitro</i>	miR-1	CM-like	downregulation of Hes-1 expression of: Nkx2.5, GATA-4, cTnT, and Cx43	Huang <i>et al.</i> , 2013 [102]
BMSC	human	synthetic nucleoside	<i>in vitro</i>	6 $\mu\text{mol/L}$ 5-AZA or 10 ng/mL TGF- β 1	CM-like	Expression of: GATA-4, Nkx2.5, Mlc-2a, actin Higher expression in AZA-group to control: Mlc-2a, Mlc-2v, cTnT	Mohanty <i>et al.</i> , 2013 [100]
EPC	human	synthetic nucleoside	<i>in vitro</i>	5-AZA	CM-like	expression of: Actn2, cTnT, cTnI and desmin	López-Ruiz <i>et al.</i> , 2014 [112]
BMSC	pig	synthetic nucleoside	<i>in vitro</i>	10 $\mu\text{mol/L}$ 5-AZA IGF-1	CM-like	Expression of: GATA-4, Nkx2.5, β -MHC and MEF2c	Li <i>et al.</i> , 2015 [99]

		lentiviral					
BMSC	rabbit	DNA Plasmid	<i>in vitro</i> / <i>in vivo</i>	GATA4, Nkx2.5 extracellular environment co-culture with RNCM	CM-like	In combination with co-culture: significantly effective and enhance the ability to repair MI	Li and Zhang, 2015 [98]
BMSC	mouse	specific culture and substrate conditions	<i>in vitro</i>	0.3 mm-thick hcECM	CM-like	no evidence of CM differentiation	Oberwallner <i>et al.</i> , 2015 [119]
BAT	mouse	specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	1 % methylcellulose/ Iscoe's Modified Dulbecco's Medium containing hematopoietic cytokines	CCS-like	regular beating expression of: Nkx2.5, GATA6, Mef2c, ANF, α -MHC, β -MHC, MLC2a, MLC2v, but not GATA4	Takahashi <i>et al.</i> , 2015 [110]
BMSC	rat	synthetic nucleoside	<i>in vitro</i> / <i>in vivo</i>	10 μ mol/L 5-AZA	CM-like	Expression of: desmin, actin and cTnT	Yang <i>et al.</i> , 2015 [97]
BMSC	canine	lentiviral	<i>in vitro</i>	Shox2 Co-culture with RNCMs	SAN-like	High levels of: Tbx3, HCN4, Cx45 Low levels of: Nkx2.5, Cx43 Able to pace RNCMs with a faster rate	Feng <i>et al.</i> , 2016 [101]
SC-ADSC VL-ADSC CA-ADSC SS-ADSC	mouse	specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	Medium suppl. with: - for vascular smooth muscle cell differentiation: TGF- β - for endothelial differentiation: hFGF, hVEGF, hIGF, AA, hEGF - for cardio- myocyte differentiation: PMA	CM-like, endotheli al cells, vascular smooth muscle cells	Highest potential with: CA-ADSC	Nagata <i>et al.</i> , 2016 [108]
DFC	human	small	<i>in vitro</i> /	10 μ M SAHA in ADMEM	CM-like	in vitro:	Sung <i>et al.</i> ,

		molecule	<i>in vivo</i>	media; following continuous culture in media containing 1 μ M of SAHA		expression of: α -SMA, TnnT2, desmin, Actc1 <i>in vivo</i> homing: 5.6 \pm 1.0% heart 3.6 \pm 1.1% liver 11.6 \pm 2.7% kidney With differences in IL-2 and IL-10	2016 [111]
E-ADSC P-ADSC O-ADSC	human	synthetic nucleoside retroviral	<i>in vitro</i>	10 μ mol/L 5-AZA or ESRRG, GATA4, MEF2C, MESP1, MYOCD, TBX5, ZFPM2	CM-like	5-AZA: no increased expression of Actn2 or cTnT 7-factor-group: E-ADSC: increased Actn2 and cTnT	Wystrychowski <i>et al.</i> , 2016 [106]
BMSC	human	synthetic nucleoside	<i>in vitro</i>	10 μ mol/L 5-AZA	CM-like	Upregulation of: Notch1, Gata4, Nkx2.5, α - actin, cTnT	Yu <i>et al.</i> , 2016 [103]
BMSC	mouse	lncRNA	<i>in vitro</i>	10 μ mol/L 5-AZA Braveheart hypoxia/reoxygenation treatment	CM-like	Expressions of: α -actin, cTnT, Nkx2.5, Gata4, Gata6, Isl-1, EMT-associated genes (Snail, Twist, N-cadherin)	Hou <i>et al.</i> , 2017 [104]

AA: ascorbic acid; Actc1: cardiac muscle alpha actin; Actn2: sarcomeric alpha-actinin; ADSC: adipose tissue-derived mesenchymal stem cells (E: epicardium, P: pericardium, O: omentum, SC: subcutaneous white adipose tissue, VL: visceral white adipose tissue; CA: cardiac brown adipose tissue, SS: subscapular brown adipose tissue); α -SMA: alpha-smooth muscle actin; BAT: brown adipose tissue derived stem cells from interscapular area; BMSC: bone marrow mesenchymal stem cells; CCS: cardiac conduction system; CM: cardiomyocyte; CMPC: cardiomyocyte progenitor cells; CPC: cardiac progenitor cells; cTnT: cardiac Troponin T; Cx43/45: Connexin43/45; DFC: dental follicle-derived mesenchymal stem cells; hCECM: human cardiac extracellular matrix; HDAC4: Histone deacetylase 4; hEGF: human epidermal growth factor; hFGF: human fibroblast growth factor; hIGF: human insulin-like growth factor; hVEGF: human vascular endothelial growth factor; IL-2/10: interleukin-2/10; lncRNA: long noncoding RNA; miR: microRNA; Mlc: myosin light chain; PMA: phorbol myristate acetate; RNCM: rat neonatal cardiomyocytes; SAHA: suberoylanilide hydroxamic acid; TGF- β : transforming growth factor- β ; TnnT2: cardiac muscle troponin T; TSA: trichostatin A; 5-AZA: 5-azacytidine

4.2. Programming of pluripotent stem cells

Pluripotent stem cells, encompassing embryonic and induced pluripotent stem cells, represent an attractive platform to study key cellular and molecular programs of early heart development. The Human Pluripotent Stem Cell registry (hPSCreg) listed a total of 1281 cell lines in August 2017 (hESC: 707, hiPSC: 574) (<http://hpscereg.eu/>). Worldwide, the majority of hESC lines are recorded in the U.S.A., while in Europe the highest numbers are in the U.K.. The number of hiPSC lines is constantly increasing, with a dramatic increase from 120 lines in January 2016.

Since their first successful differentiation towards cardiomyocytic phenotypes was demonstrated in 1991, ESCs have grown to become an invaluable *in vitro* model to study cardiac development [120]. Currently, the number of publications relying on murine and human ESCs is increasing daily - a selected portion of these published over the past five years is shown in Table 2. Importantly, gene expression analysis has revealed a unique profile for each individual hESC line [121,122], which obviously results in variable self-renewal behavior and differentiation preferences [123,124]. Moreover, even the *in vitro* “micro-environment” of each laboratory can exert a strong impact on the cell line’s gene expression signature [125].

To overcome ethical concerns as well as the poor accessibility of ESCs, iPSCs have become a major focus of interest since their seminal description a decade ago [126,127]. Representative recent studies using murine and human iPSCs for cardiogenic differentiation are outlined in Table 3.

The starting cell types for iPSC generation are available on a large scale from various easily accessible sources. Furthermore, autologous material enables the production of patient-specific iPSCs which can be expected to become highly relevant for personalized therapy as well as *in vitro* drug testing. The retained epigenetic memory of such cells, demonstrated by the incomplete reprogramming of non-CG methylation as well as differences in CG methylation and histone modifications [128] are important considerations. They represent a drawback on the one hand, by leading to intra-line variability within clones from a single subject, but can also be construed as advantageous with respect to the cells’ enhanced ability to differentiate preferentially into their cell type of origin [129,130]. Thus, iPSCs derived from murine neonatal ventricular myocytes display a higher propensity towards spontaneous differentiation into beating CM compared to iPSCs derived from other somatic cells (e.g. tail-tip fibroblasts) [131]. In addition, the re-programming strategy towards the iPS cell stage itself has both substantial influence on programming efficiency, and affects the genetic profile of iPSCs themselves, resulting in high line-to-line variability. To date, the application of synthetic modified mRNA has the highest programming efficiency (~4.4 %) using the TFs Oct4, Sox2, Klf4, c-Myc, Lin28 in combination with valproic acid [132]. Moreover, another study claimed “foot-print free” non-integrative mRNA-based reprogramming of somatic cells and subsequent effective differentiation towards a CM-like phenotype including sarcomeric marker expression and appropriate specific responses to pharmacological modulation [133]. In this regard, it has been shown that iPS-derived CMs can exhibit residual transgene expression of Oct4 and Nanog after lentiviral-mediated transduction [134], which has the potential to lead to tumor formation.

The multifaceted powerful potential of iPSC-derived cells has helped foster numerous preclinical studies, with the therapeutic effect of their derivatives largely ascribed to their paracrine effects in supporting ischemic tissue [135]. With regard to ESC, at present only a single phase I clinical trial using human ESC-derived CD15⁺Isl1⁺ progenitors for transplantation in severe heart failure is actively recruiting (ESCORT).

Apart from the cellular origin, concepts regarding differentiation of various PSCs are highly dependent on insights gained from study of natural embryonic development, to enable the driving of cell fate alongside time-, space- and signaling-dependent patterns in order to overcome hurdles associated with species-specification and inter-personal variations. The direct application of PSCs as purely undifferentiated cells is unfeasible due to their high teratogenic potential *in vivo* [136,137]. Despite experimental progress with PSC-derived CMs, achieving functional maturation of these cells has so far proved elusive *in vitro* [138]. This is likely to reflect the current infeasibility of precisely mimicking the entire natural microenvironment, including topographical, electrical, adhesive, mechanical, biochemical, and cell–cell interaction cues [139].

In this chapter we will discuss the two main emerging strategies for efficient forward programming towards CM-like cells: i) molecular programming using forced exogenous overexpression of lineage-specific TFs and miRNAs (4.2.1); and ii) targeted differentiation via provision of optimized culture conditions (4.2.2). Furthermore, specific concepts will be considered regarding selection- (4.2.3) as well as maturation- (4.2.4) strategies to enhance the quality of the final cell product.

4.2.1. Molecular programming

Early molecular programming studies using ESCs have demonstrated MesP1 to be an essential cardiac fate determinant [140–143]. Recent *in vivo* studies have confirmed the positive effect of MesP1 CPCs in promoting cardiovascular repair of murine hearts [144]. Moreover, gain-and-loss-of function experiments have disclosed a major role for miR-322/-503 in a MesP1 progenitor population in regulating early cardiac fate decision by negatively effecting neuroectoderm differentiation [145]. Thus, MesP1⁺ mesodermal progenitors represent a heterogeneous population bearing a context-dependent potential to differentiate into cardiac, hematopoietic and skeletal myogenic progenitors [146–148], which is also the case for Flk1⁺ mesodermal cells [149]. Numerous factors affect MesP1 and subsequent cardiac TF expression, such as Bry⁺ [141], Cited2 [150], CIBZ (BTB domain-containing zinc finger protein) [151] and Fndc5 (Fibronectin type III domain-containing 5 protein (also known as: peroxisomal protein (PEP)) [152]. Another group of RNAs, the so called long noncoding RNAs (lncRNAs) play a still incompletely understood role during cardiac development; for instance, the lncRNA Braveheart has been demonstrated to act as an epigenetic modulator upstream of MesP1 using multiple ESCs [153].

Forced overexpression of key fate-determining factors has also been applied to the generation of SAN-like cells from ESCs, using the TFs Tbx3 [89], Shox2 [154] and Isl1 [155], thereby directing cell fate towards a pacemaker-like phenotype. This topic is discussed in more detail below (4.4).

Data on molecular programming using forced exogenous overexpression of lineage-specific TFs are so far restricted to ESCs – to date; no attempts have been described in the literature for iPSCs.

4.2.2. Targeted differentiation

There are currently numerous direct programming protocols using PSCs which include a focus on continual optimization and refinement of the targeted differentiation process with respect to time- and dose-dependent application of cardiogenic modulators (Figure 2). This may allow precise manipulation of PSCs through

activation or inhibition of diverse implicated molecular pathways. While the protocols schematically presented in Figure 2 have some similarities in common such as respective pathway activation or inhibition, they differ with respect to specific timeframes and overall duration of cell culture required.

Such approaches start, after preliminary ROCK inhibition [156–160], with initial activation of Wnt signaling using via Wnt activators such as CHIR99021 or direct application of Wnt3 in order to induce a mesodermal CPC population [144,156,157,160–162], together with the addition of Activin A and BMPs (bone morphogenetic proteins) [144,158,159,161,163–166]. Substances administered in the second step depend upon the desired lineage specification to either a working myocardial or a conduction system cellular phenotype. CM-like induction requires the inhibition of Wnt signaling via Wnt inhibitors [156,159–161], such as IWR1 and IWP2, as well as the addition of various growth factors (GFs), such as FGF (fibroblast GF) and VEGF (vascular endothelial GF) [157,159,161,163,165]. In addition, modulation of MAPK signaling, using SB203580 [133] and PD98059 [167] (both MAPK inhibitors), or Rho-kinase inhibitors (H1152) [158] are utilised. In contrast, differentiation towards SAN-like cells mandates inhibition of GF-, including Activin- and Nodal-signaling using inhibitors such as PD 173074 (FGF signaling inhibitor) and SB-431542 (Activin/Nodal/TGF β signaling inhibitor) [159].

The final cellular product varies among protocols with respect to marker gene expression, however, each approach results in expression profiles partly specific for CM-like or SAN-like cells, including a reported >90 % cTnT⁺ cells [157], >90 % CD31⁺/VE-cadherin⁺ ECs [164] or 35-40 % double positive cTnT⁺/MF20⁺ cells [165]. In addition, targeted differentiation-derived pacemaker-like cells exhibit specific action potential profiles [159]. Enhanced proliferation (up to 14-fold) of ESC-derived CM-like cells has been obtained through the addition of specific GSK3 and CaMKII inhibitors, as well as ERK activators [168]. Furthermore, epigenetic modulators have been used such as ascorbic acid [169–171], which, when applied during a specific time-frame from day 0 to day 2 of differentiation, leads to a 2-4-fold increase in cardiogenesis [169].

Such *in vitro* differentiation protocols approximate the highly sensitive and finely-tuned interplay of signaling pathways required for healthy embryonic development. Their success is critically dependent upon careful, albeit protracted, step-by-step protocol optimization.

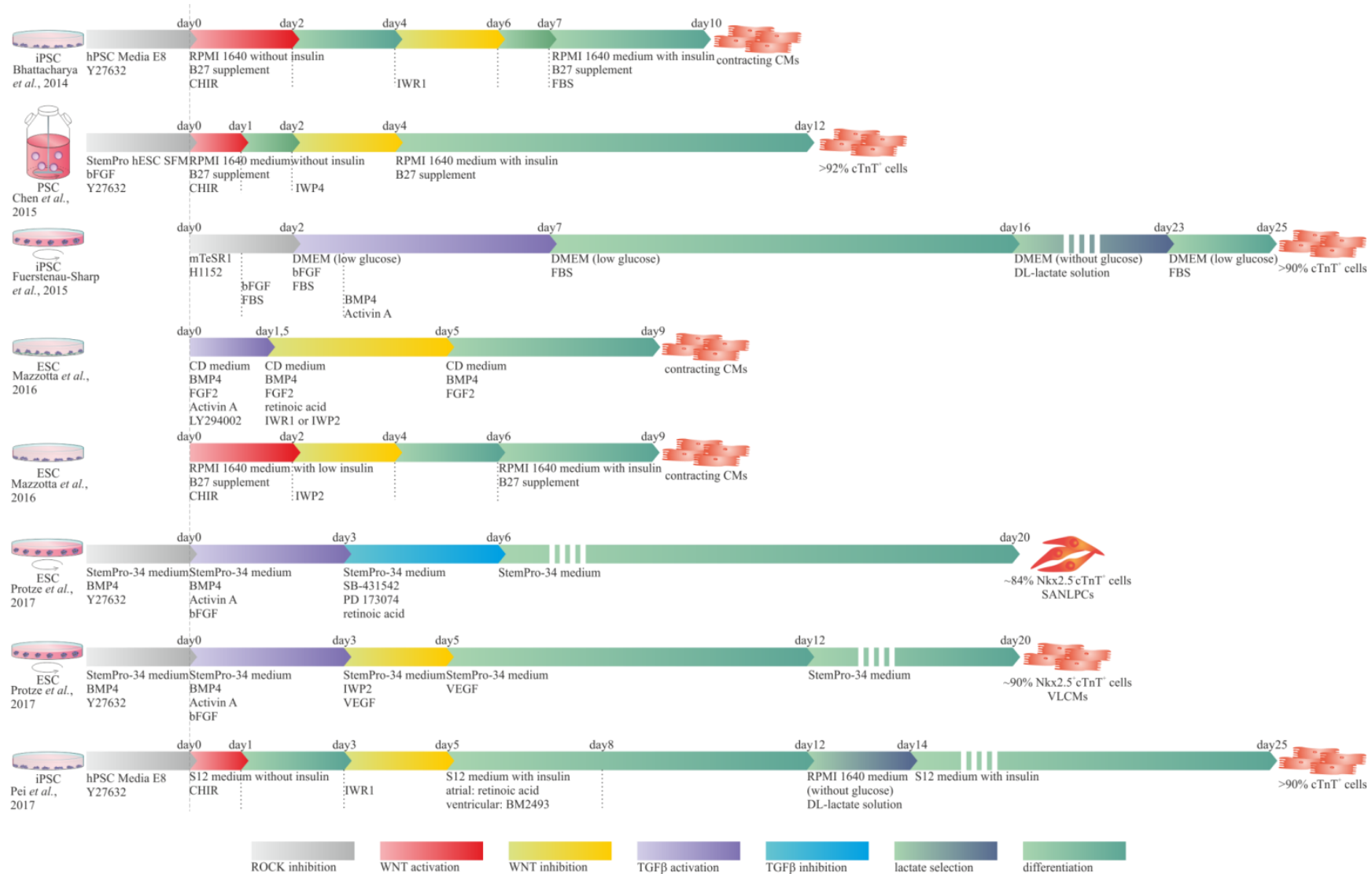


Figure 2: Schematic timescale for targeted differentiation of human PSCs. This cartoon summarizes various strategies and protocols of pluripotent stem cell (ESC, iPSC) differentiation towards cardiomyocytic cells, relying on time- and dose-dependent application of cardiogenic modulators without any nucleic acid introduction or factor overexpression.

4.2.3. Selection-strategies

Notwithstanding the existence of *in vitro* approaches described above, purity of PSC-CMs is still a major issue, with the choice of suitable and highly reliable surface marker for use in standard flow cytometry or magnetic isolation procedures for differentiating CMs still under debate. Accordingly, purification strategies based on cardiac specific intra-cellular marker expression remain an important approach.

Stable PSC lines containing: i) α MHC (99 % MHC⁺ cells) [171,172] or VE-cadherin (47 % cTnT⁺ cells) [173] promotor-linked antibiotic resistance genes have been used to efficiently select α MHC⁺ cells or a cardiac and endothelial dual-progenitor population; ii) α MHC promotor-linked green fluorescence protein (GFP) gene [168], enabling GFP⁺ flow cytometric selection. Both methods have the disadvantage of requiring stable exogenous DNA introduction. iii) Another approach, applied by Bao's group, enables the purification of CMs through specific molecular beacons (MBs) which target mRNAs, such as MBs targeting myosin heavy chain 6/7 (97 % cTnT⁺ cells) [174] or NPPA (92 % α -actinin⁺ cells) [175] mRNA. Additional approaches used include the selection of CM-like cells via: iv) mitochondria-specific fluorescent dyes (99 % α -actinin⁺ cells) [176]; or v) antibodies against partially cardiomyocyte-specific markers, e.g. SIRPA (signal-reduced protein alpha) (98 % cTnT⁺ cells) [177], EMILIN2 (elastin microfibril interface 2) (no qualitative statement about α -actinin⁺ or cTnT⁺ cell yield)[178], or VCAM1 (vascular cell adhesion molecule 1) (95 % cTnT⁺ cells) [179]. A consequence of the increasing mitochondrial-to-cell volume ratio during CM development is the metabolic substrate shift from glucose and lactate in early developmental stages to the primary reliance on fatty acid oxidation characterizing adult mature CMs [180]. These changes have been applied in non-genetic metabolic purification strategies (98 % α -actinin⁺ cells) [158,160,181,182]. Initial approaches used glucose-deplete/lactate-enriched media, achieving quite homogenous populations and increased purity [158,160]. In this regard, Kuppusamy *et al.* demonstrated a positive impact on cardiac maturation exerted by the let-7 family of microRNAs which are associated with metabolic energetics in maturing CM [183]. Overall, at present, other than standard promotor-linked selection approaches, the remaining novel selection strategies still require further evidence of reproducibility in independent laboratories using different PS cell lines.

An important consideration is that current PSC-derived CMs represent a mixture of nodal-, atrial-, ventricular-like and early-intermediate immature phenotypes, as evident from electrophysiological and pharmacological studies [89,134]. Transplantation of such CM mixtures could induce significant arrhythmia [184], thus further purification will be an indispensable prerequisite for clinical translation.

4.2.4. Maturation-strategies

In general, all published reports have - to a greater or lesser extent - generated an immature and physiologically incomplete CM phenotype, reflecting the heterogeneity of PSCs used as well as the distinction between human and murine cell lines [138]. A variety of substrates and protocol modifications are under close scrutiny with the goal of obtaining improvements in cardiogenic differentiation during culture to enable safe bench-to-bedside implementation.

Differential effects can arise from the cultivation procedure itself, including an important influence of cultured temperature and distinctions between use of 2D monolayer or 3D EB formation. Low oxygen preconditioning (2 % O₂) can impact on lineage commitment [185], with murine ESCs displaying significantly increased expression of the early differentiation markers FGF5 and Eomes consistent with preferred differentiation towards mesodermal and endodermal lineages. In contrast, culturing human PSCs under low oxygen tension prior to spontaneous differentiation in EBs primes commitment to an ectodermal lineage, indicated by significant induction of β 3-tubulin and Nestin [185].

An important but contentious issue is the use of either diverse co-culture systems or an adjusted material surface. PSC-derived CMs cultured on fibronectin-coated micro-grooved polydimethylsiloxane (PDMS) scaffolds exhibit a more organized sarcomeric structure, together with a more homogenous alignment and improved sarcoplasmic reticulum-based Ca²⁺ cycling [186]. Angelo's group introduced a biomimetic aligned nanofibrous cardiac patch which resembles the extracellular matrix of decellularized myocardium from rats [187]. The material used, namely polylactide-co-glycolide (PLGA), is an FDA approved therapeutic device due to its biodegradability and biocompatibility and has great potential to form the basis of an implantable cardiac patch. Moreover, this anisotropic environment additionally results in symmetric alignment of iPSC-derived CMs. ESC differentiation on intermediate stiffness polyacrylate (PA) hydrogel substrate resulted in only a slight enhancement of differentiation in comparison to common (rigid) polystyrene tissue culture [188]. Promising approaches using matrix-free, GMP-compliant culture protocols have yielded 94 % cTnT⁺ cells [157], which may facilitate advance towards clinical use.

hESCs co-cultured with AKT-activated endothelial cells led to an improvement in Nkx2.5⁺ cell yield as well as faster beating frequencies compared to hESCs cultured on Matrigel alone [189]. These findings concur with observations of other groups exploiting the benefits of co-culture [119,171,190]. Co-culture with MEF or SNL feeder cells has yielded better results than Matrigel for the majority of cell lines investigated [190]. Other approaches rely on matrix-cell-composites, such as BCTs (bioartificial cardiac tissue: cells plus liquid collagen type I plus Matrigel) [171], or the application of cardiac extracellular matrix [119]. In order to mimic endogenous tissue with blood capillary networks, Akashi's group have developed a vascularized 3D-iPSC-CM tissue, which may provide more comprehensive data in the field of drug screening [191]. Another technique relies on the co-culture of human cardiac microvascular endothelial cells (hCMVECs) and hMSCs in combination with induced pluripotent stem cell-derived embryonic cardiac myocytes (hiPSC-ECMs) which also aims at generating *in vitro* vascularized cardiac tissue scaffolds [192]. The considerable potential importance of culturing hiPSC-CMs as human 3D heart tissues to overcome species-dependent discrepancies of CM behavior has also been highlighted, particularly with respect to preclinical drug screening [193].

To ensure valid comparisons between techniques, a standard characterization procedure is essential. However, to date the expression of a multitude of TF or surface markers have been interrogated at quite diverse time points. However, consistency with respect to both time and marker information is very important, substantiated by recently published data revealing time-dependent morphological and electrophysiological alterations of iPSC-derived CMs [194]. Reliance on 2D morphological analysis alone to determine CM growth and maturation has been suggested to be insufficient, with clear volume differences apparent with hypertrophic stimulation or long-term culture using 3D z-stack myofilament analysis [195]. In addition, the

success of engraftment will be dependent on the state of the applied CMs, as has been demonstrated using an ischemic heart model in immuno-deficient mice [196].

To minimize risk of off-target effects attributable to incomplete knowledge of iPSC-derived CM behavior, their response to pharmacological manipulation is of central importance. Measurement on Multi-Electrode-Arrays (MEAs) are increasingly used for cell characterization and drug screening [197–199]. The use of voltage-sensitive fluorescent dyes (VSDs), such as di-4-ANEPPS, will also likely facilitate the investigation of action potential characteristics as has been demonstrated for commercially available hiPSC-derived cardiomyocytes (“iCells” and “Cor.4U”) post-substance administration [200]. Monitoring of iPSC-CMs under conditions simulating ischemia is a prerequisite to correctly evaluate the impact of hypoxia and nutrient deprivation on future cell replacement therapies. In this regard, Brodarac *et al.* demonstrated poorer tolerance of murine iPSC-CMs to hypoxia and nutrient deprivation compared to neonatal murine cardiomyocytes, with a significantly higher proportion of poly-caspase-active, 7-aminoactinomycin D-positive and TUNEL-positive cells [201].

In summary, much remains to be done before safe cardiac cell replacement therapy based on PSC strategies becomes a clinical reality, in particular the introduction of GMP-compliant standards underlying precise DNA-, viral- and xeno-free protocols for the generation of mature functional CMs.

Table 2: Overview of recently published programming strategies of ESCs toward diverse cardiovascular subtypes

Cell origin	Host	Delivery system	<i>in vivo</i> / <i>in vitro</i>	Factor / Substances	Target cell type	Special features	Literature
ESC(HES-2, H1, H9)	human	specific culture conditions	<i>in vitro</i>	day 0-1: 0.5 ng/mL of BMP-4 day 1-4: 10 ng/mL BMP-4, 5 ng/mL human bFGF, and 6 ng/mL Activin A day 4-8: basal medium containing 10 ng/mL VEGF, 150 ng/mL Dkk-1 day 8-end: basal medium with 10 ng/mL VEGF, 10 ng/mL human bFGF MEF-free and serum-free hESC adherent culture under cGMP and cGLP conditions	CM-like	27 % cTnT ⁺ expression of: sMHC, β MHC, Isl-1, Nkx2.5, MYH6, Tnnt2, Myl2, and Myl7	Chen <i>et al.</i> , 2012 [163]
ESC (H7)	human	specific culture conditions	<i>in vitro</i>	1) MEF and SNL feeder cell layers + conventional SC culture medium containing ko-SR 2) bFGF 3) Matrigel matrix + commercial mTeSR1 medium	CM-like	most efficient protocol: MEF and SNL feeder cell layers + conventional SC culture medium containing ko-SR least efficient protocol: Matrigel matrix + commercial mTeSR1 medium; neural lineage induction	Ojala <i>et al.</i> , 2012 [190]
ESC (m: J1; h:H1)	mouse / human	specific purification method	<i>in vitro</i> / <i>in vivo</i>	CM-specific MBs	CM-like	<i>in vitro</i> : myosin heavy chain-MB: ~97 % cTnT ⁺ cells <i>in vivo</i> : improved cardiac function, without tumor formation after 4 weeks	Ban <i>et al.</i> , 2013 [174]
ESC	mouse	specific culture conditions	<i>in vitro</i>	GSK3 inhibitor P38 MAPK inhibitors CaMKII inhibitors ERK activators	CM-like	ERK activators, CaMKII inhibitors: proliferative effects only on CMs in early developmental stage GSK3 inhibitor (BIO, CHIR), ERK activator (5 μ M SU1498), CaMKII inhibitor (5 μ M	Uosaki <i>et al.</i> , 2013 [168]

						KN93): induced cell cycle progression in CM, resulting in CM proliferation	
ESC	mouse	specific culture conditions	<i>in vitro</i>	44 cytokines/signaling molecules on day 3 of diff	CPC (Nkx2.5 ⁺)	IGF1, IGF2, insulin, Wnt3a: significantly increase CPC formation IGF, insulin: promote Bry ⁺ mesodermal cell proliferation Activin A, BMP2 or BMP4: decrease CPC formation	Engels <i>et al.</i> , 2014 [162]
ESC	mouse	specific culture conditions	<i>in vitro</i>	2 % O ₂ preconditioning (3 passages) of ESCs diff as EBs in 20 % O ₂	CM-like	significant increased expression of early differentiation markers FGF5, Eomes increased gene expression of Eomes, Goosecoid, Bry, AFP, Sox17, FoxA2, and protein expression of Bry, Eomes, Sox17, FoxA2 - diff into mesodermal and endodermal lineages	Fynes <i>et al.</i> , 2014 [185]
ESC	human	specific culture conditions	<i>in vitro</i>	2 % O ₂ preconditioning (3 passages) of ESCs diff as EBs in 20 % O ₂	CM-like	decreased expression of early differentiation markers FGF5, Eomes increased gene expression Nestin, β 3-tubulin – diff into ectodermal lineage	Fynes <i>et al.</i> , 2014 [185]
ESC (H9)	human	specific substrate mechanics	<i>in vitro</i>	1) TCPS 2) PA hydrogel substrate	CM-like	intermediate stiffness of PA hydrogel yielded slightly higher cTnT ⁺ cells without significant difference to TCPS	Hazeltine <i>et al.</i> , 2014 [188]
ESC (GSES)	mouse	DNA-Plasmid specific purification method	<i>in vitro</i>	Tbx3 Myh6-promoter-based antibiotic selection	SAN-like	>80 % physiologically and pharmacologically functional pacemaker cells with highly increased beating rates (300–400 bpm)	Jung <i>et al.</i> , 2014 [89] Rimmbach <i>et al.</i> , 2015 [90]
ESC	mouse	lentiviral	<i>in vitro</i>	Fndc5	CM-like	sign. expression of: Flk1, Isl1, Nkx2.5, Gata4, Mef2C, α -MHC, cTnT, α -actinin, SM22 α , α -SMA	Rabiee <i>et al.</i> , 2014 [152]
ESC (H7, ESI-017)	human	specific culture and substrate conditions	<i>in vitro</i>	matrix-free, scalable, and GMP-compliant process culture: including first CHIR and second IWP-4 induction	CM-like	>90 % pure CM; 1.5 to 2 $\times 10^9$ CM/L (up to 1 L spinner flasks) ESI-017: 6 μ M CHIR - ~91 % cTnT ⁺ H7: 12 μ M CHIR - ~92 % cTnT ⁺	Chen <i>et al.</i> , 2015 [157]

ESC	mouse	lentiviral	<i>in vitro</i> / <i>in vivo</i>	Nkx2.5 Isl1	CM-like SAN-like	Overexpression of Nkx2.5: inhibition of Isl1 expression Overexpression of Isl1: enhanced specification of cardiac progenitors, earlier cardiac differentiation, and increased cardiomyocyte number, upregulation of nodal-specific genes (e.g. Hcn4), downregulation of transcripts of working myocardium	Dorn <i>et al.</i> , 2015 [155]
ESC (R1)	mouse	adenoviral	<i>in vitro</i>	Shox2	SAN-like	increase in Cx45, decrease in Cx43, Nkx2.5 SHOX2-EBs beat spontaneously (83±7 % versus 15±6 %) pacemaker-like AP profile (62 %)	Ionta <i>et al.</i> , 2015 [154]
ESC (CGR8, αPIG44)	mouse	specific culture conditions	<i>in vitro</i>	100 μM AA	CM-like	AA application from day 0-2 increases cardiogenesis 2-4-fold day 5: increased expression of genes associated with angiogenesis, blood vessel development, hematopoiesis/erythropoiesis, Bry, Mef2c, Myl7	Ivanyuk <i>et al.</i> , 2015 [169]
ESC (H7)	human	specific purification method	<i>in vitro</i>	CM-specific MBs	CM-like	NPPA-MB: ~92 % α-actinin ⁺ cells	Jha <i>et al.</i> , 2015 [175]
ESC (α-PIG)	mouse	specific culture and substrate conditions	<i>in vitro</i>	0.3 mm-thick hcECM	CM-like	hcECM supported proliferation significantly increased expression of: Myh6, Tnnt2, Nkx2.5 Matrigel or Geltrex use did not induce cardiac-specific markers	Oberwallner <i>et al.</i> , 2015 [119]
ESC (derived from C57BL/6 mouse strain)	mouse	specific culture conditions	<i>in vitro</i>	10 μM resveratrol	CM-like	promotes mESC differentiation towards CM enhanced beating properties of EBs significantly higher expression of: Nkx2.5, Mef2c, Tbx5, dHand2, αMHC, Cx43, cTnI	Ding <i>et al.</i> , 2016 [202]
ESC	mouse	DNA-Plasmid	<i>in vitro</i>	CIBZ	CM-like	CIBZ depletion: induced expression of Bry, MesP1, Gata6, Sox17 CIBZ overexpression: decreased expression	Kotoku <i>et al.</i> , 2016 [151]

						of Bry, MesP1, Flk1 Isl1, Gata4, Mhc, cTnl; Significant suppression of beating EBs	
ESC (in-house and E14tg2a)	mouse	specific culture conditions	<i>in vitro</i>	monolayer culture without feeder cells day 0-1: IMDM/Ham's F12, N2 supplement B27 supplement, 0.5 mM AA, 4.5×10^{-4} M MTG day 1-3: 8 ng/mL Activin A, 0.5 ng/mL BMP4, 5 ng/mL hVEGF day 3-13: StemPro-34 SF medium, 0.5 mM AA, 5 ng/mL hVEGF, 10 ng/mL bFGF, 50 ng/mL hFGF10	CM-like	60 % CM 35-40 % cTnT ⁺ /MF20 ⁺ E14tg2a: more efficient CM-like cell yield with 5 ng/mL Activin A	Kokkinopoulos <i>et al.</i> , 2016 [165]
ESC	mouse	specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	25 ng/ml Activin, 20 ng/ml BMP2, 20 ng/ml BMP4, 100 ng/ml DLL1, 10 ng/ml bFGF, 10 ng/ml FGF8, 20 ng/ml Tgfb, 100 ng/ml Wnt3a, 5 μ M IWR1, 5 μ M SB431542	MesP1-CPC	BMP4 exposure: day 0-4 highest improvement of MesP ⁺ cells (29.6 %) BMP4 + IWR1: MesP ⁺ cells (13.8 %), however differentiate more efficiently into cardiac myocytes In vivo: injection of day 5 MesP1-CPCs led to improved survival of MI mice and decreased scar formation	Liu <i>et al.</i> , 2016 [144]
ESC (E14T)	mouse	specific purification method	<i>in vitro</i> / <i>in vivo</i>	selection based on VE-cadherin promoter	CEDP	Differentiation into: ~ 47 % cTnT ⁺ and ~28 % VE-cadherin ⁺ cells	Maltabe <i>et al.</i> , 2016 [173]
ESC (H7 and H9)	human	specific culture conditions	<i>in vitro</i>	1) day 0-1.5: BMP4, FGF2, Activating A, LY294002 day 1.5-5: BMP4, FGF2, RA, IWR1/IWP2 day 5-9: BMP4, FGF2 2) day 0-2: CHIR99021, day 2-4: IWP2	CM-like	WNT3, WNT8: regulation of Bry expression and mesoderm induction (via FZD7 + canonical Wnt signaling) WNT5A/5B: regulation of MesP1 expression and cardiovascular development (via ROR2 + noncanonical Wnt signaling) WNT2, WNT5A/5B, WNT11: regulation of late functional CM diff (via FZD4, FZD6 + noncanonical Wnt signaling)	Mazzotta <i>et al.</i> , 2016 [161]

ESC (5)	mouse	DNA-Plasmid	<i>in vitro</i>	CITED2	CM-like	<i>Cited2</i> depletion: significantly decreased expression of Brachyury, Mesp1, Isl1, Gata4, Tbx5 <i>Cited2</i> overexpression: stimulation of Brachyury, Mesp1, Isl1, Gata4, Tbx5, Myh6, cTnT; protein interaction with Isl1	Pacheco-Leyva <i>et al.</i> , 2016 [150]
ESC	mouse	miRNA lentiviral	<i>in vitro</i>	miR-322/-503	CM-like	Highest enriched miRNA in Mesp1 lineage (miR-322/-503) Celf1 is a direct target of miR-322/-503 miR-322/-503 selectively inhibits neuroectoderm differentiation	Shen <i>et al.</i> , 2016 [145]
ESC	human	specific culture conditions	<i>in vitro</i>	monolayer-directed differentiation protocol different concentrations of activin A and BMP4	CM-like EC-like	generation of distinct CVP populations following derivation of cardiogenic versus hemogenic mesoderm >90 % CD31 ⁺ /VE-cadherin ⁺ ECs	Palpant <i>et al.</i> , 2017 [164]
ESC (HES3-Nkx2.5 ^{gfp/w} , HES2)	human	specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	EB formation, 5 % O ₂ (d0-12) Generation of VLCMs: day 0-3: 10 ng/mL rhBMP4, 6 ng/mL rhActivinA, 5 ng/mL rhbFGF day 3-5: 0.5 μM IWP2, 10 ng/mL rhVEGF day 5-12: 5 ng/mL rhVEGF day 12-20: without additional factors day 20: FACS sorting (NKX2.5:GFP ⁺ SIRPA ⁺ CD90 ⁻) Generation of SANLPCs: day 0-3: 3 ng/mL rhBMP4, 2 ng/mL rhActivinA, 5 ng/mLrhbFGF day 3-6: 2.5 ng/mL rhBMP4, 5.4 μM SB-431542, 0.25 μM Retinoic Acid (HES3: 480–960 nM PD 173074 at day 4, HES2 at day 3)	CM-like SAN-like	FGF pathway blocks the development of NKX2-5 ⁺ CM Marker expression of the SAN lineage (TBX18, SHOX2, TBX3), typical pacemaker action potentials (90 %), ion current profiles and chronotropic response	Protze <i>et al.</i> , 2017 [159]

				day 6-20: 5 ng/mL rhVEGF day 20: FACS sorting (NKX2.5:GFP ⁻ SIRPA ⁺ CD90 ⁻)			
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AA: ascorbic acid; β MHC: β myosin heavy chain; bFGF: basic fibroblast growth factor; BMP: bone morphogenetic protein; bpm: beats per minute; Bry: Brachyury; CAMKII: Ca²⁺/calmodulin-dependent protein kinase II; CEDP: cardiac and endothelial dual-progenitor population; CHIR: CHIR99021, Wnt activator; CIBZ: BTB domain-containing zinc finger protein; CM: cardiomyocytes; CPC: cardiac progenitor cell; cTnC1: cardiac troponin C1; CVP: cardiovascular progenitor cell; diff: differentiation; EB: embryoid bodies; EC: endothelial cell; ERK: extracellular signal-regulated kinase; ESC: embryonic stem cells; Fndc5: Fibronectin type III domain-containing 5 protein (also known as: peroxisomal protein (PEP)); GSK3: glycogen synthase kinase-3; hcECM: human cardiac extracellular matrix; IWP: WNT inhibitor; ko-SR: knockout serum replacement; LY294002: phosphoinositide 3-kinase inhibitor; MAPK: p38 mitogen-activated protein kinase; MB: molecular beacon; MTG: monothioglycerol; PA: polyacrylamide; PD 173074: FGF signaling inhibitor; SAN: sinoatrial node; SB-431542: Activin/Nodal/TGF β signaling inhibitor; sMHC: sarcomeric myosin heavy chain; TCPS: tissue culture polystyrene; Tnnt2: cardiac troponin T2; VEGF: vascular endothelial growth factor; (x): number of cell lines used.

Table 3: Overview of recently published programming strategies of iPSCs toward diverse cardiovascular subtypes

Cell origin	Host	Delivery system	<i>in vivo</i> / <i>in vitro</i>	Factor / Substances	Target cell type	Special features	Literature
iPSC	mouse and human	specific culture conditions	<i>in vitro</i>	16 cytokines and chemical components	CM-like	AA: CM enhancement in 11 lines Application of 50 µg/mL AA during day 2-6: 7.3-fold (miPSC) and 30.2-fold (hiPSC) enhanced yield through specific increase in the proliferation of CPCs via the MEK-ERK1/2 pathway	Cao <i>et al.</i> , 2012 [203]
iPSC (UTA.00112. hFF, UTA.04602. WT, UTA.00525.L QT2, UTA.00106.h FF)	human	specific culture conditions	<i>in vitro</i>	1) MEF and SNL feeder cell layers + conventional SC culture medium containing ko-SR 2) bFGF 3) Matrigel matrix + commercial mTeSR1 medium	CM-like	most efficient protocol: MEF and SNL feeder cell layers + conventional SC culture medium containing ko-SR least efficient protocol: Matrigel matrix + commercial mTeSR1 medium; neural lineage induction exception: UTA.04602.WT line - highest amount of beating areas on Matrigel in mTeSR1	Ojala <i>et al.</i> , 2012 [190]
iPSC (BJ1-iPS10)	human	specific selection method	<i>in vitro</i> / <i>in vivo</i>	CM-specific MBs	CM-like	<i>in vitro</i> : myosin heavy chain-MB: ~97 % cTnT ⁺ cells <i>in vivo</i> : improved cardiac function, without tumor formation after 4 weeks	Ban <i>et al.</i> , 2013 [174]
iPSC	human	long-term culture	<i>in vitro</i>	EB-formation 360 day culture	CM-like	day 14: immature high-density Z-bands day 180: mature Z-, A-, H-, and I-bands day 360: M-, Z-, A-, H-, and I-bands	Kamakura <i>et al.</i> , 2013 [204]
iPSC	mouse and human	specific culture conditions antibiotic selection	<i>in vitro</i>	αMHC-antibiotic resistance gene EB formation BCT (cells + liquid collagen type I and Matrigel) AA static stretch	CM-like	structurally and functionally homogenous syncytium enhanced the contractility of murine and human BCTs (active tension of 4.4 mN/mm ²)	Kensah <i>et al.</i> , 2013 [171]
iPSC (from: skeletal muscle PC,	mouse	retroviral specific culture	<i>in vitro</i>	16 h after EB seeding onto gelatin: exposure to hypoxia (3 % O ₂) for 24 h	CM-like	EBs normoxic conditions: ~59 % beating EBs hypoxic conditions: abolished beating, significant increased expression of Bry and	Medley <i>et al.</i> , 2013 [205]

skeletal muscle fibroblasts)		conditions				Isl1, HIF-1 α ; accumulation of HIF-1 α and β -catenin in nuclear protein extracts, suggesting involvement of the Wnt/beta-catenin pathway	
iPSC	human	specific culture conditions	<i>in vitro</i>	day 0-1: 6 μ M CHIR day 2-3: diff medium day 4-5: 5 μ M IWR-1 day 6-7: diff medium day 8-end: diff medium + insulin	CM-like	expression of: IRX4, MLC2v, MLC2a, TNNT3, and TNNT2	Bhattacharya <i>et al.</i> , 2014 [156]
iPSC (from BJ fibroblasts)	human	mRNA reprogramming specific culture conditions	<i>in vitro</i>	day 0-8: EBs in low adhesion culture dish, 5 μ M SB203580 day 9-week 8: EBs plated on 0.1 % gelatin coated dishes, without SB203580	CM-like	day 11-14 until day 50-60: rhythmically contracting areas (55-75 %) day 6: expression of Isl1, Kdr, Mef2C, NKx2.5, Gata4, Tbx3/5/20 day 8, 14: expression of Tnni2, Mlc2a/v, Myh7, Myl3/4, Ca _v 1.3, Hcn4, Serca2a, Ryr2 specific response to pharmacological substance administration	Mehta <i>et al.</i> , 2014 [133]
iPSC (IMR90)	human	specific culture conditions	<i>in vitro</i>	day 20-27: 20 ng/mL T3	CM-like	increased cardiomyocyte size, anisotropy, and sarcomere length 2-fold higher force per-beat	Yang <i>et al.</i> , 2014 [206]
iPSC (from Dr. Joseph Wu, Stanford)	human	specific culture and substrate conditions	<i>in vitro</i>	matrix-free, scalable, and GMP-compliant process culture: including first CHIR and second IWP-4 induction	CM-like	>90 % pure CM; 1.5 to 2 $\times 10^9$ CM/L (up to 1 L spinner flasks) relationship between aggregate size and CHIR concentration 6 μ M CHIR 94 % cTnT ⁺	Chen <i>et al.</i> , 2015 [157]
iPSC (from activated T cells, hPBMC)	human	retroviral specific culture conditions metabolic selection (lactate)	<i>in vitro</i>	day 1-13: 5 % O ₂ and 7 % CO ₂ day 1-2: 50 % mTeSR, 45 % DMEM (low glucose), 5 % FBS, 1 μ M H1152, 100 ng/mL bFGF day 2: 2/3 medium change to: DMEM (low glucose), 10 % FBS, 50 ng/mL bFGF day 3-7: DMEM (low	CM-like	before selection: 30-45 % CM, expression of cTnT and MLC2v after lactate selection: 90 % CM, electrophysiological characterization: ventricular, atrial and nodal-like action potentials	Fuerstenau-Sharp <i>et al.</i> , 2015 [158]

				glucose), 10 % FBS, 50 ng/mL bFGF, 6 ng/mL activating A, 10 ng/mL BMP4 day 8-13: DMEM (low glucose), 10 % FBS day 14-end: 20 % O ₂ and 7 % CO ₂ day 16-18: DMEM (no glucose), 1 % sodium DL- lactate solution			
iPSC	mouse	specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	αMHC-GFP EB formation diff. after EB: 1 week treatment with 1 % DMSO and/or 100 ng/mL NRG-1β	CM-like	significantly higher expression of: Gata4, Gata6, αMhc, Myhl7, Myl3, cTnnc1, Ryr2, Serca2a ventricular-like AP preserves cardiac function in mouse model	Iglesias-García <i>et al.</i> , 2015 [207]
iPSC (TiB7.4, AT25)	mouse	specific culture conditions	<i>in vitro</i>	100 μM AA	CM-like	AA application from day 0-2 increases cardiogenesis 2-4-fold	Ivanyuk <i>et al.</i> , 2015 [169]
iPSC (DF19-9 –11T.H)	human	RNA	<i>in vitro</i>	EB-formation diff after EB: transfection with CIR RNA	CM-like	spindle-shaped cells with characteristic expression of: cTnT, tropomyosin, α-actinin	Kochegarov <i>et al.</i> , 2015 [208]
iPSC (AT25)	mouse	specific culture and substrate conditions	<i>in vitro</i>	0.3 mm-thick hcECM	CM-like	hcECM supported the proliferation significantly increased expression of: Myh6, Tnnt2, Nkx2.5 Matrigel, Geltrex: did not induce cardiac- specific markers	Oberwallner <i>et al.</i> , 2015 [119]
iPSC	mouse	lentiviral	<i>in vitro</i>	telomerase-competent cell lines (TRF1 expression) AA	CM-like	iPSCs ^{highTRF1} : differentiate earlier and more efficiently into CMs iPSC ^{lowTRF1} : differentiate very efficiently to the ectoderm lineage AA: increase CM yield with iPSC ^{highTRF1}	Aguado <i>et al.</i> , 2016 [170]
iPSC (Duanqing Pei, Chinese Academy of Sciences)	mouse	specific selection method	<i>in vitro</i>	0.001 – 0.2 μmol/L EPI 10 μmol/L phentolamine 5 μmol/L propranolol 10 μmol/L phenylephrine 10 μmol/L clonidine	CM-like	EPI and activation of α1-AR: enhancement of CM differentiation via MEK-ERK1/2 signaling	Li <i>et al.</i> , 2016 [167]

				1 μ mol/L isoproterenol 10 μ mol/L PD98059			
iPSC (from: MEFs)	mouse	retroviral specific culture conditions	<i>in vitro</i>	EB formation medium containing: DMEM, 15% fetal bovine serum, 0.2 mmol/l L-glutamine, 0.1 mmol/l nonessential amino acids, and 0.1 mmol/l β -mercaptoethanol	CM-like	After EB formation: expression of Flk1, α -actinin, α -MHC, cTnT, Cx43, Nkx2.5 active beating: 360 bpm incompletely differentiated iPS cells: teratoma formation after transplantation into a SCID mouse model of MI	Wang <i>et al.</i> , 2016 [209]
iPSC (from foreskin tissue)	human	specific culture conditions metabolic selection (lactate)	<i>in vitro</i>	chemical-defined and albumin-free medium day 0-1: S12 without insulin medium, 4-8 μ M CHIR day 1-3: S12 without insulin medium day 3-5: S12 without insulin medium, 5 μ M IWR-1 day 5-end: S12 with insulin medium, atrial diff: 1 μ M RA, ventricular diff: 1 μ M BMS493	CM-like Atrial-like	highly homogenous atrial and ventricular myocytes in a scalable fashion with normal electrophysiological properties	Pei <i>et al.</i> , 2017 [160]

AA: ascorbic acid; AP: action potential; AR: adrenergic receptor; BCT: bioartificial cardiac tissue; bpm: beats per minute; BMS493: RA inhibitor; Bry: Brachyury; CHIR: CHIR99021 (GSK-3 inhibitor, Wnt activator); CIR: specific fetal cardiac-inducing RNA (fragment of N-sulfoglucosaminesulfohydrolase and the caspase recruitment domain family member 14 precursor); Cx43: connexin 43; clonidine: selective α 2-AR agonist; cTnT: cardiac troponin T; DMSO: dimethyl sulfoxide; EPI: epinephrine; hcECM: human cardiac extracellular matrix; hPBMC: human peripheral blood mononuclear cells; H1152: Rho-kinase (ROCK) inhibitor; IRX4: iroquois-class homeodomain protein IRX-4; isoproterenol: β -AR agonist; IWP-4: WNT inhibitor; IWR-1: WNT inhibitor; MEF: mouse embryonic fibroblast; MLC2a: myosin regulatory light chain 2, atrial isoform; MLC2v: myosin regulatory light chain 2, ventricular/cardiac muscle isoform; NRG-1 β : Neuregulin-1 β ; PC: progenitor cells; PD98059: MEK1/2 inhibitor; MI: myocardial infarction; phenylephrine: selective α 1-AR agonist; phentolamine: α -AR antagonist; propranolol: β -AR antagonist; RA: retinoic acid; SB203580: MAPK signaling inhibitor; SCID: severe combined immunodeficiency; TNNT3: cardiac troponin I; Tnnt2: cardiac troponin T2; TRF1: shelterin-complex protein; T3: Tri-iodo-L-thyronine.

4.3. Direct reprogramming of somatic cells

Another ambitious approach to cell replacement therapy aims to avoid the relatively uncontrollable pluripotent state, instead choosing to elicit a cell fate switch through the direct conversion of terminally differentiated somatic cells towards mature cell types of interest representing the same germ layer, or even across germ layers. Such an approach has a number of prerequisites for efficient and feasible reprogramming, including epigenetic modulation and lineage-specific intervention.

As early as 1987, murine fibroblasts were successfully converted into skeletal muscle cells using only a single key TF, namely MyoD [210]. Subsequently, numerous publications have reported direct conversion - with one or a number of TFs combined - into several somatic cell types, such as insulin⁺ β -cells, hepatocytes, osteoblasts, hematopoietic lineage cells, neurons and cardiomyocytes (summarized in [211]). However, the quality of the cells obtained was highly variable. While, thus far, no single master regulator has been discovered to efficiently induce the switch of fully differentiated somatic cells towards a mature induced cardiomyocyte (iCM) lineage, promising results in the field of direct reprogramming have been made and will be discussed in this section.

The concept of patient-specific lineage-conversion holds enormous potential for future clinical applications including: i) elucidating individual disease pathogenesis; ii) lower risk of tumorigenesis and inflammation after cell transplantation compared with PSCs; iii) avoiding the need for transplantation through the possibility to directly convert resident cells; and iv) avoidance of ethical concerns regarding cellular source. Despite the many advantages, the actual consequences of massive fibroblast-to-myocyte programming *in situ* remain unknown and may potentially be detrimental to cardiac function [212].

Contemporary research efforts focus on readily available murine cell types, with murine neonatal cardiac fibroblasts (CFs) currently the most efficient somatic cell source for direct reprogramming based on their heterogeneity and plasticity, as well as their resistance to the hypoxic environment of the injured myocardium [213–215]. Another important source are murine embryonic fibroblasts [216–220]. However, neither of these two cell types are readily accessible from humans with few results so far reported using such human cell types [221,222]. In contrast to iPSC strategies, a cell fate switch is largely achieved through forced exogenous overexpression of lineage-specific TFs, with Gata4, Tbx5 and Mef2c the most frequently used TFs in combination [219,223–229], or at least a critical part of a more complex composition [216,217,220,221,230,231] (Table 4). However, the reported marker gene expression patterns obtained vary widely between different laboratories and starting material; e.g. 30 % [227] or 35 % [223] cTnT+ cells, 10-15 % iCM [224], 3 % [225] or 20 % [229] α MHC+ cells. Moreover, the cells display only marginal similarity to mature CM based on their molecular and electrophysiological phenotype [223]. Such low efficiencies may reflect insufficient construct design with respect to expression stoichiometry, with a tailored ratio of protein expression – constituting higher protein levels of Mef2c in combination with lower levels of Gata4 and Tbx5 – shown to enhance programming efficiency [228]. Several approaches now aim to improve reprogramming efficiency as well as maturation of iCMs by adding further TFs of the cardiac lineage such as Hand2 [216,217,219,220,222], together with signaling modulators such as inhibitors of TGF- β (A83-01, SB431542) [217,230], WNT (XAV939) [230] or ROCK (SR-3677, Thiazovivin, Y-27632) [217].

A number of pro-cardiogenic microRNAs have been identified [232–234] and applied in combination with TFs [217,222] or as sole modulators [235–237] for direct re-programming. MicroRNAs, for example microRNA-1, can interact with myogenic TFs such as SRF (serum response factor), Mef2c, MyoD or Nkx2.5 in a regulatory

loop as repressors and cooperators [238–240]. MicroRNA-1 negatively impacts the Notch signaling pathway via direct repression of Dll1 [241] and its downstream factor Hes1 [102], resulting in expression of Gata4, Nkx2.5 and Myogenin. A combination of miR-1/-122/-208/499 and JAK inhibitor I has been demonstrated to induce a cell fate switch towards a cardiac-like phenotype with 28 % α MHC+ cells *in vitro* and improved cardiac outcomes *in vivo* [235–237].

One approach focuses on the use of a chemical cocktail to convert murine embryonic fibroblasts to iCMs with spindle, rod and round shaped morphologies [218]. The cells generated manifest action potentials of atrial- and ventricular-like cells. Notwithstanding the heterogeneity of the cells obtained, this method offers a potential alternative to genome integrative methods which may prove safer. However, the effective time windows for each chemical modulator, which include signaling pathway activators/inhibitors or epigenetic regulators, has to be clearly defined to achieve optimal results [220].

While progress has been made, so far the iCMs obtained are still immature in phenotype and inhomogeneous as a population, lacking the terminal structural and electrophysiological characteristics of authentic adult CMs.

Table 4: Overview of recently published direct reprogramming strategies of somatic cells toward diverse cardiovascular subtypes

Cell origin	Host	Delivery system	<i>in vivo</i> / <i>in vitro</i>	Factor / Substances	Target cell type	Special features	Literature
neonatal cardiac fibroblasts	mouse	retroviral or lentiviral	<i>in vitro</i> / <i>in vivo</i>	Gata4, Mef2c, Tbx5	CM-like	30 % cTnT ⁺ cells (to a lesser extent in tail-tip fibroblasts) Upregulation of: Myh6, Actc1, Actn2, Nppa	Ieda <i>et al.</i> , 2010 [227]
neonatal cardiac fibroblasts	mouse	lentiviral microRNA specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	miR-1/-122/-208/499 JAK inhibitor I	CM-like	day 3: 2- to 3-fold upregulation of Mef2c , Tbx5 , Hand2 , Nkx2.5 , Gata4 day 6: expression of cTnT, cTnI, sarcomeric actinin reprogramming efficiency: 1.13–5.28 % in non-JAK inhibitor I-treated cells with JAK inhibitor I: 28 % α MHC ⁺ cells enhanced cardiac function in mouse model	Jayawardena <i>et al.</i> , 2012 and 2014 [235,236]
fibroblasts (from hESCs; H9)	human	retroviral	<i>in vitro</i>	EGFP, ESRRG, GATA4, MEF2C, MESP1, TBX5, MYOCD, ZFPM2 SIS3	CM-like	20 % functional Ca ²⁺ transients cardiac marker expression of: cTnT, α -Actinin, ACTC1, ACTN2, MYH6, MYL2, MYL7, TNNT2, NPPA, PLN, and RYR2 SIS3 significantly decreases α MHC ⁺ cells	Fu <i>et al.</i> , 2013 [221]
tail tip and embryonic fibroblasts (B6;129S4)	mouse	retroviral	<i>in vitro</i>	M3 domain of mouse MyoD fused on carboxy-terminus of Mef2c, Gata4, Hand2, Tbx5 GSK126 (day 1-4), UNC0638 (day 3-7)	CM-like	Reprogramming efficiency: MM ₃ -GHT: 3.5 % (> 15-fold increase) MM ₃ -GHT + GSK126: further increase to control 2.1-fold (most efficient combination) MM ₃ -GHT + UNC0638: further increase to control 2-fold	Hirai <i>et al.</i> , 2013 [219] and 2014 [220]
NRVM	rat	adenoviral	<i>in vitro</i> / <i>in vivo</i>	Tbx18	SAN-like	downregulation of Cx43 pacemaker-like AP profile (9.2 %)	Kapoor <i>et al.</i> , 2013 [242]
neonatal foreskin and adult fibroblasts	human	retroviral	<i>in vitro</i>	Gata4, Hand2, Tbx5, myocardin, miR-1/-133 Culture time: 4-11 weeks	CM-like	~35 % tropomyosin ⁺ cells ~20 % cTnT ⁺ cells	Nam <i>et al.</i> , 2013 [222]
CM	pig	adenoviral	<i>in vitro</i> /	Tbx18	SAN-like	mean HR was higher in TBX18-transduced	Hu <i>et al.</i> , 2014

			<i>in vivo</i>			animals sympathetic predominance in the TBX18-transduced group TBX18-transduced animals had persistent and stable activity	[243]
embryonic fibroblasts (C57BL/6)	mouse	chemical cocktail	<i>in vitro</i>	on Matrigel 2-stage protocol: day 0-16: CRM (knockout DMEM, 15 % FBS, and 5 % KSR, 0.5 % N2, 2 % B27, 1 % Glutamax, 1 % NEAA, 0.1 mM β -mercaptoethanol, 50 μ g/ml AA , 100 units/ml penicillin, 100 μ g/ml streptomycin) + CRFVPT (10 μ M CHIR (C), 10 μ M RepSox (R), 50 μ M Forskolin (F), 0.5 mM VPA (V), 5 μ M Parnate, (P), 1 μ M TTNPB (T)) day 17-end: CMM (DMEM medium, 15 % FBS, 2i (3 μ M CHIR, 1 μ M PD0325901), 1000 units/mL LIF, 50 μ g/mL AA, and 1 μ g/mL insulin)	CM-like	morphology: spindle shape, rod shape or round shape spontaneously beating activity: increases from day 8 cardiac marker expression of: Mef2c, α - Actinin, Gata4, cTnT, Nkx2.5, α -MHC, N- cadherin, Cx43, cTnl action potential of atrial- and ventricular- like CMs	Fu <i>et al.</i> , 2015 [218]
cardiac fibroblasts	mouse	retroviral antibiotic selection	<i>in vitro</i>	Gata4, Mef2c, Tbx5	CM-like	stoichiometry of G, M, T protein expression influences reprogramming efficiency high Mef2c and low Gata4, Tbx5 most efficient	Wang <i>et al.</i> , 2015 [228]
embryonic fibroblasts	mouse	retroviral	<i>in vitro</i>	Gata4, Hand2, Mef2c, Tbx5, miR-1/-133, Y-27632, Thiazovivin, SR-3677, A83- 01	CM-like	spontaneously beating activity without signaling inhibitors: GHMT > day 21, GHMT+miR-1/-133 > day 8 ROCK inhibitors enhance reprogramming of MEFs TGF- β inhibitors enhance reprogramming of MEFs most efficiently	Zhao <i>et al.</i> , 2015 [217]

embryonic, adult cardiac, tail tip	mouse	retroviral	<i>in vitro</i>	Gata4, Hand2, Mef2c, Tbx5, Akt1	CM-like	spontaneously beating activity: MEFs > day 7 (50 % > day 21), CFs > day 14, TTFs > day 21; responsive to β -adrenoreceptor pharmacologic modulation, polynucleated, and hypertrophic	Zhou <i>et al.</i> , 2015 [216]
neonatal cardiac fibroblasts	mouse	retroviral (TFs) and lentiviral (shRNA)	<i>in vitro</i>	Gata4, Mef2c, Tbx5 shRNA of 35 selected components of chromatin modifying or remodeling complexes	CM-like	Bm1 downregulation significantly enhanced CM generation	Zhou <i>et al.</i> , 2016 [231]
neonatal cardiac fibroblasts	mouse	TFs specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	Gata4, Mef2c, Tbx5 SB431542 XAV939	CM-like	8-fold increased reprogramming efficiency beating cells 1 week after reprogramming enhanced cardiac function in mouse model	Mohamed <i>et al.</i> , 2017 [230]

AA: ascorbic acid; Actc1: cardiac α -actin; Actn2: actinin α 2; AP: action potential; Akt1: Akt1/protein kinase B; A83-01: TGF- β inhibitor; CHIR: CHIR99021 (GSK-3 inhibitor, Wnt activator); CM: cardiomyocytes; GSK126: Enhancer of Zeste Homolog 2 (Ezh2) inhibitor; KSR: knockout serum replacement; Myh6: α -myosin heavy chain; NEAA: non-essential amino acid; Nppa: natriuretic peptide precursor type A; NRVM: neonatal rat ventricular myocytes; PD0325901: MEK1/2 inhibitor; RepSox: TGF- β 1 inhibitor; SAN: sino-atrial-nodal cells; SB431542: TGF- β inhibitor; shRNA: small hairpin RNA; SIS3: SMAD3 inhibitor (activated downstream of TGF β signaling); SR-3677: ROCK inhibitor; TF: transcription factor; Thiazovivin: ROCK inhibitor; TTNPB: analog of retinoic acid; UNC0638: G9a and GLP inhibitor; VPA: valproic acid (histone deacetylase inhibitor); XAV939: WNT inhibitor; Y-27632: ROCK inhibitor.

4.4. Programming of cardiac conduction system cells

As outlined earlier, the availability of highly specific cardiomyocyte subtypes is critical for future tailored cell therapy of cardiovascular disease. The treatment of rhythm disorders by cardiac cell therapy will require a highly pure population of cells belonging to the cardiac conduction system [89,244–247]. As a corollary, while a mixture of cardiomyocyte subtypes is capable of coupling to working myocardium and setting the pace *in vivo* in a porcine model of complete atrioventricular block, the resulting rhythms are neither stable nor reliably exceed junctional escape rhythm rates [248].

4.4.1. Composition of the cardiac conduction system

Automaticity of the heart beat is crucial for life: the heart's regular contractile activity results from electrical impulses initiated and conducted by highly specialized cells within the heart which form the cardiac conduction system (CCS) [249–251].

The initial impulse is generated in a small number (~10,000) of highly specialized pacemaker myocytes which form the sinoatrial node (SAN) [242]. The SAN is located at the junction of the right atrium and the superior vena cava [249,250]. SAN cells differ from working myocardial cells in their content of ion channels and gap junction proteins [252,253]. In particular they are rich in hyperpolarization-activated cyclic nucleotide-gated cation channel 4 (HCN4) and t-type calcium channel (Ca_v3.1). SAN cells lack natriuretic factor (ANF) and the gap junction proteins connexin43 (Cx43, predominant in ventricular and atrial cells) and connexin40 (Cx40, expressed in atrial working myocardium) [249,252,253]. In contrast, the main gap junction protein in the SAN is connexin45 (Cx45). The membrane voltage clock (cyclic activation and deactivation of membrane ion channels, resulting in part from f-channel conductance of the “funny current”) and the subsarcolemmal Ca²⁺ clock (resulting from rhythmic spontaneous sarcoplasmic reticulum Ca²⁺ release) function synergistically to generate SAN automaticity [251]. An illustration of the major differences between these cells is that the upstroke of the action potential (AP) in SAN results from Ca²⁺-channels as opposed to voltage-gated Na⁺-channels typical of working myocardial cells [253].

The impulse that emerges then propagates rapidly and anisotropically through atrial myocardium until it reaches the atrioventricular node (AVN) where the signal is delayed. Atrial and ventricular myocardium are electrically isolated from each other, allowing the atria to contract first, a physiological requirement for optimal ventricular filling prior to ventricular systole [249,250].

The electrical impulse then proceeds through the ventricular conduction system (VCS). The latter consists of a fast-conducting atrioventricular bundle (AVB) (or His bundle), left and right bundle branches (BBs) and the Purkinje fiber network. The AVB commences at the AVN and proceeds through the ventricular septum, where it sub-divides into the right and left BBs. The BBs themselves divide into Purkinje fibers, which are spread over the entirety of the left and right ventricles, enabling synchronous biventricular contraction [249,250,254].

While AVN cells resemble those of the SAN, cells of the VCS differ significantly from both SAN and ventricular working myocardial cells. In particular, while depolarization involves voltage-gated Na⁺-channels, the “funny current” is still present in the VCS [253]. Similarly, the gap junction proteins are different, with Cx40 required in VCS cells to facilitate high conduction velocity from Purkinje fibers to ventricular cells [252].

Overall, a high degree of coordination is required between all these different cell types to ensure normal stability of cardiac rhythm and rate. One manifestation of a primary disturbance in cardiac pacemaker function is the so called “sick sinus syndrome” (SSS) [255].

4.4.2. Manifestations of sick sinus syndrome

SSS, or sinoatrial disease, refers to a chronic clinical syndrome reflecting SAN dysfunction which has a variety of causes [255]. It results in a variety of abnormalities, including sinus bradycardia, sinus pauses, sinus arrest and sinoatrial exit block and may result in chronotropic incompetence, i.e. insufficient augmentation of heart rate to meet physiological requirements during exercise or other stress [256]. In up to half of cases, SSS may be accompanied by paroxysmal atrial tachycardia and AVN conduction disturbance, as part of the tachycardia-bradycardia syndrome [255–259]. The rhythm disturbances can result in a variety of symptoms, including palpitations, lightheadedness, shortness of breath, exercise intolerance, fatigue and frank syncope [255].

While SSS occurs predominantly in the elderly, it is prevalent at all ages [255,257–259]. In young adults and children, SSS commonly results from post-operative atrial trauma or inherited disease. In the elderly, SSS may reflect progressive age-related attrition in SAN cell number [258], or result from a distinct disease process such as atherosclerosis. The sinus node is supported by the right coronary artery whose compromise transiently with ischaemia or through actual infarction can result in permanent SAN dysfunction [255,259]. Familial SSS has been described to arise from genetic mutations, characteristically originating from alterations in one of three genes [255]: two of these, HCN4 and SCN5A (sodium channel, voltage-gated, type V, alpha subunit), are crucial for transmembrane ion exchange and thus highly relevant for action potential generation. The third gene implicated, MYH6 (myosin, heavy chain 6, cardiac muscle, alpha) plays an essential role in myosin formation to support cardiomyocyte contractility [255].

At present, symptomatic SSS is treated by implantation of an electrical pacemaker device, regardless of underlying aetiology, and constitutes one of the major indications for permanent pacemaker implantation globally (30-50 % of all cases) [255,257–260].

While the advent of implantable permanent pacemakers has revolutionized the management of life-threatening or highly symptomatic bradycardias, dramatically improving symptoms, quality of life and, in specific cases, prolonging survival [261] it bears some limitations. These can include the possibility of device infection (which can necessitate removal of the entire pacing system), limited battery lifespan (necessitating intermittent generator changes in those who are pacemaker dependent at specific intervals), lead damage or vessel thrombosis, dyssynchronous electromechanical activation and incomplete recapitulation of physiological heart rate increments and interference with external electromagnetic devices [262]. While many of these are theoretical or of small consequence at the individual patient level, their occurrence (particularly device infection) can be serious and can pose a relevant cumulative risk in certain groups, e.g. the paediatric pacing-dependent population.

To circumvent the concerns inherent to implantable electronic devices, a number of quite promising approaches towards engineering a biological pacemaker have recently appeared. These employ two principle strategies [260]: virus-based gene transfer aimed at converting resident cardiac cells into cells with pacemaker properties [242,243,263]; or, a cell-based strategy, in which *in-vitro* pre-processed cells are transplanted into

the heart as pacemakers [89,159,264,265]. Of note, when generated from patient-derived iPSCs, such cells may become of great importance for personalized *in vitro* drug testing.

4.4.3. Modification and direct reprogramming of working myocardial cells

Generation of CCS cells follows similar principles to those used for working cardiomyocyte (re-)programming. However, the programming factors need to be carefully selected for this highly specific purpose. TFs such as T-box 3 (Tbx3), T-box 18 (Tbx18), short stature homeobox 2 (Shox2) or ISL LIM homeobox 1 (Isl1) play critical roles in the intrinsic development of PCs, but are absent or highly downregulated in the other cardiomyocyte subtypes [155,266–269]. The expression of ion channel components such as HCN4, calcium voltage-gated channel subunit alpha1 D (Cacna1d, Ca_v1.3), or the calcium voltage-gated channel subunit alpha1 G (Cagna1g, Ca_v3.1) also differs between PCs and the working myocardium, as do gap junction proteins such as Cx45 and Connexin30.2 (Cx30.2), both expressed instead of Cx43 [270–277].

The TF Tbx3 functions as transcriptional repressor during embryonic development, preventing expression of TFs typical for the working myocardium and thereby formation of working myocardial CMs. While Tbx3 is not solely responsible for SAN formation, it imposes a pacemaker gene program [269]. Accordingly, use of Tbx3 for direct reprogramming of resident myocardium cells appears attractive and has been evaluated in two different studies of Tbx3 overexpression in murine hearts. However, neither Tbx3 expression in atrial myocardium [267], nor tamoxifen-induced expression of Tbx3 in whole working myocardium [263] leads to fully functional PCs. While both reports demonstrate that Tbx3 can partially induce a number of pacemaker-related genes, the resulting cells still differ significantly from native pacemaker cells (PMCs) with regard to their overall expression patterns. In the first study, SAN specific markers were found to be upregulated such as Hcn4, Cx30.2 and Lbh. Similarly, atrial specific markers were downregulated, including natriuretic peptide A (Nppa), Cx40, Cx43 and sodium voltage-gated channel alpha subunit 5 (Scn5a, Nav1.5) [267]. The second study recapitulated the expression of some of these genes in isolated atrial cells (e.g. Lbh, Nppa, Cx43 and Cx40), but others reacted differently, such as Hcn4 which exhibited an unaltered expression pattern. Further, in the atrium another member of the cyclic nucleotide gated potassium channel family (Hcn1) was upregulated, while Hcn4 was actually downregulated in ventricular cells. Remarkably, the expression level of the TF NK2 homeobox 5 (Nkx2-5), which plays a pivotal role in working myocardium, was not modified by Tbx3 expression in these cells [263]. Overall, these findings suggest that Tbx3 alone is insufficient to convert working myocardium into PCs.

A related SAN-specification factor, Tbx18, has been tested after initial experiments with rat neonatal ventricular myocytes [242]. In contrast to other TFs tested (Shox2, Tbx3, Tbx5 and Tbx20), only Tbx18 transduction has been shown to significantly increase the number of spontaneously beating cultures. The resulting cells exhibit a more pacemaker-like morphology, as well as enhanced HCN4 expression and pacemaker-like cellular automaticity. Although the beating frequency of the transduced cells was double that of control cells, it was still far lower than that observed *in vivo* in rat hearts (95 bpm vs. 350bpm) [242]. Importantly, the effect of Tbx18 has not only been demonstrated *in vitro*, but also reported in a large-animal model *in vivo* using adenoviral gene transfer. After pilot experiments in guinea pig hearts [242], a consecutive study evaluated the ability of Tbx18-expressing adenovirus injected into the interventricular septum of pigs to rescue induced complete heart block [243]. Examination of both animal models revealed evidence of partial transformation into pacemaker-like cells after transduction, as reflected by upregulation of Hcn4 and downregulation of working myocardial genes (Cx43 and Nkx2-5). In addition, ventricular ectopic beats were

induced in both guinea pig and pigs hearts [242,243], with automaticity of the pig heart largely independent of the backup implanted electronic pacemaker for the short duration of the study [243]. Single cell analysis of the transduced guinea pig heart indicated the effect of Tbx18-expression to be only very transient: after 6 weeks, less than one third of transduced cells retained their pacemaker-like-morphology. However, due to limitations with respect to the recovery of single cells from the pig heart, insights into *in situ* reprogramming that could be gained from single cell analysis were lacking. Consistent with the temporal time course of expression of adenoviral vectors, SAN functional testing using electronic burst ventricular pacing revealed a rapid recovery in Tbx18-transduced animals at day 8, which increased to levels comparable with the control group (percutaneous GFP injected) by 2 weeks [243]. While the study describes the first partially successful *in situ* reprogramming towards a biological pacemaker in a clinically relevant large animal model, the long term effects of Tbx18-based reprogramming of working myocardium remain to be determined [242,243]. In this regard, a recent study used two independent Cre/loxP-mediated conditional transgenic mouse models to express Tbx18 in the atrial and ventricular myocardium during fetal development to further investigate the ability of Tbx18 to convert working myocardium into pacemaker cells [278]. Ectopic expression of Tbx18 was discernible from E12.5 or E14.5 and caused right ventricular hypoplasia, atrial dilatation and ventricular septal defects. In contrast to the experiments of the Marban group, no upregulation in expression of SAN-related genes was found in working myocardium, despite downregulation in chamber specific genes such as Cx40 and Nav1.5. Notably, Tbx18 expression also induced ectopic expression of atrial and ventricular marker genes, including Nppa in the ventricles and myosin, light polypeptide 2, regulatory, cardiac, slow (Myl2, Mlc2v) and myosin, heavy polypeptide 7, cardiac muscle, beta (Myh7) in the atria [278]. It remains to be seen whether the contrasting outcomes of the two studies arise from application of Tbx18 in different species [243,278], or from differing expression time points in the heart (fetal [278] vs. adult [243]). Such considerations will need to be carefully addressed to obviate potential side effects before Tbx18 overexpression can be used to generate a biological pacemaker in patients.

Based on the experience with directly reprogramming fibroblasts into spontaneously beating cells, it seems likely that such an approach can be further developed to generate distinct cardiomyocyte subtypes, including pacemaker cells [279]. To identify possible TFs for such reprogramming, a study examined an initial group of 20 candidates by transducing them into embryonic fibroblasts of a mouse line expressing GFP under the control of Hcn4 regulatory promoter regions. An iterative process of successive omission of candidates expendable for EGFP expression led to the definition of the smallest group of factors promoting reporter expression, namely: Tbx5, Tbx3, Gata6 and either Retinoic acid receptor, gamma (Rarg) or Retinoid X receptor, alpha (Rxra). However, induction of significant Hcn4 expression alone does not seem sufficient to generate functional pacemaker cells as no spontaneous beating activity was observed, nor were the cells excitable via depolarization stimuli [279]. Moreover, in further experiments fibroblast transduction experiments using the “classical” cardiomyocyte reprogramming factors Gata4, Hand2, Mef2C and Tbx5, some of the reprogrammed cells expressed Hcn4-GFP. More detailed analysis revealed that this approach yielded multiple potential cardiac cell types: atrial-like, pacemaker-like and ventricular-like. Together, these data suggest that reprogramming of fibroblasts into PMC may be feasible in principle, but is still currently far from being reliably established.

4.4.4. Nodal cell programming of adult stem cells

Several studies have described diverse modifications of adult stem cells for pacemaker cell generation. These have primarily employed mesenchymal stem cells [101,280–291] derived from canine [101,281–284], rat

[285,292], rabbit [289–291] or human [280,286,287] tissue. Additional reports describe using adipose tissue-derived stem cells [288,293]. Most groups using ASCs chose to overexpress an Hcn-family member [280,282–287,289–291] to drive cell fate towards a nodal phenotype, while others used TFs such as Shox2 [101,281]. These have been combined with other treatments such as 5-Azacytidine [288] or electric-pulse current stimulation (EPCS) [281,282]. While utilizing divergent experimental setups, the scientific findings are quite comparable. Depending on the respective experiment, the resulting cells have displayed some nodal cell properties, for example measurable funny current (I_f) which could be enhanced with EPCS or with isoproterenol and blocked with cesium [281–283,287,290]. Moreover, expression of typical pacemaker genes like Cx45, Hcn4, Tbx3 are observed to increase, while genes associated with working myocardium, such as Cx43 and Nkx2-5, are downregulated [101,281,282]. Furthermore, a change in cell morphology towards a more pacemaker-like phenotype has been reported [101,281,288]. Co-culture of the modified ASCs with myocytes from newborn mice, regardless of origin, has led to increased beating frequency of the neonatal myocytes when compared to co-cultures with unmodified ASCs [101,287,290].

For *in vivo* testing, cells have been transplanted preferentially into canine hearts [283,287]. After induction of heart block, two publications describe the appearance of ventricular escape rhythms observed via ECG recordings [283,287]. However, this required vagal stimulation to induce sinus arrest to be apparent [283], and was associated with higher escape frequencies than control cell transplantation [287]. In all of these studies the lack of resulting cellular autonomous activity is strikingly consistent [101,280–292].

One report describes spontaneous activity of transformed ASCs derived from brown fat: interestingly, this phenomenon seems to reflect a reaction of the cells to the cultivation media as no genetic modification was applied [293]. While analysis of ultrastructural, proteomic, electrophysiological and pharmacological parameters of the resulting beating cells indicated the presence of some pacemaker-like features, further investigation is required, particularly over more prolonged culture periods [293].

In summary, significant further efforts are required to enable reprogramming of true nodal cells from ASCs.

4.4.5. Forward programming of pluripotent stem cells into nodal cells

PSCs represent a suitable source for any desired distinct cell type based on their unlimited differentiation potential. Concomitantly, this same unlimited differentiation potential represents a major obstacle to obtaining only a particular cell type. Thus a key challenge for the field is how to force PSCs exclusively towards a desired lineage.

The spontaneous differentiation rate of nodal cells from murine PSCs typically does not exceed ~1 %. While great improvements have been made recently with regard to the differentiation of human PSCs into cardiac cell phenotypes using specific culture conditions [161,188,190,294,295], the typical proportion of rare nodal cell types elicited still needs to be accurately defined.

Since the first description of mouse embryonic stem cells being differentiated into cardiomyocytes for the first time in 1991 [120], studies have examined the composition of different beating cells and attempted to specifically direct fate during differentiation. Besides considering cellular morphology and canonical marker expression patterns, the importance of recapitulating electrophysiological properties has been increasingly recognized. If one uses classical random differentiation protocols, the cells obtained represent a variety of

kinds of cardiomyocyte cell types: nodal, atrial, ventricular and immature cardiomyocytes [245]. Accordingly, a key focus of interest has been how to reliably influence cell fate during differentiation. At present, there are three main strategies to enhance the proportion of nodal cell types within culture: stimulation via intrinsic culture conditions, enrichment via selection and forced overexpression of specific TFs.

With respect to the first strategy, the small molecule compound EBIO (1-ethyl-2-benzimidazolinone), a small-/intermediate-conductance Ca^{2+} -activated potassium channel modulator, has been postulated to increase the formation of nodal cells from murine ES cells. While application of EBIO to ES cells has been reported to lead to induction of sino-atrial and reduction in chamber-specific myocardial programs, the resultant cells had low beating frequencies, with no confirmation of ability to pace myocardium, or electrophysiological discrimination between mature pacemaker cells and those of an early/intermediate cell type which also spontaneously contract [296]. Interestingly, a recent study addressed the influence of EBIO on human PSC differentiation, shedding light on a mechanism of action via lineage-specific effects. While addition of EBIO resulted in dose-dependent enrichment of cardiomyocytes, with increased nodal- and atrial-like phenotypes, the effect was mainly attributable to a EBIO-induced severe reduction in cell survival, thereby favouring cardiac progenitor cell preservation [297].

Recently, a promising study described the generation of hPSC-derived pacemaker cells using a specific differentiation protocol combined with surface marker selection based on SIRPA (signal-regulatory protein alpha) [159]. SIRPA represents a cell-surface marker suitable to isolate populations of cardiomyocytes from hPSCs [177]. In combination with a transgene-independent differentiation protocol [159], the resulting hPSC-derived SAN-like cardiomyocyte cells fulfill a number of typical pacemaker features; in particular they are capable of pacing host tissue post-transplantation into the apex of rat hearts. However, further points will need to be addressed to better understand the cell generated: early/intermediate cell types were not taken into account despite the fact that the funny channel densities reported resemble those of immature cells, with action potential curves revealing clear plateau phases; an investigation of the characteristic Ca^{2+} release from sarcoplasmic reticulum; an examination of the specific morphology of single cells, specifically for typical spindle- or spider-shaped cells. An additional concern is the observed atypical expression of Cx43 and Cx40 [159].

A separate study [298] based on surface marker purification to isolate SAN progenitors used the activated leukocyte cell adhesion molecule (Alcam, CD166 antigen) during murine ESC differentiation. While the cells obtained display some pacemaker characteristics, selection via Alcam appears to be extremely dependent on time point and species. Thus, cell sorting at different differentiation time points yielded different degrees of sarcomeric α -actinin expression, a marker of cardiac-committed cells [298]. An earlier study using ALCAM as an expression marker in human embryonic stem cells revealed that the enriched cells manifested an embryonic cardiomyocyte phenotype [299]. Notably, only about 10% of Alcam-selected cells retained Hcn4 expression after 3 weeks in culture, a finding which may reflect a maturation process of initially Hcn4 positive early/intermediate CMs towards principally working myocardial cells over this timeframe [298].

In a further setting, the transcription factor Shox2 has been used with the goal of generating nodal cells from murine ESCs. Hashem and Claycomb transfected cells with a plasmid bearing a neomycin resistance gene controlled via the Shox2 promoter. Subsequent neomycin application during differentiation led to an almost pure population of Shox2 positive cells. Analysis of expression signatures revealed intrinsic nodal

characteristics (Tbx3, HCN4, Cx45, Ca_v1.3, Ca_v3.1) as well as expression of other cardiac marker genes (Tbx5, Mlc2v, Cx43). However, while the cells were spontaneously active, no additional functional data were shown [300]. In a similar approach, Ionta *et al.* used Shox2 overexpression in mouse embryonic stem cells to force cells into a nodal cell lineage [264]. However, while the resulting cells were spontaneously active, the beating frequencies were below 80 bpm and therefore did not exceed those of WT-ES cell derived CM [264]. Consequently, it is unclear whether these cells represent functional pacemaker cells.

Our group has combined overexpression of the highly conserved key nodal cell inducer, Tbx3, with a neomycin resistance gene under control of the well-established α MHC-promoter. This approach leads to small aggregates consisting of ~300 - 500 cells, which we term “induced sino-atrial bodies” (iSABs). iSABs exhibit high beating frequencies of between 400-500 bpm *in vitro*, thereby for the first time truly corresponding to those of a murine heart and even exceeding *in vitro* cultivated nodal cells derived from mouse SAN. Evidence from extensive analysis of these cells, including confocal laser scanning microscopy, FACS, single-cell patch clamping (Figure 3 A), funny channel density measurements and Ca²⁺ imaging reveals that iSABs consistently represent over 80 % mature functional nodal cells, with the remainder constituting immature nodal cells. Additional single cell analysis identifies characteristic spindle (Figure 3 B) and spider (Figure 3 C) cell morphology. To further address the pacing potential of iSABs, we employed the *ex vivo* model system of cultivated mouse ventricular slices. Remarkably, iSABs were capable of integrating into these slices, retaining their spontaneous activity and pacing the heart slices to result in robust contraction. We confirmed functional coupling to the slices using calcium-transient analysis, which revealed synchronization between iSABs and slices. Therefore, we have introduced, for the first time, highly pure PSC-derived nodal tissue which is functional on the physiological level *in vitro*, as well as in an *ex vivo* model [89]. Recently, we utilized iSABs as an *in vitro* model system to help decipher the role of the γ 2 subunit of AMP-activated protein kinase (AMPK) in the regulation of SAN biology and infer a role for AMPK in control of mammalian intrinsic heart rate [301]. An important next step will be to determine the ability of iSABs to pace cardiac tissue *in vivo* and to prove whether this approach can be extended to human PSCs.

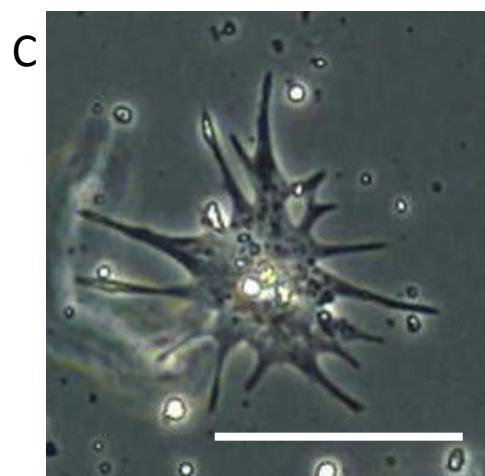
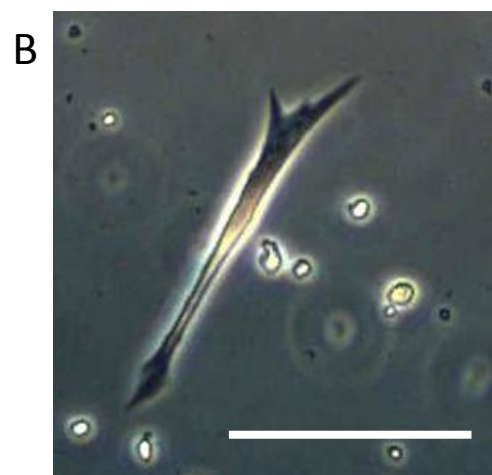
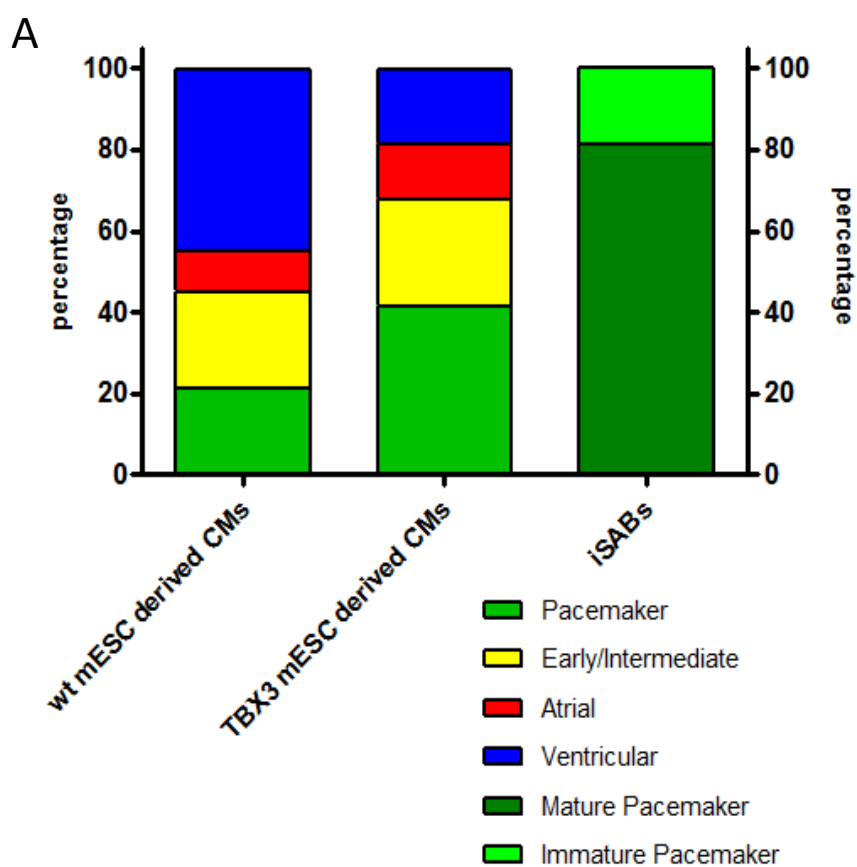


Figure 3: Properties of iSAB derived single cells. (A) Cardiac subtype distribution based on single cell patch clamp analysis and funny channel density measurements. Morphology of iSAB derived representative (B) spindle and (C) spider cells; scale bar 100 μ m.

4.4.6. Systems-based network approaches to enhance nodal cell programming

Further improvement of nodal cell programming will require an in depth understanding of underlying gene regulatory mechanisms. Given the complexity of the cardiovascular system and cardiovascular diseases, systems-based approaches play an increasingly important role in elucidating interactions between underlying traits and processes by using multiple ‘omics’ layers [302]. Such systems-based approaches are global analyses in which the different molecular levels are investigated and then integrated into qualitative and quantitative mathematical models (e.g. Boolean models, ordinary differential equations, network analysis concepts, etc.), providing an additional layer of understanding for cardiomyocyte dynamics [303]. Hence we used our recently developed iSABs, representing the first highly pure, stem cell programming-derived nodal cell tissue, to define the pacemaker transcriptome [91]. We highlight up-to-date systems-based approaches and compare our findings with the existing literature on a multi-level scale to verify the overall quality of the iSAB model system and address its potential transferability towards the human SAN (Figure 4).

The first step in such a systems-based data analysis procedure is to define the model system and available experimental input data (e.g. different SC-derived cardiomyocyte subtypes characterized by RNA-Seq data). Recent advances in high-throughput sequencing (HTS) have emphasized the important and versatile roles of coding and non-coding RNAs, including quantification of splice variants or identification of novel ncRNAs, during cardiac development. We used our RNA-Seq workflow, called *TRAPLINE*, to define the differences in transcriptomes between iSABs and randomly distributed mESC-derived cardiomyocyte subtypes [89,91]. Using the Galaxy framework, the numerous embedded data analysis workflows guarantees simple access, easy extension and flexible adaption of computational tools to individual needs, as well as sophisticated analyses that do not require in-depth command-line knowledge [304,305]. Data analysis with *TRAPLINE* results in a set of differentially expressed genes, their corresponding protein-protein interactions, splice variants, promoter activity and predicted miRNA-target interactions [91].

A central concept in systems biology is that networks, rather than classic linear pathways, underlie biological processes. The concept of biological networks arose when classic signaling pathways were represented as graphs in which the components (i.e. expressed gene transcripts) were termed nodes and their interactions (i.e. genes encoding transcription factors or protein-protein interactions) were called links or edges [306]. Demonstrating the general cellular differences between the two cell types through analysis of the transcriptome, we used the Biological Networks Gene Ontology tool (*BiNGO*) to determine the Gene Ontology (GO) terms significantly overrepresented in a set of significantly upregulated transcripts in iSABs (Figure 4A) [307]. These GO terms represent the accumulated biological processes of the significantly overexpressed transcripts. While the underlying statistical p-value, false discovery rate and family-wise error rate provide a good first impression of a specific functional category, it is important to also check the functional categories in the entire GO hierarchy. Thus it is highly likely that when a whole branch of the GO hierarchy is highlighted as being significantly overrepresented, the most intensely colored nodes furthest down the hierarchy can be expected to be the most biologically relevant ones.

The second step of the analysis characterizes the relevant components of the system (e.g. the actual set of significant differentially expressed gene transcripts driving the enhanced cardiac rhythm). A prominent method is that of gene set enrichment analysis (GSEA), performed by *ClueGo* [308]. The gene sets are analyzed on the basis of prior biological knowledge, such as use of GO or signaling pathways such as *Wikipathways*. Using

statistical tests like the Fisher exact test, one can then ask whether the genes are enriched within a collection of pathways. Such analyses are highly dependent on using current versions of curated sets of GO annotations. Only annotated transcripts and ncRNAs can be integrated through GWAS analyses. Newly discovered transcripts or ncRNAs have to be characterized beforehand by other experiments or *in silico* prediction simulations [309,310]. One approach to overcome this limitation and independently link coding and non-coding transcripts without using annotations is the use of weighted correlation network analysis to identify clusters of highly correlated genes or an intramodular hub gene [311]. As described earlier, in using iSABs to define a function for AMPK in intrinsic heart rate regulation [301], we constructed a gene co-expression network from the iSAB transcriptome and identified *Prkag2* in a module highly interconnected with known pacemaker-relevant genes. Hierarchical clustering and classical multi-dimensional scaling revealed, in common with the SAN transcriptome, that this *Prkag2*-containing module signified an important signaling hub with significant connectivity to genes vital for normal SAN function [301].

The third analysis step is to determine how the identified gene transcripts interact with each other or regulate other relevant interaction partners. This can be done by using data mining approaches [312] and databases such as BioGrid and String to incorporate resulting protein-protein interactions (PPTs) into the network, which is subsequently investigated for underlying dynamics and the molecular enrichment among genes within the network mathematically (i.e. how it responds to various perturbations and interconnects with other data layers). Based on the combined use of the BioGrid and String databases, we have obtained a network with 8,120 nodes and 55,720 edges, representing the interactome. After applying the tool *KeyPathwayMiner* [313], we were able to identify the most important subnetworks within the constructed interactome model to demonstrate known molecular interactions between significantly upregulated genes (Figure 4B). Once such a network is developed and available for the researcher, more sophisticated mathematical models can be applied to the network. Based on the input datatype available, one can employ ordinary differential equations (ODE), discrete modeling or hybrid modeling (composed of ODE and logic sub-modules) as a strategy to handle large scale, non-linear biochemical networks [314]. It has been shown that these kinds of network approaches are able to identify a specific regulatory core within a large gene network and, moreover, to predict receptor signatures associated with certain diseases [315]. Nevertheless, such *in silico* simulations and subsequent signature predictions still need experimental validation.

Ultimately, the information obtained at each analysis step is combined to draw conclusions about the complex behavior of the stem cell-derived cardiac pacemaker model and compared to current knowledge about the human SAN (Figure 4C). The results we have obtained for phenotypic associations such as contraction, electrophysiology, metabolism and differentiation factors, are in line with the current literature about the SAN. The knowledge gained from such systems-based analyses will be crucial for further optimization of cell programming and purification [91].

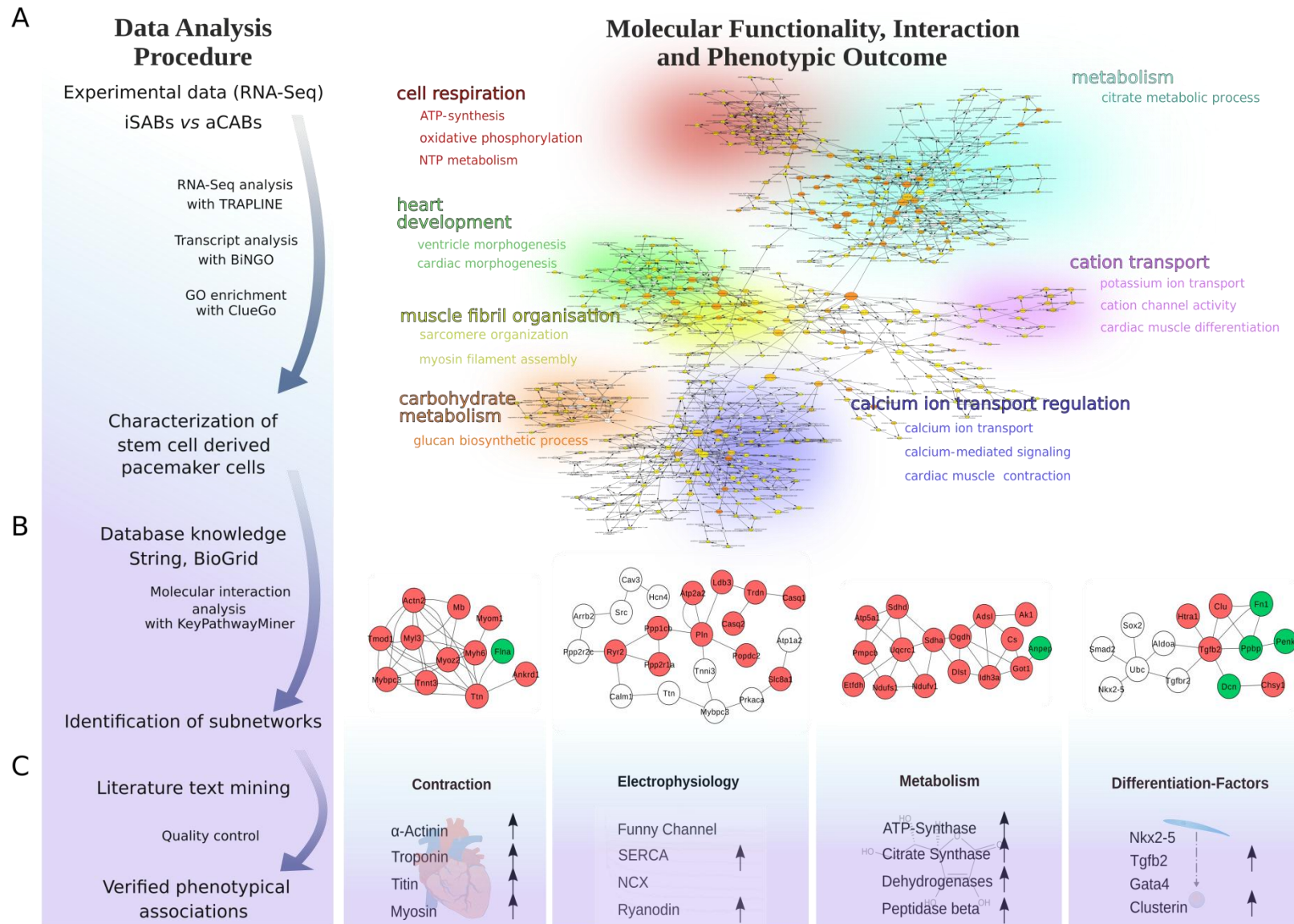


Figure 4: Systems-based data analysis procedure for the identification of molecular functionalities, interactions and phenotypic associations applied to stem cell-derived cardiac cell types by using RNA-Seq data. (A) Calculation of overrepresented GO terms using the Cytoscape applications BiNGO and ClueGo. **(B)** Identified subnetworks obtained after KeyPathwayMiner analysis of the former constructed interactome network. Red represents the upregulated transcripts within iSABs and green represents the downregulated transcripts. The edges (lines between encircled genes) are experimentally verified interactions obtained from String and BioGrid. **(C)** Summary of the upregulated factors identified in the data and the literature for processes within contraction, electrophysiology, metabolism and differentiation.

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Figure 1

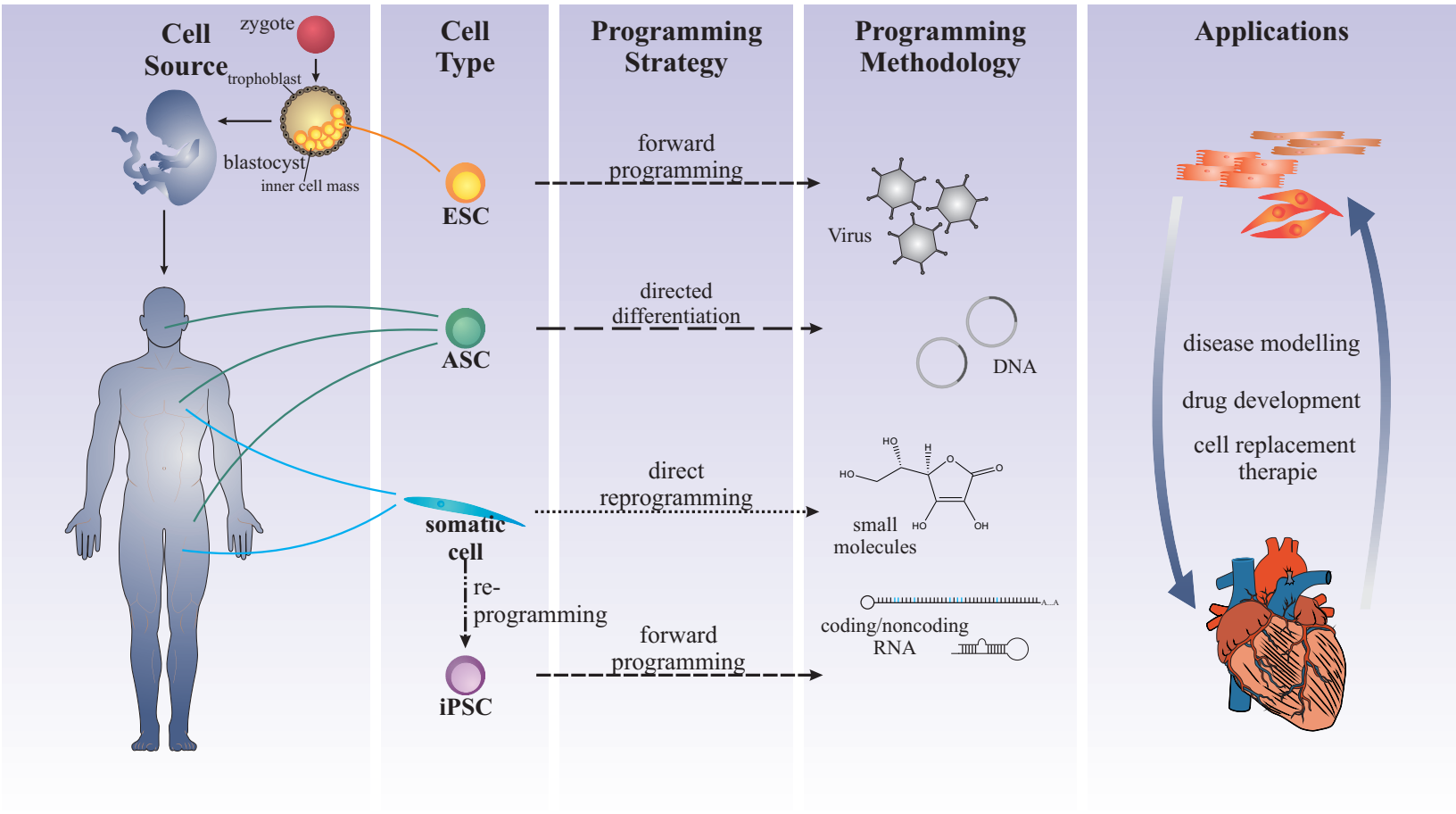


Figure 2

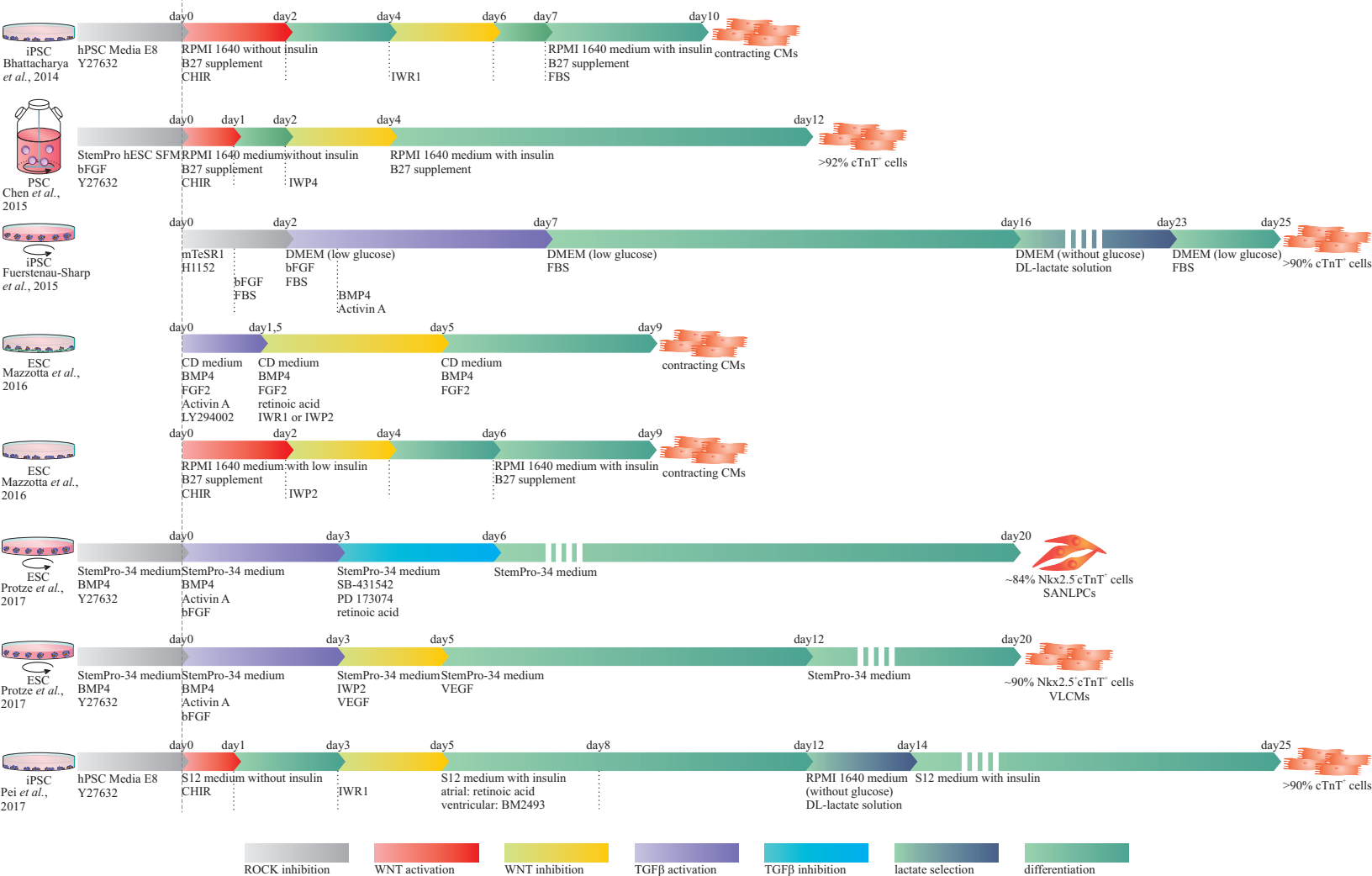


Figure 3

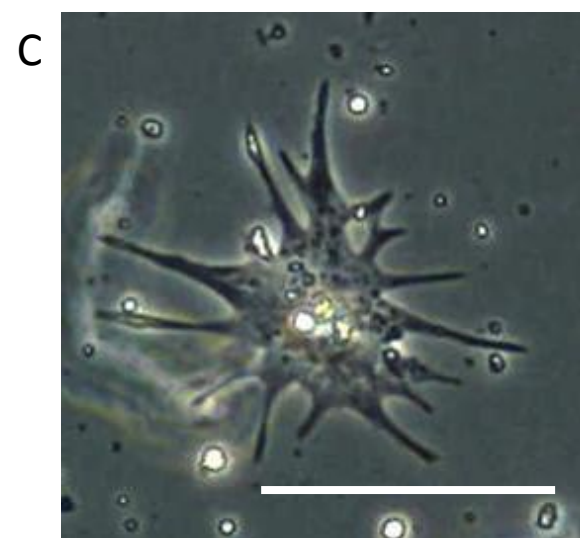
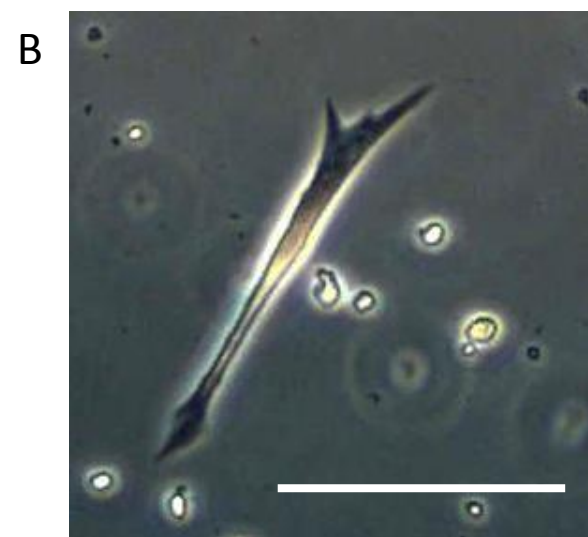
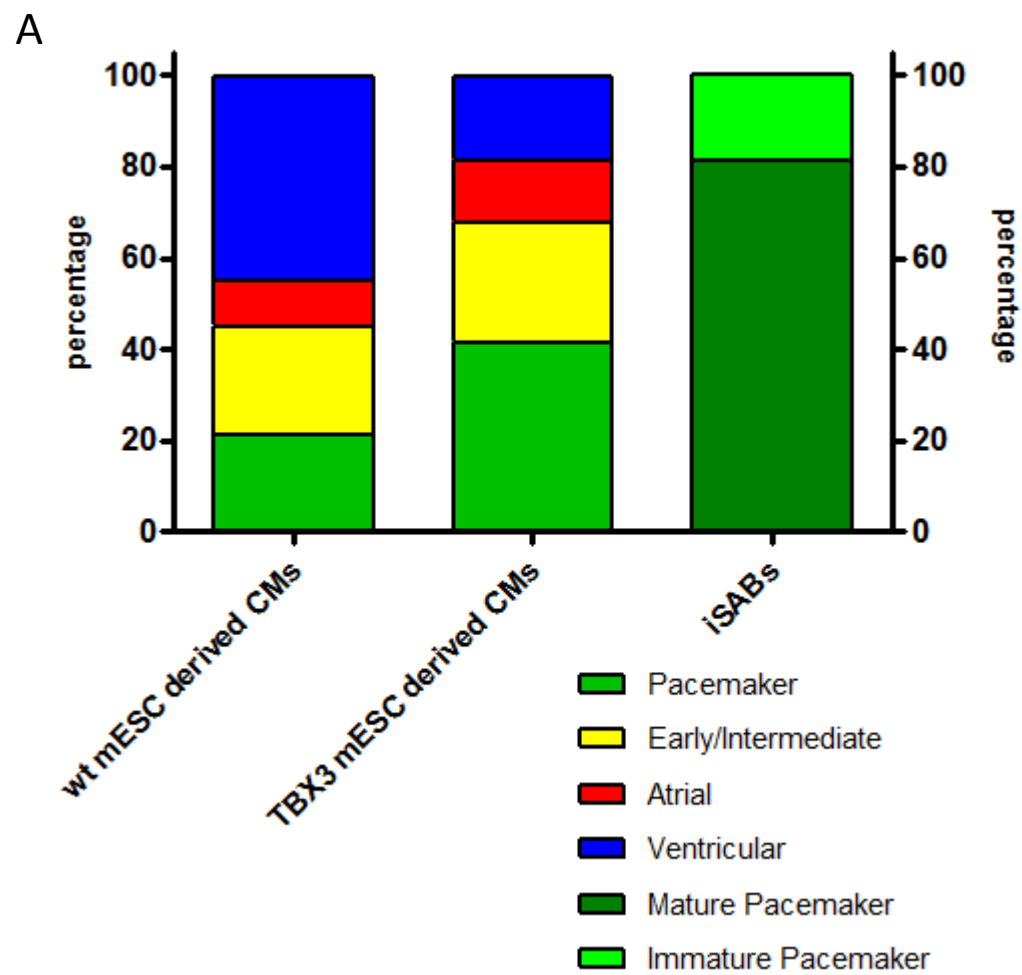
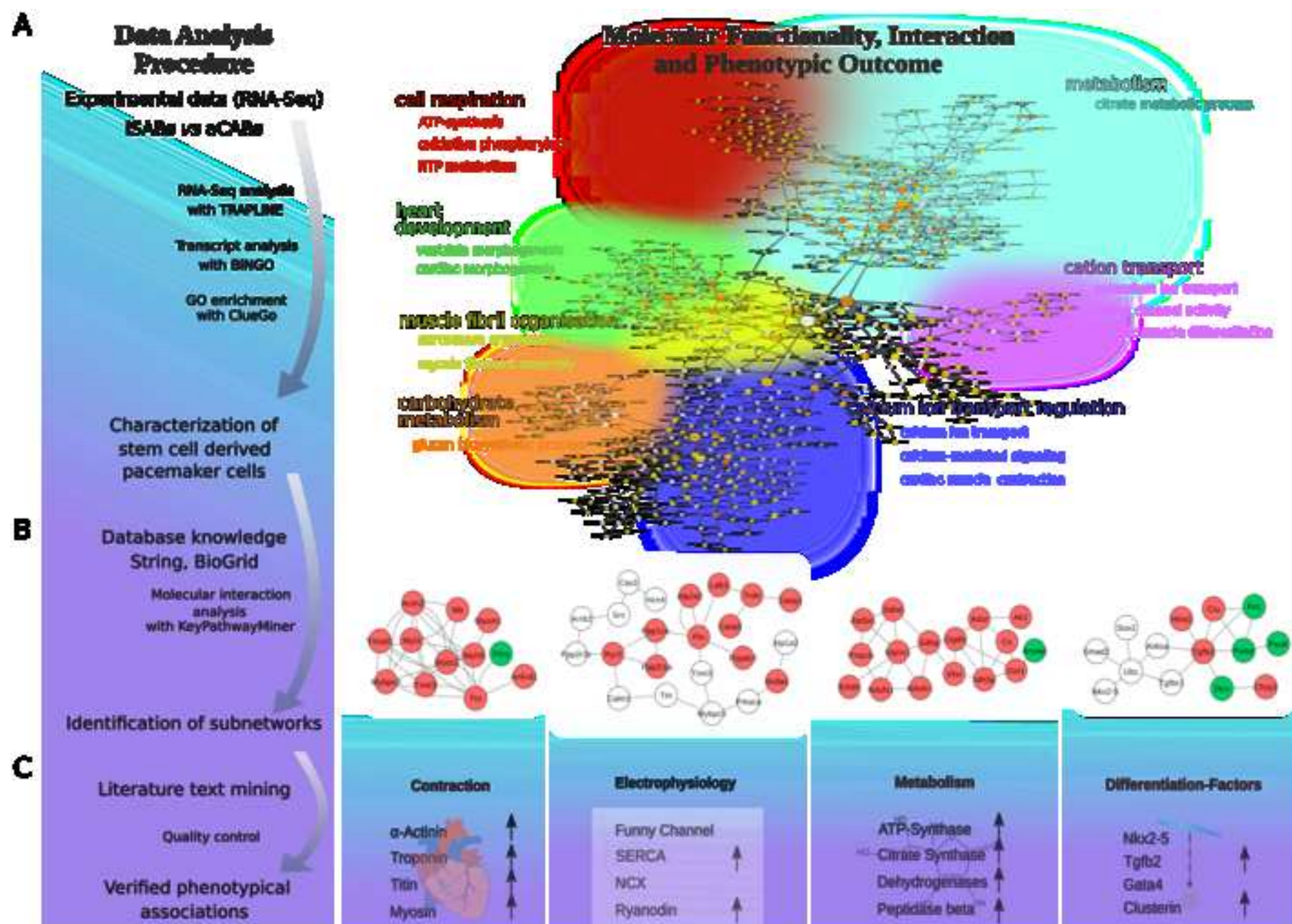


Figure 4
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List of abbreviations

ADSC	adipose tissue-derived mesenchymal stem cell
Alcam	activated leukocyte cell adhesion molecule
AMI	acute myocardial infarction
ANF	natriuretic factor
AP	action potential
ASC	adult stem cell
AV	atrioventricular
AVB	atrioventricular bundle
AVN	atrioventricular node
BB	bundle branch
BCT	bioartificial cardiac tissue
bHLH	basic helix-loop-helix
Bry	Brachyury
CABG	coronary artery bypass graft
Ca_v1.3	calcium voltage-gated channel subunit alpha1 D
Ca_v3.1	calcium voltage-gated channel subunit alpha1 G
CCS	cardiac conduction system
CF	cardiac fibroblast
CHD	congenital heart disease
CM	cardiomyocyte
CMPC	cardiomyocyte progenitor cell
CMVEC	cardiac microvascular endothelial cell
CPC	cardiac progenitor cell
CS	conduction system
CV	cardiovascular
CVD	cardiovascular disease
Cx30.2	connexin30.2
Cx40	connexin40
Cx43	connexin43
Cx45	connexin45
ECG	electrocardiogram
EMILIN2	elastin microfibril interface 2
EPC	endothelial progenitor cell
EPCS	electric-pulse current stimulation
ESC	embryonic stem cell
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FHF	first (primary) heart field
GF	growth factor
GFP	green fluorescence protein
GO	gene ontology
HCN4	hyperpolarization-activated cyclic nucleotide-gated cation channel 4
hPSCreg	Human Pluripotent Stem Cell registry

HTS	high-throughput sequencing
ICM	induced cardiomyocyte
iPSC	induced pluripotent stem cell
iSAB	induced sino-atrial body
Isl1	ISL LIM homeobox 1
JNK	c-Jun N-terminal kinase
LVEF	left ventricular ejection fraction
MAPK	mitogen-activated protein kinase
MB	molecular beacons
MEA	multi-electrode-array
Mlc2v	myosin, light polypeptide 2, regulatory, cardiac, slow
MSC	mesenchymal stem cell
MYH6	myosin, heavy chain 6, cardiac muscle, alpha
Myh7	myosin, heavy polypeptide 7, cardiac muscle, beta
Na_v1.5	sodium voltage-gated channel alpha subunit 5
Nkx2-5	NK2 homeobox 5
Nppa	natriuretic peptide A
ODE	ordinary differential equation
PA	polyacrylate
PDMS	polydimethylsiloxane
PLGA	polylactide-co-glycolide
PMC	pacemaker cell
PPT	protein-protein interaction
PSC	pluripotent stem cell
Rarg	retinoic acid receptor, gamma
ROCK	rho-associated, coiled-coil containing protein kinase
Rxra	retinoid X receptor, alpha
SA	sino-atrial
SAN	sinoatrial node
SCD	sudden cardiac death
SCN5A	sodium channel, voltage-gated, type V, alpha subunit
SHF	second heart field
Shox2	short stature homeobox 2
SIRPA	signal-reduced protein alpha
SSS	sick sinus syndrome
Tbx18	T-box 18
Tbx3	T-box 3
TF	transcription factor
THF	tertiary heart field
VCAM1	vascular cell adhesion molecule 1
VCS	ventricular conduction system
VEGF	vascular endothelial growth factor
wt	wild-type

Table 1

Table 1: Overview of recently published programming strategies of ASCs toward diverse cardiovascular subtypes

Cell origin	Host	Delivery system	<i>in vivo</i> / <i>in vitro</i>	Factor / Substances	Target cell type	Special features	Literature
CMPC	human	synthetic nucleoside protein	<i>in vitro</i>	5 μ mol/L 5-AZA following 1 ng/mL TGF- β 1	CM-like	spontaneously beating myocytes; gap-junctional communication and action potentials of maturing cardiomyocytes	Goumans <i>et al.</i> , 2007 [117]
ADSC	rat	protein	<i>in vitro</i>	10 ng/mL TGF- β 1	CM-like	Actin, cMhc	Gwak <i>et al.</i> , 2009 [107]
ADSC	human	synthetic nucleoside	<i>in vitro</i>	10 μ mol/L 5-AZA or 100 ng/mL TSA or Co-culture with RNCM or modified cardiomyogenic medium	CM-like	Highest expression in direct contact co-culture with RNCM: Actin, Gata4, Nkx2.5, cTnT spontaneous contractions synchronous Ca ²⁺ transient	Choi <i>et al.</i> , 2010 [109]
CMPC	human	miRNA	<i>in vitro</i>	miR-1 and miR-499	CM-like	Repression of HDAC4 and Sox6 Enhanced cardiomyogenesis	Sluijter <i>et al.</i> , 2010 [118]
CMPC	human	miRNA	<i>in vitro</i> / <i>in vivo</i>	miR-499	CM-like	Repression of Sox6 and Rod1 Enhanced cardiomyogenesis	Hosoda <i>et al.</i> , 2011 [116]
ADSC	rat	synthetic nucleoside	<i>in vitro</i>	Planat-Bérnard or 5 μ mol/L 5-AZA	CM-like	no spontaneous contraction Actn2, Cx-43	Carvalho <i>et al.</i> , 2012 [105]
BMSC	mouse	miRNA (Lentiviral)	<i>in vitro</i>	miR-1	CM-like	downregulation of Hes-1 expression of: Nkx2.5, GATA-4, cTnT, and Cx43	Huang <i>et al.</i> , 2013 [102]
BMSC	human	synthetic nucleoside	<i>in vitro</i>	6 μ mol/L 5-AZA or 10 ng/mL TGF- β 1	CM-like	Expression of: GATA-4, Nkx2.5, Mlc-2a, actin Higher expression in AZA-group to control: Mlc-2a, Mlc-2v, cTnT	Mohanty <i>et al.</i> , 2013 [100]
EPC	human	synthetic nucleoside	<i>in vitro</i>	5-AZA	CM-like	expression of: Actn2, cTnT, cTnI and desmin	López-Ruiz <i>et al.</i> , 2014 [112]
BMSC	pig	synthetic	<i>in vitro</i>	10 μ mol/L 5-AZA	CM-like	Expression of: GATA-4, Nkx2.5, β -MHC and	Li <i>et al.</i> , 2015

		nucleoside lentiviral		IGF-1		MEF2c	[99]
BMSC	rabbit	DNA Plasmid	<i>in vitro</i> / <i>in vivo</i>	GATA4, Nkx2.5 extracellular environment co-culture with RNCM	CM-like	In combination with co-culture: significantly effective and enhance the ability to repair MI	Li and Zhang, 2015 [98]
BMSC	mouse	specific culture and substrate conditions	<i>in vitro</i>	0.3 mm-thick hcECM	CM-like	no evidence of CM differentiation	Oberwallner <i>et al.</i> , 2015 [119]
BAT	mouse	specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	1 % methylcellulose/ Iscoe's Modified Dulbecco's Medium containing hematopoietic cytokines	CCS-like	regular beating expression of: Nkx2.5, GATA6, Mef2c, ANF, α -MHC, β -MHC, MLC2a, MLC2v, but not GATA4	Takahashi <i>et al.</i> , 2015 [110]
BMSC	rat	synthetic nucleoside	<i>in vitro</i> / <i>in vivo</i>	10 μ mol/L 5-AZA	CM-like	Expression of: desmin, actin and cTnT	Yang <i>et al.</i> , 2015 [97]
BMSC	canine	lentiviral	<i>in vitro</i>	Shox2 Co-culture with RNCMs	SAN-like	High levels of: Tbx3, HCN4, Cx45 Low levels of: Nkx2.5, Cx43 Able to pace RNCMs with a faster rate	Feng <i>et al.</i> , 2016 [101]
SC-ADSC VL-ADSC CA-ADSC SS-ADSC	mouse	specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	Medium suppl. with: - for vascular smooth muscle cell differentiation: TGF- β - for endothelial differentiation: hFGF, hVEGF, hIGF, AA, hEGF - for cardio- myocyte differentiation:	CM-like, endotheli al cells, vascular smooth muscle cells	Highest potential with: CA-ADSC	Nagata <i>et al.</i> , 2016 [108]

				PMA			
DFC	human	small molecule	<i>in vitro</i> / <i>in vivo</i>	10 μ M SAHA in ADMEM media; following continuous culture in media containing 1 μ M of SAHA	CM-like	in vitro: expression of: α -SMA, TnnT2, desmin, Actc1 <i>in vivo</i> homing: 5.6 \pm 1.0% heart 3.6 \pm 1.1% liver 11.6 \pm 2.7% kidney With differences in IL-2 and IL-10	Sung <i>et al.</i> , 2016 [111]
E-ADSC P-ADSC O-ADSC	human	synthetic nucleoside retroviral	<i>in vitro</i>	10 μ mol/L 5-AZA or ESRRG, GATA4, MEF2C, MESP1, MYOCD, TBX5, ZFPM2	CM-like	5-AZA: no increased expression of Actn2 or cTnT 7-factor-group: E-ADSC: increased Actn2 and cTnT	Wystrychowski <i>et al.</i> , 2016 [106]
BMSC	human	synthetic nucleoside	<i>in vitro</i>	10 μ mol/L 5-AZA	CM-like	Upregulation of: Notch1, Gata4, Nkx2.5, α -actin, cTnT	Yu <i>et al.</i> , 2016 [103]
BMSC	mouse	lncRNA	<i>in vitro</i>	10 μ mol/L 5-AZA Braveheart hypoxia/reoxygenation treatment	CM-like	Expressions of: α -actin, cTnT, Nkx2.5, Gata4, Gata6, Isl-1, EMT-associated genes (Snail, Twist, N-cadherin)	Hou <i>et al.</i> , 2017 [104]

AA: ascorbic acid; Actc1: cardiac muscle alpha actin; Actn2: sarcomeric alpha-actinin; ADSC: adipose tissue-derived mesenchymal stem cells (E: epicardium, P: pericardium, O: omentum, SC: subcutaneous white adipose tissue, VL: visceral white adipose tissue; CA: cardiac brown adipose tissue, SS: subscapular brown adipose tissue); α -SMA: alpha-smooth muscle actin; BAT: brown adipose tissue derived stem cells from interscapular area; BMSC: bone marrow mesenchymal stem cells; CCS: cardiac conduction system; CM: cardiomyocyte; CMPC: cardiomyocyte progenitor cells; CPC: cardiac progenitor cells; cTnT: cardiac Troponin T; Cx43/45: Connexin43/45; DFC: dental follicle-derived mesenchymal stem cells; hcECM: human cardiac extracellular matrix; HDAC4: Histone deacetylase 4; hEGF: human epidermal growth factor; hFGF: human fibroblast growth factor; hIGF: human insulin-like growth factor; hVEGF: human vascular endothelial growth factor; IL-2/10: interleukin-2/10; lncRNA: long noncoding RNA; miR: microRNA; Mlc: myosin light chain; PMA: phorbol myristate acetate; RNCM: rat neonatal cardiomyocytes; SAHA: suberoylanilide hydroxamic acid; TGF- β : transforming growth factor- β ; TnnT2: cardiac muscle troponin T; TSA: trichostatin A; 5-AZA: 5-azacytidine

Table 2

Table 1: Overview of recently published programming strategies of ESCs toward diverse cardiovascular subtypes

Cell origin	Host	Delivery system	<i>in vivo</i> / <i>in vitro</i>	Factor / Substances	Target cell type	Special features	Literature
ESC(HES-2, H1, H9)	human	specific culture conditions	<i>in vitro</i>	day 0-1: 0.5 ng/mL of BMP-4 day 1-4: 10 ng/mL BMP-4, 5 ng/mL human bFGF, and 6 ng/mL Activin A day 4-8: basal medium containing 10 ng/mL VEGF, 150 ng/mL Dkk-1 day 8-end: basal medium with 10 ng/mL VEGF, 10 ng/mL human bFGF MEF-free and serum-free hESC adherent culture under cGMP and cGLP conditions	CM-like	27 % cTnT ⁺ expression of: sMHC, β MHC, Isl-1, Nkx2.5, MYH6, Tnnt2, Myl2, and Myl7	Chen <i>et al.</i> , 2012 [163]
ESC (H7)	human	specific culture conditions	<i>in vitro</i>	1) MEF and SNL feeder cell layers + conventional SC culture medium containing ko-SR 2) bFGF 3) Matrigel matrix + commercial mTeSR1 medium	CM-like	most efficient protocol: MEF and SNL feeder cell layers + conventional SC culture medium containing ko-SR least efficient protocol: Matrigel matrix + commercial mTeSR1 medium; neural lineage induction	Ojala <i>et al.</i> , 2012 [190]
ESC (m: J1; h:H1)	mouse / human	specific purification method	<i>in vitro</i> / <i>in vivo</i>	CM-specific MBs	CM-like	<i>in vitro</i> : myosin heavy chain-MB: ~97 % cTnT ⁺ cells <i>in vivo</i> : improved cardiac function, without tumor formation after 4 weeks	Ban <i>et al.</i> , 2013 [174]
ESC	mouse	specific culture	<i>in vitro</i>	GSK3 inhibitor P38 MAPK inhibitors	CM-like	ERK activators, CaMKII inhibitors: proliferative effects only on CMs in early	Uosaki <i>et al.</i> , 2013 [168]

		conditions		CaMKII inhibitors ERK activators		developmental stage GSK3 inhibitor (BIO, CHIR), ERK activator (5 μ M SU1498), CaMKII inhibitor (5 μ M KN93): induced cell cycle progression in CM, resulting in CM proliferation	
ESC	mouse	specific culture conditions	<i>in vitro</i>	44 cytokines/signaling molecules on day 3 of diff	CPC (Nkx2.5 ⁺)	IGF1, IGF2, insulin, Wnt3a: significantly increase CPC formation IGF, insulin: promote Bry ⁺ mesodermal cell proliferation Activin A, BMP2 or BMP4: decrease CPC formation	Engels <i>et al.</i> , 2014 [162]
ESC	mouse	specific culture conditions	<i>in vitro</i>	2 % O ₂ preconditioning (3 passages) of ESCs diff as EBs in 20 % O ₂	CM-like	significant increased expression of early differentiation markers FGF5, Eomes increased gene expression of Eomes, Goosecoid, Bry, AFP, Sox17, FoxA2, and protein expression of Bry, Eomes, Sox17, FoxA2 - diff into mesodermal and endodermal lineages	Fynes <i>et al.</i> , 2014 [185]
ESC	human	specific culture conditions	<i>in vitro</i>	2 % O ₂ preconditioning (3 passages) of ESCs diff as EBs in 20 % O ₂	CM-like	decreased expression of early differentiation markers FGF5, Eomes increased gene expression Nestin, β 3-tubulin – diff into ectodermal lineage	Fynes <i>et al.</i> , 2014 [185]
ESC (H9)	human	specific substrate mechanics	<i>in vitro</i>	1) TCPS 2) PA hydrogel substrate	CM-like	intermediate stiffness of PA hydrogel yielded slightly higher cTnT ⁺ cells without significant difference to TCPS	Hazeltine <i>et al.</i> , 2014 [188]
ESC (GSES)	mouse	DNA-Plasmid specific purification method	<i>in vitro</i>	Tbx3 Myh6-promoter-based antibiotic selection	SAN-like	>80 % physiologically and pharmacologically functional pacemaker cells with highly increased beating rates (300–400 bpm)	Jung <i>et al.</i> , 2014 [89] Rimmbach <i>et al.</i> , 2015 [90]
ESC	mouse	lentiviral	<i>in vitro</i>	Fndc5	CM-like	sign. expression of: Flk1, Isl1, Nkx2.5, Gata4, Mef2C, α -MHC, cTnT, α -actinin, SM22 α , α -SMA	Rabiee <i>et al.</i> , 2014 [152]

ESC (H7, ESI-017)	human	specific culture and substrate conditions	<i>in vitro</i>	matrix-free, scalable, and GMP-compliant process culture: including first CHIR and second IWP-4 induction	CM-like	>90 % pure CM; 1.5 to 2 × 10 ⁹ CM/L (up to 1 L spinner flasks) ESI-017: 6 μM CHIR - ~91 % cTnT ⁺ H7: 12 μM CHIR - ~92 % cTnT ⁺	Chen <i>et al.</i> , 2015 [157]
ESC	mouse	lentiviral	<i>in vitro</i> / <i>in vivo</i>	Nkx2.5 Isl1	CM-like SAN-like	Overexpression of Nkx2.5: inhibition of Isl1 expression Overexpression of Isl1: enhanced specification of cardiac progenitors, earlier cardiac differentiation, and increased cardiomyocyte number, upregulation of nodal-specific genes (e.g. Hcn4), downregulation of transcripts of working myocardium	Dorn <i>et al.</i> , 2015 [155]
ESC (R1)	mouse	adenoviral	<i>in vitro</i>	Shox2	SAN-like	increase in Cx45, decrease in Cx43, Nkx2.5 SHOX2-EBs beat spontaneously (83±7 % versus 15±6 %) pacemaker-like AP profile (62 %)	Ionta <i>et al.</i> , 2015 [154]
ESC (CGR8, αPIG44)	mouse	specific culture conditions	<i>in vitro</i>	100 μM AA	CM-like	AA application from day 0-2 increases cardiogenesis 2-4-fold day 5: increased expression of genes associated with angiogenesis, blood vessel development, hematopoiesis/erythropoiesis, Bry, Mef2c, Myl7	Ivanyuk <i>et al.</i> , 2015 [169]
ESC (H7)	human	specific purification method	<i>in vitro</i>	CM-specific MBs	CM-like	NPPA-MB: ~92 % α-actinin ⁺ cells	Jha <i>et al.</i> , 2015 [175]
ESC (α-PIG)	mouse	specific culture and substrate conditions	<i>in vitro</i>	0.3 mm-thick hcECM	CM-like	hcECM supported proliferation significantly increased expression of: Myh6, Tnnt2, Nkx2.5 Matrigel or Geltrex use did not induce cardiac-specific markers	Oberwallner <i>et al.</i> , 2015 [119]

ESC (derived from C57BL/6 mouse strain)	mouse	specific culture conditions	<i>in vitro</i>	10 μ M resveratrol	CM-like	promotes mESC differentiation towards CM enhanced beating properties of EBs significantly higher expression of: Nkx2.5, Mef2c, Tbx5, dHand2, α MHC, Cx43, cTnC1	Ding <i>et al.</i> , 2016 [202]
ESC	mouse	DNA-Plasmid	<i>in vitro</i>	CIBZ	CM-like	CIBZ depletion: induced expression of Bry, MesP1, Gata6, Sox17 CIBZ overexpression: decreased expression of Bry, MesP1, Flk1 Isl1, Gata4, Mhc, cTnl; Significant suppression of beating EBs	Kotoku <i>et al.</i> , 2016 [151]
ESC (in-house and E14tg2a)	mouse	specific culture conditions	<i>in vitro</i>	monolayer culture without feeder cells day 0-1: IMDM/Ham's F12, N2 supplement B27 supplement, 0.5 mM AA, 4.5×10^{-4} M MTG day 1-3: 8 ng/mL Activin A, 0.5 ng/mL BMP4, 5 ng/mL hVEGF day 3-13: StemPro-34 SF medium, 0.5 mM AA, 5 ng/mL hVEGF, 10 ng/mL bFGF, 50 ng/mL hFGF10	CM-like	60 % CM 35-40 % cTnT ⁺ /MF20 ⁺ E14tg2a: more efficient CM-like cell yield with 5 ng/mL Activin A	Kokkinopoulos <i>et al.</i> , 2016 [165]
ESC	mouse	specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	25 ng/ml Activin, 20 ng/ml BMP2, 20 ng/ml BMP4, 100 ng/ml DLL1, 10 ng/ml bFGF, 10 ng/ml FGF8, 20 ng/ml Tgfb, 100 ng/ml Wnt3a, 5 μ M IWR1, 5 μ M SB431542	MesP1-CPC	BMP4 exposure: day 0-4 highest improvement of MesP ⁺ cells (29.6 %) BMP4 + IWR1: MesP ⁺ cells (13.8 %), however differentiate more efficiently into cardiac myocytes In vivo: injection of day 5 MesP1-CPCs led to improved survival of MI mice and decreased scar formation	Liu <i>et al.</i> , 2016 [144]
ESC (E14T)	mouse	specific purification	<i>in vitro</i> / <i>in vivo</i>	selection based on VE-cadherin promoter	CEDP	Differentiation into: ~ 47 % cTnT ⁺ and ~28 % VE-cadherin ⁺ cells	Maltabe <i>et al.</i> , 2016 [173]

		method					
ESC (H7 and H9)	human	specific culture conditions	<i>in vitro</i>	1) day 0-1.5: BMP4, FGF2, Activating A, LY294002 day 1.5-5: BMP4, FGF2, RA, IWR1/IWP2 day 5-9: BMP4, FGF2 2) day 0-2: CHIR99021, day 2-4: IWP2	CM-like	WNT3, WNT8: regulation of Bry expression and mesoderm induction (via FZD7 + canonical Wnt signaling) WNT5A/5B: regulation of MesP1 expression and cardiovascular development (via ROR2 + noncanonical Wnt signaling) WNT2, WNT5A/5B, WNT11: regulation of late functional CM diff (via FZD4, FZD6 + noncanonical Wnt signaling)	Mazzotta <i>et al.</i> , 2016 [161]
ESC (5)	mouse	DNA-Plasmid	<i>in vitro</i>	CITED2	CM-like	<i>Cited2</i> depletion: significantly decreased expression of Brachyury, Mesp1, Isl1, Gata4, Tbx5 <i>Cited2</i> overexpression: stimulation of Brachyury, Mesp1, Isl1, Gata4, Tbx5, Myh6, cTnT; protein interaction with Isl1	Pacheco-Leyva <i>et al.</i> , 2016 [150]
ESC	mouse	miRNA lentiviral	<i>in vitro</i>	miR-322/-503	CM-like	Highest enriched miRNA in Mesp1 lineage (miR-322/-503) Celf1 is a direct target of miR-322/-503 miR-322/-503 selectively inhibits neuroectoderm differentiation	Shen <i>et al.</i> , 2016 [145]
ESC	human	specific culture conditions	<i>in vitro</i>	monolayer-directed differentiation protocol different concentrations of activin A and BMP4	CM-like EC-like	generation of distinct CVP populations following derivation of cardiogenic versus hemogenic mesoderm >90 % CD31 ⁺ /VE-cadherin ⁺ ECs	Palpant <i>et al.</i> , 2017 [164]
ESC (HES3-Nkx2.5 ^{gfp/w} , HES2)	human	specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	EB formation, 5 % O ₂ (d0-12) Generation of VLCMs: day 0-3: 10 ng/mL rhBMP4, 6 ng/mL rhActivinA, 5 ng/mL rhbFGF day 3-5: 0.5 μM IWP2, 10 ng/mL rhVEGF	CM-like SAN-like	FGF pathway blocks the development of NKX2-5 ⁺ CM Marker expression of the SAN lineage (TBX18, SHOX2, TBX3), typical pacemaker action potentials (90 %), ion current profiles and chronotropic response	Protze <i>et al.</i> , 2017 [159]

				day 5-12: 5 ng/mL rhVEGF day 12-20: without additional factors day 20: FACS sorting (NKX2.5:GFP ⁺ SIRPA ⁺ CD90 ⁻) Generation of SANLPCs: day 0-3: 3 ng/mL rhBMP4, 2 ng/mL rhActivinA, 5 ng/mLrhbFGF day 3-6: 2.5 ng/mL rhBMP4, 5.4 μM SB-431542, 0.25 μM Retinoic Acid (HES3: 480–960 nM PD 173074 at day 4, HES2 at day 3) day 6-20: 5 ng/mL rhVEGF day 20: FACS sorting (NKX2.5:GFP ⁺ SIRPA ⁺ CD90 ⁻)		
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AA: ascorbic acid; βMHC: β myosin heavy chain; bFGF: basic fibroblast growth factor; BMP: bone morphogenetic protein; bpm: beats per minute; Bry: Brachyury; CAMKII: Ca²⁺/calmodulin-dependent protein kinase II; CEDP: cardiac and endothelial dual-progenitor population; CHIR: CHIR99021, Wnt activator; CIBZ: BTB domain-containing zinc finger protein; CM: cardiomyocytes; CPC: cardiac progenitor cell; cTnC1: cardiac troponin C1; CVP: cardiovascular progenitor cell; diff: differentiation; EB: embryoid bodies; EC: endothelial cell; ERK: extracellular signal-regulated kinase; ESC: embryonic stem cells; Fndc5: Fibronectin type III domain-containing 5 protein (also known as: peroxisomal protein (PEP)); GSK3: glycogen synthase kinase-3; hcECM: human cardiac extracellular matrix; IWP: WNT inhibitor; ko-SR: knockout serum replacement; LY294002: phosphoinositide 3-kinase inhibitor; MAPK: p38 mitogen-activated protein kinase; MB: molecular beacon; MTG: monothioglycerol; PA: polyacrylamide; PD 173074: FGF signaling inhibitor; SAN: sinoatrial node; SB-431542: Activin/Nodal/TGFβ signaling inhibitor; sMHC: sarcomeric myosin heavy chain; TCPS: tissue culture polystyrene; Tnnt2: cardiac troponin T2; VEGF: vascular endothelial growth factor; (x): number of cell lines used.

Table 1: Overview of recently published programming strategies of iPSCs toward diverse cardiovascular subtypes

Cell origin	Host	Delivery system	<i>in vivo</i> / <i>in vitro</i>	Factor / Substances	Target cell type	Special features	Literature
iPSC	mouse and human	specific culture conditions	<i>in vitro</i>	16 cytokines and chemical components	CM-like	AA: CM enhancement in 11 lines Application of 50 µg/mL AA during day 2-6: 7.3-fold (miPSC) and 30.2-fold (hiPSC) enhanced yield through specific increase in the proliferation of CPCs via the MEK-ERK1/2 pathway	Cao <i>et al.</i> , 2012 [203]
iPSC (UTA.00112. hFF, UTA.04602. WT, UTA.00525.L QT2, UTA.00106.h FF)	human	specific culture conditions	<i>in vitro</i>	1) MEF and SNL feeder cell layers + conventional SC culture medium containing ko-SR 2) bFGF 3) Matrigel matrix + commercial mTeSR1 medium	CM-like	most efficient protocol: MEF and SNL feeder cell layers + conventional SC culture medium containing ko-SR least efficient protocol: Matrigel matrix + commercial mTeSR1 medium; neural lineage induction exception: UTA.04602.WT line - highest amount of beating areas on Matrigel in mTeSR1	Ojala <i>et al.</i> , 2012 [190]
iPSC (BJ1-iPS10)	human	specific selection method	<i>in vitro</i> / <i>in vivo</i>	CM-specific MBs	CM-like	<i>in vitro</i> : myosin heavy chain-MB: ~97 % cTnT ⁺ cells <i>in vivo</i> : improved cardiac function, without tumor formation after 4 weeks	Ban <i>et al.</i> , 2013 [174]
iPSC	human	long-term culture	<i>in vitro</i>	EB-formation 360 day culture	CM-like	day 14: immature high-density Z-bands day 180: mature Z-, A-, H-, and I-bands day 360: M-, Z-, A-, H-, and I-bands	Kamakura <i>et al.</i> , 2013 [204]
iPSC	mouse and human	specific culture conditions antibiotic selection	<i>in vitro</i>	αMHC-antibiotic resistance gene EB formation BCT (cells + liquid collagen type I and Matrigel) AA static stretch	CM-like	structurally and functionally homogenous syncytium enhanced the contractility of murine and human BCTs (active tension of 4.4 mN/mm ²)	Kensah <i>et al.</i> , 2013 [171]

iPSC (from: skeletal muscle PC, skeletal muscle fibroblasts)	mouse	retroviral specific culture conditions	<i>in vitro</i>	16 h after EB seeding onto gelatin: exposure to hypoxia (3 % O ₂) for 24 h	CM-like	EBs normoxic conditions: ~59 % beating EBs hypoxic conditions: abolished beating, significant increased expression of Bry and Isl1, HIF-1 α ; accumulation of HIF-1 α and β -catenin in nuclear protein extracts, suggesting involvement of the Wnt/beta-catenin pathway	Medley <i>et al.</i> , 2013 [205]
iPSC	human	specific culture conditions	<i>in vitro</i>	day 0-1: 6 μ M CHIR day 2-3: diff medium day 4-5: 5 μ M IWR-1 day 6-7: diff medium day 8-end: diff medium + insulin	CM-like	expression of: IRX4, MLC2v, MLC2a, TNNT3, and TNNT2	Bhattacharya <i>et al.</i> , 2014 [156]
iPSC (from BJ fibroblasts)	human	mRNA reprogramming specific culture conditions	<i>in vitro</i>	day 0-8: EBs in low adhesion culture dish, 5 μ M SB203580 day 9-week 8: EBs plated on 0.1 % gelatin coated dishes, without SB203580	CM-like	day 11-14 until day 50-60: rhythmically contracting areas (55-75 %) day 6: expression of Isl1, Kdr, Mef2C, NKx2.5, Gata4, Tbx3/5/20 day 8, 14: expression of Tnni2, Mlc2a/v, Myh7, Myl3/4, Ca _v 1.3, Hcn4, Serca2a, Ryr2 specific response to pharmacological substance administration	Mehta <i>et al.</i> , 2014 [133]
iPSC (IMR90)	human	specific culture conditions	<i>in vitro</i>	day 20-27: 20 ng/mL T3	CM-like	increased cardiomyocyte size, anisotropy, and sarcomere length 2-fold higher force per-beat	Yang <i>et al.</i> , 2014 [206]
iPSC (from Dr. Joseph Wu, Stanford)	human	specific culture and substrate conditions	<i>in vitro</i>	matrix-free, scalable, and GMP-compliant process culture: including first CHIR and second IWP-4 induction	CM-like	>90 % pure CM; 1.5 to 2 $\times 10^9$ CM/L (up to 1 L spinner flasks) relationship between aggregate size and CHIR concentration 6 μ M CHIR 94 % cTnT ⁺	Chen <i>et al.</i> , 2015 [157]
iPSC (from activated T cells, hPBMc)	human	retroviral specific culture conditions	<i>in vitro</i>	day 1-13: 5 % O ₂ and 7 % CO ₂ day 1-2: 50 % mTeSR, 45 % DMEM (low	CM-like	before selection: 30-45 % CM, expression of cTnT and MLC2v after lactate selection: 90 % CM, electrophysiological characterization:	Fuerstenau-Sharp <i>et al.</i> , 2015 [158]

		metabolic selection (lactate)		glucose), 5 % FBS, 1 μ M H1152, 100 ng/mL bFGF day 2: 2/3 medium change to: DMEM (low glucose), 10 % FBS, 50 ng/mL bFGF day 3-7: DMEM (low glucose), 10 % FBS, 50 ng/mL bFGF, 6 ng/mL activating A, 10 ng/mL BMP4 day 8-13: DMEM (low glucose), 10 % FBS day 14-end: 20 % O ₂ and 7 % CO ₂ day 16-18: DMEM (no glucose), 1 % sodium DL-lactate solution		ventricular, atrial and nodal-like action potentials	
iPSC	mouse	specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	α MHC-GFP EB formation diff. after EB: 1 week treatment with 1 % DMSO and/or 100 ng/mL NRG-1 β	CM-like	significantly higher expression of: Gata4, Gata6, α Mhc, Myhl7, Myl3, cTnnc1, Ryr2, Serca2a ventricular-like AP preserves cardiac function in mouse model	Iglesias-García <i>et al.</i> , 2015 [207]
iPSC (TiB7.4, AT25)	mouse	specific culture conditions	<i>in vitro</i>	100 μ M AA	CM-like	AA application from day 0-2 increases cardiogenesis 2-4-fold	Ivanyuk <i>et al.</i> , 2015 [169]
iPSC (DF19-9 –11T.H)	human	RNA	<i>in vitro</i>	EB-formation diff after EB: transfection with CIR RNA	CM-like	spindle-shaped cells with characteristic expression of: cTnT, tropomyosin, α -actinin	Kochegarov <i>et al.</i> , 2015 [208]
iPSC (AT25)	mouse	specific culture and substrate conditions	<i>in vitro</i>	0.3 mm-thick hcECM	CM-like	hcECM supported the proliferation significantly increased expression of: Myh6, Tnnt2, Nkx2.5 Matrigel, Geltrex: did not induce cardiac-specific markers	Oberwallner <i>et al.</i> , 2015 [119]
iPSC	mouse	lentiviral	<i>in vitro</i>	telomerase-competent cell	CM-like	iPSCs ^{highTRF1} : differentiate earlier and more	Aguado <i>et al.</i> ,

				lines (TRF1 expression) AA		efficiently into CMs iPSC ^{lowTRF1} : differentiate very efficiently to the ectoderm lineage AA: increase CM yield with iPSC ^{highTRF1}	2016 [170]
iPSC (Duanqing Pei, Chinese Academy of Sciences)	mouse	specific selection method	<i>in vitro</i>	0.001 – 0.2 µmol/L EPI 10 µmol/L phentolamine 5 µmol/L propranolol 10 µmol/L phenylephrine 10 µmol/L clonidine 1 µmol/L isoproterenol 10 µmol/L PD98059	CM-like	EPI and activation of α1-AR: enhancement of CM differentiation via MEK-ERK1/2 signaling	Li <i>et al.</i> , 2016 [167]
iPSC (from: MEFs)	mouse	retroviral specific culture conditions	<i>in vitro</i>	EB formation medium containing: DMEM, 15% fetal bovine serum, 0.2 mmol/l L-glutamine, 0.1 mmol/l nonessential amino acids, and 0.1 mmol/l β-mercaptoethanol	CM-like	After EB formation: expression of Flk1, α-actinin, α-MHC, cTnT, Cx43, Nkx2.5 active beating: 360 bpm incompletely differentiated iPS cells: teratoma formation after transplantation into a SCID mouse model of MI	Wang <i>et al.</i> , 2016 [209]
iPSC (from foreskin tissue)	human	specific culture conditions metabolic selection (lactate)	<i>in vitro</i>	chemical-defined and albumin-free medium day 0-1: S12 without insulin medium, 4-8 µM CHIR day 1-3: S12 without insulin medium day 3-5: S12 without insulin medium, 5 µM IWR-1 day 5-end: S12 with insulin medium, atrial diff: 1 µM RA, ventricular diff: 1 µM BMS493	CM-like Atrial-like	highly homogenous atrial and ventricular myocytes in a scalable fashion with normal electrophysiological properties	Pei <i>et al.</i> , 2017 [160]

AA: ascorbic acid; AP: action potential; AR: adrenergic receptor; BCT: bioartificial cardiac tissue; bpm: beats per minute; BMS493: RA inhibitor; Bry: Brachyury; CHIR: CHIR99021 (GSK-3 inhibitor, Wnt activator); CIR: specific fetal cardiac-inducing RNA (fragment of N-sulfoglucosaminesulfohydrolase and the caspase

recruitment domain family member 14 precursor); Cx43: connexin 43; clonidine: selective α_2 -AR agonist; cTnT: cardiac troponin T; DMSO: dimethyl sulfoxide; EPI: epinephrine; hcECM: human cardiac extracellular matrix; hPBMC: human peripheral blood mononuclear cells; H1152: Rho-kinase (ROCK) inhibitor; IRX4: iroquois-class homeodomain protein IRX-4; isoproterenol: β -AR agonist; IWP-4: WNT inhibitor; IWR-1: WNT inhibitor; MEF: mouse embryonic fibroblast; MLC2a: myosin regulatory light chain 2, atrial isoform; MLC2v: myosin regulatory light chain 2, ventricular/cardiac muscle isoform; NRG-1 β : Neuregulin-1 β ; PC: progenitor cells; PD98059: MEK1/2 inhibitor; MI: myocardial infarction; phenylephrine: selective α_1 -AR agonist; phentolamine: α -AR antagonist; propranolol: β -AR antagonist; RA: retinoic acid; SB203580: MAPK signaling inhibitor; SCID: severe combined immunodeficiency; TNNT3: cardiac troponin I; Tnnt2: cardiac troponin T2; TRF1: shelterin-complex protein; T3: Tri-iodo-L-thyronine.

Table 4

Table 1: Overview of recently published direct reprogramming strategies of somatic cells toward diverse cardiovascular subtypes

Cell origin	Host	Delivery system	<i>in vivo</i> / <i>in vitro</i>	Factor / Substances	Target cell type	Special features	Literature
neonatal cardiac fibroblasts	mouse	retroviral or lentiviral	<i>in vitro</i> / <i>in vivo</i>	Gata4, Mef2c, Tbx5	CM-like	30 % cTnT ⁺ cells (to a lesser extent in tail-tip fibroblasts) Upregulation of: Myh6, Actc1, Actn2, Nppa	Ieda <i>et al.</i> , 2010 [227]
neonatal cardiac fibroblasts	mouse	lentiviral microRNA specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	miR-1/-122/-208/499 JAK inhibitor I	CM-like	day 3: 2- to 3-fold upregulation of Mef2c , Tbx5 , Hand2 , Nkx2.5 , Gata4 day 6: expression of cTnT, cTnI, sarcomeric actinin reprogramming efficiency: 1.13–5.28 % in non-JAK inhibitor I-treated cells with JAK inhibitor I: 28 % α MHC ⁺ cells enhanced cardiac function in mouse model	Jayawardena <i>et al.</i> , 2012 and 2014 [235,236]
fibroblasts (from hESCs; H9)	human	retroviral	<i>in vitro</i>	EGFP, ESRRG, GATA4, MEF2C, MESP1, TBX5, MYOCD, ZFPM2 SIS3	CM-like	20 % functional Ca ²⁺ transients cardiac marker expression of: cTnT, α -Actinin, ACTC1, ACTN2, MYH6, MYL2, MYL7, TNNT2, NPPA, PLN, and RYR2 SIS3 significantly decreases α MHC ⁺ cells	Fu <i>et al.</i> , 2013 [221]
tail tip and embryonic fibroblasts (B6;129S4)	mouse	retroviral	<i>in vitro</i>	M3 domain of mouse MyoD fused on carboxy-terminus of Mef2c, Gata4, Hand2, Tbx5 GSK126 (day 1-4), UNC0638 (day 3-7)	CM-like	Reprogramming efficiency: MM ₃ -GHT: 3.5 % (> 15-fold increase) MM ₃ -GHT + GSK126: further increase to control 2.1-fold (most efficient combination) MM ₃ -GHT + UNC0638: further increase to control 2-fold	Hirai <i>et al.</i> , 2013 [219] and 2014 [220]
NRVM	rat	adenoviral	<i>in vitro</i> / <i>in vivo</i>	Tbx18	SAN-like	downregulation of Cx43 pacemaker-like AP profile (9.2 %)	Kapoor <i>et al.</i> , 2013 [242]
neonatal foreskin and	human	retroviral	<i>in vitro</i>	Gata4, Hand2, Tbx5, myocardin, miR-1/-133	CM-like	~35 % tropomyosin ⁺ cells ~20 % cTnT ⁺ cells	Nam <i>et al.</i> , 2013 [222]

adult fibroblasts				Culture time: 4-11 weeks			
CM	pig	adenoviral	<i>in vitro</i> / <i>in vivo</i>	Tbx18	SAN-like	mean HR was higher in TBX18-transduced animals sympathetic predominance in the TBX18-transduced group TBX18-transduced animals had persistent and stable activity	Hu <i>et al.</i> , 2014 [243]
embryonic fibroblasts (C57BL/6)	mouse	chemical cocktail	<i>in vitro</i>	on Matrigel 2-stage protocol: day 0-16: CRM (knockout DMEM, 15 % FBS, and 5 % KSR, 0.5 % N2, 2 % B27, 1 % Glutamax, 1 % NEAA, 0.1 mM β -mercaptoethanol, 50 μ g/ml AA , 100 units/ml penicillin, 100 μ g/ml streptomycin) + CRFVPT (10 μ M CHIR (C), 10 μ M RepSox (R), 50 μ M Forskolin (F), 0.5 mM VPA (V), 5 μ M Parnate, (P), 1 μ M TTNPB (T)) day 17-end: CMM (DMEM medium, 15 % FBS, 2i (3 μ M CHIR, 1 μ M PD0325901), 1000 units/mL LIF, 50 μ g/mL AA, and 1 μ g/mL insulin)	CM-like	morphology: spindle shape, rod shape or round shape spontaneously beating activity: increases from day 8 cardiac marker expression of: Mef2c, α -Actinin, Gata4, cTnT, Nkx2.5, α -MHC, N-cadherin, Cx43, cTnl action potential of atrial- and ventricular-like CMs	Fu <i>et al.</i> , 2015 [218]
cardiac fibroblasts	mouse	retroviral antibiotic selection	<i>in vitro</i>	Gata4, Mef2c, Tbx5	CM-like	stoichiometry of G, M, T protein expression influences reprogramming efficiency high Mef2c and low Gata4, Tbx5 most efficient	Wang <i>et al.</i> , 2015 [228]
embryonic	mouse	retroviral	<i>in vitro</i>	Gata4, Hand2, Mef2c, Tbx5,	CM-like	spontaneously beating activity without	Zhao <i>et al.</i> ,

fibroblasts				miR-1/-133, Y-27632, Thiazovivin, SR-3677, A83-01		signaling inhibitors: GHMT > day 21, GHMT+miR-1/-133 > day 8 ROCK inhibitors enhance reprogramming of MEFs TGF- β inhibitors enhance reprogramming of MEFs most efficiently	2015 [217]
embryonic, adult cardiac, tail tip	mouse	retroviral	<i>in vitro</i>	Gata4, Hand2, Mef2c, Tbx5, Akt1	CM-like	spontaneously beating activity: MEFs > day 7 (50 % > day 21), CFs > day 14, TTFs > day 21; responsive to β -adrenoreceptor pharmacologic modulation, polynucleated, and hypertrophic	Zhou <i>et al.</i> , 2015 [216]
neonatal cardiac fibroblasts	mouse	retroviral (TFs) and lentiviral (shRNA)	<i>in vitro</i>	Gata4, Mef2c, Tbx5 shRNA of 35 selected components of chromatin modifying or remodeling complexes	CM-like	Bm1 downregulation significantly enhanced CM generation	Zhou <i>et al.</i> , 2016 [231]
neonatal cardiac fibroblasts	mouse	TFs specific culture conditions	<i>in vitro</i> / <i>in vivo</i>	Gata4, Mef2c, Tbx5 SB431542 XAV939	CM-like	8-fold increased reprogramming efficiency beating cells 1 week after reprogramming enhanced cardiac function in mouse model	Mohamed <i>et al.</i> , 2017 [230]

AA: ascorbic acid; Actc1: cardiac α -actin; Actn2: actinin α 2; AP: action potential; Akt1: Akt1/protein kinase B; A83-01: TGF- β inhibitor; CHIR: CHIR99021 (GSK-3 inhibitor, Wnt activator); CM: cardiomyocytes; GSK126: Enhancer of Zeste Homolog 2 (Ezh2) inhibitor; KSR: knockout serum replacement; Myh6: α -myosin heavy chain; NEAA: non-essential amino acid; Nppa: natriuretic peptide precursor type A; NRVM: neonatal rat ventricular myocytes; PD0325901: MEK1/2 inhibitor; RepSox: TGF- β 1 inhibitor; SAN: sino-atrial-nodal cells; SB431542: TGF- β inhibitor; shRNA: small hairpin RNA; SIS3: SMAD3 inhibitor (activated downstream of TGF β signaling); SR-3677: ROCK inhibitor; TF: transcription factor; Thiazovivin: ROCK inhibitor; TTNPB: analog of retinoic acid; UNC0638: G9a and GLP inhibitor; VPA: valproic acid (histone deacetylase inhibitor); XAV939: WNT inhibitor; Y-27632: ROCK inhibitor.